ROYAUME DU MAROC
Université Mohammed V - RABAT -
جامعة محمد الخامس – الرباط –
Faculté des sciences
كلية العلوم
CENTRE D'ETUDES DOCTORALES - SCIENCES ET TECHNOLOGIES

N° d'ordre 3734

THESE

En vue de l'obtention du : ***DOCTORAT***

Centre de Recherche : Biotechnologies Végétales et Microbiennes, Biodiversité et Environnement
Structure de Recherche : Équipe de Microbiologie et Biologie Moléculaire
Discipline : Biologie
Spécialité : Cancérologie et Biologie Moléculaire

Présentée et soutenue le 24/12/2022 par :

Melle. HAFIDI ALAOUI Chaimae

Molecular biomarkers and scoring models assessment for better management of bladder cancer patients in Morocco

Devant le Jury

Youssef BAKRI	PES, Université Mohammed V, Faculté des Sciences, Rabat	Président / Rapporteur
Nadia DAKKA	PES, Université Mohammed V, Faculté des Sciences, Rabat	Rapporteur/ Examinatrice
Mohcine BENNANI MECHITA	PES, Université Abdelmalek Essaadi, Faculté des Sciences et Techniques, Tanger	Rapporteur/ Examinateur
Mohammed OUKABLI	PES, Université Mohammed V, Faculté de Médecine et de Pharmacie, Rabat	Examinateur
Imane CHAOUI	Expert, Dr, Centre National de l'Energie, des Sciences et des Techniques Nucléaires, Rabat	Examinatrice
Mohammed EL MZIBRI	Expert, Dr, Centre National de l'Energie, des Sciences et des Techniques Nucléaires, Rabat	Co-encadrant
Mohammed ATTALEB	Expert, Dr, Centre National de l'Energie, des Sciences et des Techniques Nucléaires, Rabat	Co-Encadrant
Abdelkarim FILALI-MALTOUF	Expert, PES, Académie Hassan II des Sciences et des Techniques, Rabat	Co-Directeur de thèse
Bouchra BELKADI	PES, Université Mohammed V, Faculté des Sciences, Rabat	Directeur de thèse

ROYAUME DU MAROC
Université Mohammed V - RABAT -
جامعة محمد الخامس – الرباط –
Faculté des sciences
كلية العلوم
CENTRE D'ETUDES DOCTORALES - SCIENCES ET TECHNOLOGIES

N° d'ordre 3734

THESE

En vue de l'obtention du : ***DOCTORAT***

Centre de Recherche : Biotechnologies Végétales et Microbiennes, Biodiversité et Environnement
Structure de Recherche : Équipe de Microbiologie et Biologie Moléculaire
Discipline : Biologie
Spécialité : Cancérologie et Biologie Moléculaire

Présentée et soutenue le 24/12/2022 par :

Melle. HAFIDI ALAOUI Chaimae

Molecular biomarkers and scoring models assessment for better management of bladder cancer patients in Morocco

Devant le Jury

Youssef BAKRI	PES, Université Mohammed V, Faculté des Sciences, Rabat	Président / Rapporteur
Nadia DAKKA	PES, Université Mohammed V, Faculté des Sciences, Rabat	Rapporteur/ Examinatrice
Mohcine BENNANI MECHITA	PES, Université Abdelmalek Essaadi, Faculté des Sciences et Techniques, Tanger	Rapporteur/ Examinateur
Mohammed OUKABLI	PES, Université Mohammed V, Faculté de Médecine et de Pharmacie, Rabat	Examinateur
Imane CHAOUI	Expert, Dr, Centre National de l'Energie, des Sciences et des Techniques Nucléaires, Rabat	Examinatrice
Mohammed EL MZIBRI	Expert, Dr, Centre National de l'Energie, des Sciences et des Techniques Nucléaires, Rabat	Co-encadrant
Mohammed ATTALEB	Expert, Dr, Centre National de l'Energie, des Sciences et des Techniques Nucléaires, Rabat	Co-Encadrant
Abdelkarim FILALI-MALTOUF	Expert, PES, Académie Hassan II des Sciences et des Techniques, Rabat	Co-Directeur de thèse
Bouchra BELKADI	PES, Université Mohammed V, Faculté des Sciences, Rabat	Directeur de thèse

Dédicace

Je dédie ce travail :

À mes chers parents : Yahia Fatima et Hafidi Alaoui Hassan

À ceux qui m'ont donné la vie, l'exemple de dévouement, et pour qui aucune dédicace ne saurait être assez éloquente pour exprimer ce que vous méritez pour l'ampleur des sacrifices que vous avez endurés pour pourvoir nous éduquer, et votre souci majeur a toujours été notre réussite. Puisse Dieu, le tout puissant, vous préservez et vous accorde santé, longue vie et bonheur. Je vous aime très fort.

À ma très chère sœur Salma

En témoignage de l'attachement, de l'amour et de l'affection que je porte pour toi. Je te souhaite le bonheur et la réussite.

À mes chers frères : hamza, Ali et Ahmed.

Merci pour votre amour fraternel que vous m'avez toujours témoigné, que ce travail soit considéré comme le vôtre.

À la mémoire de mes grands parents

À ma Grand-mère, mes Tantes, mes Oncles, Cousins et Cousines

Merci pour l'attention que vous m'avez toujours accordée Soyez assurés de mon affection.

À mes chers amis

Je remercie mes amis Hajar, Oumaima, Safae, Meryem, Kaouar, Jawad, Younes pour vos encouragements et votre soutien. Que ce travail soit l'expression de notre amitié, je vous souhaite tout ce qu'il y a de mieux dans la vie.

À toute l'équipe d'unité de Biologie et recherche médicale au CNSTEN

Pour leur gentillesse et leur présence et qui ont fait part de leurs conseils et avis dans la préparation de ce projet.

À mon cher Professeur FILALI-MALTOUF Abdelkarim

Que je remercie chaleureusement pour son encouragement et qui a assuré un enseignement riche et efficace au cycle de Master. Veuillez trouver ici l'expression de ma profonde admiration pour toutes vos qualités scientifiques et humaines. J'espère être à la hauteur de vos attentes.

Foreword

This research work presented in this thesis was performed at the Biology and Medical Research Unit of the National Center for Energy Sciences and Nuclear Techniques (CNESTEN) in collaboration with the Microbiology and Molecular Biology Laboratory, Faculty of Sciences, Mohammed V University, Rabat, Morocco.

It was directed for Three-years academics from 2017-2018 through 2019-2020 by Professor **Filali-Maltouf Abdelkarim** and from 2020-2021 to 2022-2023 by Professor **BELKADI Bouchra** at Microbiology and Molecular Biology Laboratory, Faculty of Sciences, Rabat-Morocco. This work was co-directed by Dr. **EL MZIBRI Mohammed** and Dr. **ATTALEB Mohammed** at the Biology and Medical Research Unit of CNESTEN, Rabat-Morocco.

Acknowledgment

My research would be so much poorer without the assistance and guidance that I have received, directly or indirectly, from many people.

To start, I would also like to acknowledge my supervisory committee member, **Pr. FILALI-MALTOUF Abdelkarim.** I sincerely thank you for always encouraging me and pushing me throughout our research program. Many thanks for all your wonderful ideas, advice, and support through the years. Your coordination was decisive in the success of this project. and **Pr. BELKADI Bouchra** for accepting the role of my advisor in the two final years of my graduate program and providing every possible help for the smooth transition. Thank you for your foresight, guidance, understanding and for providing me with all the tools and resources needed for my thesis project.

Secondly, I would like to thank **Dr. ATTALEB Mohammed** for accepting me to contribute to this project as a student, for his patience and support during the course of my Ph.D. studies, and for all your advice which was not only important for my project but will be also crucial for my future research career.

I would like to express my gratitude to **Dr. EL MZIBRI Mohammed**, for providing me with a nice research environment in the laboratory; your helpful suggestions and critiques have enlightened me a lot in moving my research project forward. you always found the time to help me in my research. Your passion and enthusiasm for science are contagious and motivated me every step of the way.

My gratitude also goes to **Pr. BAKRI Youssef,** the jury president, your presence as the committee member was a great honor for us. Thank you for your dedicated guidance and suggestions.

I would like to thank **Pr. DAKKA Nadia** for evaluating my work and for serving on the committee member.

I would like to express my deepest gratitude to **Pr. BENNANI MECHITA Mohcine,** whose guidance, support and encouragement have been invaluable on the day of my PhD thesis defense. Many thanks, Professor, for the scientific discussion, your recommendations and your suggestions.

I would like to thank my examiner, **Pr. Mohamed OUKABLI,** for engaging with my thesis diligently and enthusiastically, and providing valuable suggestions.

I would also like to extend my gratitude to the thesis examiner, **Dr. Imane CHAOUI,** you were easily accessible and ready to assist throughout this project. Also, your suggestions brought in threads of thought that made my research so much richer and my dissertation something I can be proud of having written.

Many thanks to all people at the HMIMV who collaborated on this project, **Pr. Abderrahmane AL BOUZIDI**, **Pr. Ahmed AMEUR**, **Pr. Mohamed OUKABLI**, **Pr. Mounia BENSAID**, **Dr Mohammed TETOU,** and **Dr Ilyass HASSAN.**

I thank all of my labmates for being so generous with their time in helping me with my studies, thank you **Meryem EL AZZOUZI**, **Hajar EL AHANIDI,** and everyone in the laboratory, including **Kaoutar BOUOTHMANY**, **Younes EL FOUNINI,** and **Rajae BENZEID** you were very helpful to me.

Lastly and most importantly, I would like to express my deepest gratitude to **my parents** for their support and unconditional love. Thanks to **Salma, Hamza, Ali, Ahmed** and all my family members, from all corners of the world, for their love and support despite the distance.

Résumé

Le cancer de la vessie (CV) est l'un des cancers les plus répandus au Maroc, compte tenu de sa lourde prise en charge. Actuellement, de nouvelles méthodes pour prédire le risque de récidive et de progression sont développées, de nouvelles combinaisons de médicaments intra-vésicaux sont testées ainsi que des tests biomoléculaires non invasifs sont en cours d'étude pour optimiser les résultats et améliorer la gestion des patients marocains atteints de CV.

Dans ce cadre nos travaux de recherche ont ciblé trois parties principales. La première a été consacrée à l'évaluation de l'utilité des modèles de notations EORTC et CUETO pour estimer les risques de récidive et de progression chez les patients atteints de CV non infiltrant le muscle. Cette étude préliminaire a montré que les deux modèles peuvent stratifier les patients en quatre groupes de risque, néanmoins le modèle de notation CUETO reste le plus performant pour prédire la récidive dans notre population. Une étude rétrospective dans une large population marocaine multicentrique et pendant une période de suivi prolongée, serait nécessaire pour la validation externe de ces modèles de notation.

La deuxième partie a été axée sur l'évaluation de la performance diagnostique et pronostique d'un panel de biomarqueurs génétiques. Nous avons observé une fréquence importante de mutations dans le gène p53 (100%), suivi de hTERT (60%), tandis que les mutations au niveau des gènes PIK3CA et AKT1 ont été considérées comme des événements rares dans notre population. La fréquence des mutations au niveau de ces gènes est élevée, leur identification est aisée dans les sédiments urinaires et avec une spécificité élevée (100%) ainsi qu'une sensibilité qui varie entre 50% et 100%. Sur la base de ces propriétés, ces biomarqueurs pourraient être pris en considération pour les investigations génétiques.

Dans la troisième partie notre intérêt s'est focalisé sur l'évaluation du statut de méthylation des gènes hTERT, TWIST1, VIM et NID2. Nos résultats ont montré que l'hyper-méthylation des gènes étudiés (90% ; 85,71%; 67,14% et 67,14%, respectivement) est un évènement fréquent dans le CV. Une spécificité de 100% et une sensibilité de 82,8% à 97,1% ont été déterminées dans les échantillons urinaires. Ces caractéristiques permettraient d'utiliser la détermination de la méthylation au niveau des gènes cités comme outil non-invasif prometteur de diagnostic et une cible thérapeutique de choix pour le traitement du CV, le suivi ainsi que l'amélioration de la prise en charge des patients atteints de CV au Maroc.

Nos travaux mettent en valeur l'importance de l'évaluation des altérations génétiques et épigénétiques et leurs apports pour le diagnostic du CV. Ces approches peuvent enrichies par l'étude de l'expression d'autres gènes impliqués dans d'autres voies de signalisation afin de mieux comprendre les mécanismes impliqués dans la carcinogenèse de la vessie.

Mots-clés : Cancer de la vessie, Diagnostic, Biomarqueurs génétiques, épigénétiques, Test non-invasif, EORTC, CUETO.

Abstract

Bladder cancer (BC) is one of the most common cancers in Morocco due to its high dependency on management. Currently, new methods to predict the risk of recurrence and progression have been developed, new intra-vesical drug combinations have been tested, and non-invasive biomolecular tests have been investigated to optimize outcomes and improve the management of Moroccan patients with BC.

In this context, our research work focused on three main parts. The first part aimed to assess the usefulness of the EORTC and CUETO scoring models for estimating the risks of recurrence and progression in non-muscle-invasive BC patients. This preliminary study showed that both models can stratify patients into four risk groups. However, the CUETO scoring model remains the best-performing model for predicting recurrence in our population. A retrospective study in a large multicenter Moroccan population and during an extended follow-up period would be necessary for external validation of these scoring models.

The second part focused on evaluating the diagnostic and prognostic performance of a panel of genetic biomarkers. We observed a high frequency of mutations in the p53 gene (100%), followed by hTERT (60%), while mutations in the PIK3CA and AKT1 genes were considered rare events in our population. Identifying these gene mutations in urine liquid biopsy demonstrates a high specificity (100%) and a sensitivity that varies between 50% and 100%. Based on these properties, these biomarkers could be considered for genetic investigations.

In the third part, our interest focused on evaluating the methylation status of hTERT, TWIST1, VIM and NID2 genes. Our results revealed that hypermethylation of the studied genes (90%; 85.71%; 67.14%, and 67.14%, respectively) is a frequent event in BC. A specificity of 100% and a sensitivity ranging from 82.8% to 97.1% were found in urine samples. These results suggest using a methylation pattern of urinary DNA as a promising non-invasive diagnostic tool and a therapeutic target for the treatment of BC, follow-up and improvement of the management of BC patients in Morocco.

Our studies highlight the importance of assessing genetic and epigenetic alterations and their contribution to the diagnosis of BC. These approaches can be enriched by studying the expression of other genes involved in other signaling pathways to better understand the mechanisms involved in bladder carcinogenesis.

Key-words: Cancer de la vessie, Diagnostic, Biomarqueurs génétiques, épigénétiques, Test non-invasif, EORTC, CUETO.

Résumé détaillé

Le cancer de la vessie est l'un des cancers les plus courants dans le monde. Les hommes sont environ 3 à 4 fois plus susceptibles de développer un cancer de la vessie que les femmes. Les principaux facteurs de risque comprennent le tabagisme, l'exposition professionnelle à des produits chimiques tels que l'arsenic et les colorants azoïques, ainsi que des antécédents familiaux de cancer de la vessie. Les symptômes peuvent inclure des douleurs abdominales, des douleurs pendant la miction, du sang dans l'urine et une faiblesse générale. Le traitement dépendra de stade et de grade du cancer, mais peut inclure la chirurgie, la chimiothérapie, l'immunothérapie et la radiothérapie.

Selon les données de l'Organisation Mondiale de la Santé (OMS) publiées en 2021, le cancer de la vessie est classé en huitième position des cancers les plus fréquents au Maroc. Cette maladie représente un problème de santé publique en raison de sa prévalence élevée et sa lourdeur prise en charge. Environ 50% à 70% des patients atteints d'un cancer de la vessie non infiltrant le muscle présentent une récidive de la maladie, ce qui nécessite souvent une surveillance et des traitements supplémentaires à long terme. De plus, le cancer de la vessie de stade avancé peut souvent progresser rapidement et se propager à d'autres organes, ce qui peut rendre la gestion de ce cancer plus difficile et complexe.

La gestion du cancer de la vessie nécessite une classification des tumeurs non infiltrant le muscle en groupe de risque, ce qui permet de définir le traitement adéquat pour chaque groupe de risque. Il existe plusieurs outils de prédiction du risque de récidive et de progression du cancer de la vessie, peuvent aider les médecins à prendre des décisions quant à la surveillance et au traitement des patients atteints de cancer de la vessie. L'outil de prédiction EORTC (Organisation européenne pour la recherche et le traitement du cancer) est un modèle qui utilise plusieurs facteurs de risque pour prédire le risque de récidive et de progression du cancer de la vessie, notamment le stade et le grade histologique de la tumeur, la taille de la tumeur, la présence de carcinome *in situ*, le taux de récidive antérieure et le nombre de tumeurs. La principale limite du modèle de notation EORTC est son incapacité à prédire la récidive et la progression chez les patients traités par le BCG. Le modèle de notation CUETO (Club Urologico Espanol de Tratamiento Oncologico) a été spécifiquement créé et validé par le Club Urologico Espanol de Tratamiento Oncologico pour surmonter ce problème, il est basé sur sept facteurs de risque : l'âge, le genre, le stade et le grade histologique de la tumeur, la présence de carcinome *in situ*, le taux de récidive antérieure et le nombre de tumeurs.

La première partie de cette étude est consacrée pour évaluer l'utilité des systèmes de notation EORTC et CUETO à prédire les risques de récidive et de la progression chez les patients marocains atteints de TVNIM. Les résultats ont clairement montré que les deux modèles de prédiction EORTC et CUETO sous-estiment les probabilités de récidive et de progression à court terme. Les deux tables surestiment les taux de récidive et de progression à 5 ans. Nous avons également observé que seul le modèle CUETO a réussi à stratifier nos patients en quatre groupes de risque de récidive (p = 0,005).

Plusieurs biomarqueurs génétiques et épigénétiques ont été récemment incorporés dans les outils de prédiction EORTC et CUETO pour améliorer la valeur prédictive de ces modèles pour les patients atteints de TVNIM. En effet, ces dernières années, Des efforts considérables ont été déployés pour identifier des biomarqueurs prédictifs et diagnostiques dans les stades précoces et avancés du cancer de la vessie afin de surmonter les limites de la cystoscopie (méthode invasive) et de la cytologie urinaire (une sensibilité limitée pour détecter les tumeurs de petite taille) qui sont les deux méthodes couramment utilisées pour le dépistage et la surveillance du cancer de la vessie. Des tests moléculaires basés sur l'analyse de l'ADN et de l'ARN peuvent être utilisés pour détecter les altérations génétiques et épigénétiques dans les cellules de la vessie, pour ensuite les introduire dans le diagnostic et le suivi afin d'améliorer la prise en charge de CV au Maroc.

Dans ce contexte, nous avons étudié le statut mutationnel des gènes *TERT*, *Tp53*, et *PIK3CA/AKT1* et le statut de méthylation des gènes *TWIST1, hTERT, VIM* et *NID2*. Ces biomarqueurs génétiques et épigénétiques pourraient améliorer de façon non invasive la détection et pourraient être intéressants comme biomarqueurs prédictifs de réponse aux thérapies ciblées dans la prise en charge du CV.

Nous avons utilisé une cohorte de 70 patients suivis pour un cancer de la vessie dans le service d'urologie de l'hôpital militaire Mohammed V, Rabat - Maroc, au cours de 2017-2019. 68 patients recrutés étaient des hommes et 2 sont des femmes, avec une sex-ratio de 34. L'âge moyen des patients était de 67 ans, allant de 47 à 85 ans. 28 patients (40%) ont été déclarés comme fumeurs actifs ou anciens fumeurs. La plupart des cas avaient un stade ≤ PT1 (74,29 %) et présentaient un grade tumoral élevé (61,43 %) au moment de diagnostic. Parmi les 56 patients présentant un type de TVNIM, 12 ont connu une récidive tumorale (23,08 %) et 5 (10 %) ont été diagnostiqués avec une progression au cours des 4 années de suivi.

Dans la deuxième partie de cette thèse, nous avons effectué une analyse du statut mutationnel de certains biomarqueurs génétiques qui sont impliqués dans la prolifération et l'invasion

tumorale. L'analyse du séquençage du gène *Tp53* a révélé des fréquences très élevées de mutations avec une prévalence élevée de 100%, et au moins une mutation a été détectée chez chaque patient, suivie par le promoteur du gène *hTERT* (60%) et le gène *PIK3CA* (12,6%). Une seule mutation pathogène E17K dans l'exon 1 du gène *AKT* a été détectée. Dans notre étude, les mutations du gène *Tp53* semblent être corrélées au grade de la tumeur, tandis qu'aucune corrélation entre les gènes *hTERT* et *PIK3CA/AKT1* et les paramètres clinico-pathologiques n'a été démontrée dans notre cohorte, cela peut être dû à la petite taille de l'échantillon.

Afin d'évaluer l'utilité du sédiment urinaire pour détecter les altérations génétiques en tant que matrice non invasif pour le diagnostic et le suivi du cancer de la vessie, nous avons exploré les cellules exfoliées présentes dans l'urine pour rechercher des mutations dans les gènes *PIK3CA, AKT1* et *TERT*. Pour ces trois gènes, nos résultats révèlent une sensibilité qui varie entre 50% et 100% et une spécificité de 100%.

Outre les marqueurs génétiques, un grand nombre des biomarqueurs épigénétiques sont proposés en permanence, et les technologies utilisées pour analyser ces biomarqueurs épigénétiques prometteurs s'améliorent et deviennent plus faciles à utiliser dans la pratique clinique. Récemment, il a été signalé que l'hyper-méthylation du promoteur des gènes *TWIST, VIM, NID2* et *hTERT* est impliquée dans la régulation de l'EMT en tant que marqueur mésenchymateux important dans divers types de tumeurs, notamment le cancer de la vessie. Cela suggère la possibilité de les utiliser comme une cible thérapeutique prometteuse pour ce cancer, en particulier dans les stades avancés.

Dans la troisième partie de cette étude, notre objectif était d'étudier le profil de méthylation des sites CpG dans les promoteurs de quatre gènes candidats en utilisant les biopsies et les sédiments urinaires de 70 patients marocains atteints de cancer de la vessie en utilisant la méthode PCR spécifique de la méthylation. Nous avons également évalué l'impact pronostique de ces marqueurs dans le CV.

Nos résultats indiquent que les fréquences de méthylation des gènes *hTERT, TWIST1, VIM* et *NID2* étaient respectivement de 90%, 85,71%, 67,14% et 67,14%. L'analyse statistique a montré une association significative entre l'hyper-méthylation du gène *TWIST1* et la récurrence du CV (p = 0,041). Nous avons analysé les échantillons d'urine appariés provenant de la même cohorte afin de définir la sensibilité et la spécificité de ces biomarqueurs qui peuvent être utiliser comme méthode de détection non invasive. Nous avons trouvé une sensibilité et une spécificité élevées pour détecter l'hyper-méthylation des gènes *TWIST1, hTERT, NID2 et VIM,* qui atteignent 91,7 % ; 97,1 % ; 84 % et 82,8 %, respectivement.

Publication List

a. Publications

Published Original Articles:

1. **Chaimae Hafidi Alaoui**, Mohammed Tetou, Ilias Hassan, Meryem El Azzouzi, Hajar El Ahanidi, Abdelkarim Filali-Maltouf, Imane Chaoui, Mounia Bensaid, Laila Benbacer, Mohamed Oukabli, Abderrahmane Al Bouzidi, Mohammed Attaleb, Mohammed El Mzibri, Ahmed Ameur, Utility of EORTC and CUETO scoring models in the estimation of recurrence and progression of non-muscle-invasive bladder cancer in Moroccan patients, Eurasian Journal of Medicine and Oncology, 2022; 6(2): 121-129. DOI: 10.14744/ejmo.2022.99203.
2. **Hafidi Alaoui, C**.; El Ahanidi, H.; El Azzouzi, M.; Filali-Maltouf, A.; Chaoui, I.; Bensaid, M.; Benbacer, L.; Tetou, M.; Hassan, I.; Oukabli, M.; Ameur, A.; Al Bouzidi, A.; Attaleb, M.; El Mzibri, M. Evaluation of TERT Promoter Mutations in Tumor Biopsies and Urine Sediment of Moroccan Bladder Cancer Patients. *Ann. Cancer Res. Therap.* 2022, *30* (1),1–7. https://doi.org/10.4993/acrt.30.1.
3. **Hafidi Alaoui, C**.; El Ahanidi, H.; El Azzouzi, M.; Belkadi Bouchra; Chaoui, I.; Bensaid, M.; Benbacer, L.; Tetou, M.; Hassan, I.; Oukabli, M.; Ameur, A.; Al Bouzidi, A.; Attaleb, M.; El Mzibri, Assessment of p53 mutations and prognosis in bladder cancer patients from Morocco. Submitted in Cancer Genetics, oct 2022.
4. El Ahanidi, H.; El Azzouzi, M.; Arrouchi, H.; **Hafidi Alaoui, C.;** Tetou, M.; Bensaid, M.; Oukabli, M.; Ameur, A.; Al Bouzidi, A.; El Mzibri, M.; Attaleb, M. AKT1 and PIK3CA Activating Mutations in Moroccan Bladder Cancer Patients' Biopsies and Matched Urine. *PAMJ* 2022, *Volume 41* (Article 59). https://doi.org/10.11604/pamj.2022.41.59.31383.
5. El Ahanidi, H.; El Azzouzi, M.; **Hafidi Alaoui, C.;** Tetou, M.; Bensaid, M.; Chaoui, I.; Benbacer, L.; Hassan, I.; Oukabli, M.; Michaud, K.; Ameur, A.; Al Bouzidi, A.; El Mzibri, M.; Jandus, C.; Attaleb, M. Immune Checkpoint and Telomerase Crosstalk Is Mediated by MiRNA-138 in Bladder Cancer. *Front. Oncol.* 2022, *11*, 795242. https://doi.org/10.3389/fonc.2021.795242.
6. El Azzouzi, M.; El Ahanidi, H.; **Hafidi Alaoui, C**.; Chaoui, I.; Benbacer, L.; Tetou, M.; Hassan, I.; Bensaid, M.; Oukabli, M.; Ameur, A.; Al Bouzidi, A.; El Mzibri, M.; Attaleb, M. Evaluation of DNA Methylation in Promoter Regions of HTERT, TWIST1, VIM and NID2 Genes in Moroccan Bladder Cancer Patients. *Cancer Genetics* 2022, *260–261*, 41–45. https://doi.org/10.1016/j.cancergen.2021.12.001.147.
7. El azzouzi, M., El ahanidi, H., **Hafidi Alaoui, C.** *et al.* Exploring urine sediments as a non-invasive method for DNA methylation detection in bladder cancer. *Afr J Urol* 28, 31 (2022). https://doi.org/10.1186/s12301-022-00298-3.

Published Reviews:

1. El Ahanidi, H.; **Hafidi Alaoui, C**.; Bensaid, M.; El Mzibri, M.; Ameur, A.; Abbar, M.; Al Bouzidi, A.; Mohammed Attaleb, M. A. Assessment Of Molecular Biomarkers For Bladder Cancer Diagnosis, Grading And Prognosis. *Frontiers in Science and Engineering* 2019, *Vol 8*, Supplment 2 (2019). https://doi.org/10.34874/IMIST.PRSM/FSEjournal-V8I2.28049.

b. Communications

1. Selected Speaker at "First International Congress of the Moroccan Society of Genomics and Human Genetics & the Third edition of the School of Immunogenetics" (19-21 December 2019).

Talk: "Molecular analysis of a panel of genes in bladder cancer cases in Morocco".

2. Selected Poster at "Summer School, Human Genome Variation: Applications In Human Medicine And Genetic Identification" (11-13 July 2019).

Poster: "Characterization of multiple somatic mutations in a panel of genes commonly mutated in bladder cancer patients".

3. Selected Poster at " 1ère Journée Scientifique de Biosciences et Biotechnologies". (28 Mai 2022)

Poster: "Assessment of p53 mutations and prognosis in bladder cancer patients from Morocco".

List of Figures

List of Tables

List of Abbreviations

AJCC	American Joint Committee on Cancer
ADC	Adenocarcinoma
AKT1	Serine-Threonine Protein Kinase
AP	Apurinic/Apyrimidinic
APC	Antigen-Presenting Cells
APE1	AP Endonuclease humane
ASCO	American Society of Clinical Oncology
ASTRO	American Society for Radiation Oncology
ATR	Ataxia telangiectasia and Rad3 related
ATM	Ataxia telangiectasia mutated
AUA	American Urological Association
BAX	BCL2 Associated X, Apoptosis Regulator
BC	Bladder Cancer
Bcl-2	B-Cell Leukemia/Lymphoma 2
BCG	Bacillus Calmette Guérin
BER	Base Excision Repair
bHLH	Basic Helix-Loop-Helix
BTA	Bladder Tumor Antigen
CANNTG	Calcitonin-negative tumor cells to the neuroendocrine-specific
C*cAFU*	Cancer Committee of the French Association of Urology
CCND1	Cyclin D1
CCNE	Cyclin E1
Cdc2	Cell division control 2
Cdc25	Cell division control 25
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
CdK2	Cyclin-dependent Kinase 2
CHEK2	Checkpoint Kinase 2
CIS	Carcinoma In Situ
CG	Cisplatin and Gemcitabine
c-Myc	Cellular Myelocytomatosis oncogene
CpG	Cytosine–phosphate–Guanine
CT	Chemotherapy
CTCF	CCCTC-Binding Factor
CTE	C-Terminal Extension
MMC	Mitomycin C
MMR	Mismatch Repair
MOMP	Major Outer Membrane Protein
MRE11	Meiotic Recombination 11 homolog A
MRI	Magnetic Resonance Imaging
MSH2	MutS Homolog 2
MSP	Methylation-specific PCR
mTOR	Mechanistic Target of Rapamycin
mUC	Metastatic Urothelial Carcinoma
MVAC	Methotrexate, Vinblastine, Adriamycin and Cisplatin
Myod1	Myogenic Differentiation 1
MZF2	Myeloid specific Zinc Finger protein 2
NER	Nucleotide Excision Repair
NFκB	Nuclear Factor kappa B
NHEJ	Non- Homologous End Joining
Nbs1	Nibrin1
NID2	Nidogen 2
NMIBC	Non-Muscle Invasive Bladder Cancer
NMP22	Nuclear Matrix Protein 22
NPC	Nasopharyngeal Cancer
PCNA	Proliferating Cell Nuclear Antigen
PD-1	Programmed Cell Death 1
PD-L1	Programmed Cell Death-Ligand 1
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-Kinase
PI3KR	PhosphoInositide-3-kinase Regulatory
PIP2	PhosPhatidylInositol (3,4)-bisphosphate
PH	Protein Kinase B
PLCG1	Phospholipase C Gamma 1
PMAIP1	Phorbol-12-Myristate-13-Acetate-Induced Protein 1
POT1	Protection of Telomere 1
POU4F2	POU Class 4 Homeobox 2
PTEN	Cytokeratin Phosphatase and TENsin homolog.
PUMA	P53 upregulated modulator of apoptosis

CTLA-4	Cytotoxic T- Lymphocyte Associated protein 4
CUETO	Club Urologico Espanol de Tratamiento Oncologico
Cytc	Cytochrome c
DBD	DNA-Binding Domain
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
DSBs	Double-Strand Breaks
E2F3	E2F Transcription Factor 3
EAU	European Association of Urology
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
EORTC	European Organisation for Research and Treatment of Cancer
ER	Estrogen Receptor
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ESCC	Esophageal Squamous Cell Carcinoma
ETS	E-Twenty Six
FAK	Focal Adhesion Kinase
FDA	Food and Drug Administration
FGFR3	Fibroblast growth factor receptor 3
GABP	GA-Ginding Protein
GADD45	Growth Arrest and DNA-Damage-inducible protein-45
GDF15	Growth Differentiation Factor 15
HAND1	Heart And Neural Crest Derivatives Expressed 1
HDAC	Histone Deacetylase
HIF1A	Hypoxia-Inducible Factor 1 subunit Alpha
HG	High Grade
HR	Homologous Recombination
HRAS	Harvey Rat Sarcoma viral oncogene homolog
HRE	Hypoxia-Response Element
Hsp90	Heat shock protein 90
hTERT	Human Telomerase Reverse Transcriptase
IARC	International Agency for Research on Cancer
ICI	Immune Checkpoint Inhibitors
IL 2	Interleukin 2
IPD	Individual Participant Data
ISUP	International Society of Urological Pathology
PUNLMP	Papillary Urothelial Neoplasia of Low Malignant Potential
RAD50	Double Strand Break Repair Protein
RAP1	Repressor Activator Protein 1
RB1	Retinoblastoma 1
Ref1	RNA and export factor binding protein 1
RHEB	Ras homolog enriched in brain
RPS6	Ribosomal protein S6
RT	Radiotherapy
RT	Reverse-Transcriptase
S6K1	Ribosomal S6 kinase 1
SAM	S-Adenyl Methionine
SAH	S-AdenosylHomocysteine
SCC	Squamous Cell Carcinoma
SIP1	SMN-Interacting Protein 1
SP1	Sphingosine-1-Phosphate
STAT	Signal Transducer and Activator of Transcription
SUO	Society of Urologic Oncology
TACC3	Transforming Acidic Coiled-Coil Containing Protein 3
TAD	Tandem Activation Domains
TNF	Tumor Necrosis Factor
TNM	Tumour, Node, Metastasis
TCC	Transitional Cell Carcinoma
TCGA	The Cancer Genome Atlas
TEN	Essential N-terminal
TERC	Human Telomerase RNA component
THOR	TERT Hypermethylated Oncological Region
TIN2	TRF1-Interacting Nuclear Factor 2
TKI	Tyrosine Kinase Inhibitor
TP53	Tumor Protein 53
TP63	Tumor Protein P63
TPP1	TIN2 and POT1 interacting Protein
TRBD	TERT RNA-binding domain
TRF1	Telomeric Repeat-Binding Factor 1
TRF2	Telomeric Repeat-Binding Factor 2
TURBT	Transurethral Resection of the Bladder Tumor
TWIST	Twist Family BHLH Transcription Factor 1
TY	Thyroglobulin type-I

KRT4	Keratin 4
KRT5	Keratin 5
LDLR	Low-Density Lipoprotein Receptor
LG	Low Grade
Mad1	MAX dimerization protein 1
MAPK	Mitogene-Activated Protein Kinase
MAX	MYC Associated Factor X
MDMia2	Mouse Double Minute 2
MEF2	Myocyte Enhancer Factor 2
MEN1	Multiple Endocrine Neoplasia type 1
MIBC	Muscle-Invasive Bladder Cancer
UC	Urothelial Carcinomas
UGS	Urogenital Schistosomiasis
VIM	Vimentin
WAF1	Wild-type Activating Fragment-1
WHO	World Health Organisation
WT1	Wilms' Tumor 1
XPE	Xeroderma Pigmentosum complementation group E
XRCC4	X-Ray Repair Cross Complementing 4
YWTD	LY. Low-density lipoprotein-receptor
ZNF154	Zinc Finger Protein 154

Summary

General introduction

Bladder cancer (BC) is considered the tenth most common cancer worldwide, and its incidence is steadily rising particularly in developed nations. More than half a million were diagnosed with BC worldwide, with a very high incidence in southern Europe and a higher mortality rate in North Africa (GLOBOCAN, 2020).

The most common type of BC is urothelial carcinoma (UC), and in this subtype, non-muscle invasive bladder cancer (NMIBC) is the most common form (Babjuk et al., 2022). This form is characterized by alterations in several molecular and pathological pathways. The genomic heterogeneity and dynamic nature of NMIBC lead to BC progression and invasion as well as frequent recurrences that can occur during therapy, making the clinical management of this BC type difficult (Têtu, 2009).

Management of BC involves risk stratification of NMIBC into risk groups, which enables the identification of the right level of care for distinct risk groups of patients. In addition, the use of risk prediction tools for recurrence and progression is recommended to enhance population health management and improve overall health outcomes (Sylvester et al., 2021). For this purpose, EORTC and CUETO scoring models have been developed for predicting recurrence and progression based on clinicopathological factors. The validation of these tools has been the focus of several groups to evaluate their usefulness in clinical practice (Sylvester et al., 2006; Fernandez-Gomez et al., 2009).

In Morocco, the stratification of patients into risk groups of recurrence and progression is done following the updated Cancer Committee of the French Association of Urology (ccAFU) guidelines (Rouprêt et al., 2020). None of the EORTC and CUETO scoring models are accepted and used in clinical practice because of the overestimation of the risk of recurrence and progression. To the best of our knowledge, no study has assessed the utility of these tools in the Moroccan population. Thus, it is crucial to evaluate the usefulness of these scoring models before introducing them into clinical practice.

Cystoscopy and urine cytology remain important methods used for diagnosing and surveillance of bladder cancer (Raitanen et al., 2001). To complement these gold standard tools in BC management , alternative tests based on the molecular approach have been developed recently, and several markers corresponding to molecular alterations have been proposed and which can

be integrated into prediction models EORTC and CUETO. These molecular abnormalities, specific to tumor cells, can result from an alteration in the nucleotide sequence (mutations: Insertions/deletions) or epigenetic mechanisms, such as acetylation, methylation or demethylation of regulatory gene sequences. However, these assays have not been used as routine clinical tests due to their relatively low sensitivities and/or specificities (Têtu, 2009).

Mutation profiling studies in bladder tumors have identified somatic genetic alterations that activate oncogenes or proto-oncogenes such as *FGFR3, HER2, CCND1, C-myc, FGF family, PIK3CA, AKT1* or inactivate tumor suppressor genes such as *p53, p21, pRB, p73, PTEN* that are involved in the modulation of neoplastic cell transformation. In addition to genetic changes, hypermethylation in the CpG islands of several gene promoters is a major event involved in the carcinogenesis process of several cancers, including BC , that show overexpression of oncogenes and the possible subsequent silencing of tumor suppressor genes (Hong-Tao et al., 2016).

Furthermore, multiple genetic and epigenetic modulations of genes activities occur simultaneously or are mutually influenced by each other. Many genetic mutations disrupt the functions of genes involved in epigenetic regulation; on the contrary, epigenetic aberrations lead to alterations in transcription (Hong-Tao et al., 2016). Therefore, understanding the genetic and epigenetic alterations in BCis highly important for developing accurate and practical methods for Research in this discipline aims to establish panels of genes that can be used as diagnostic and prognostic biomarkers and for the identification of novel therapeutic targets for more streamlined management of patients with BC (Califf, 2018).

In this context, the purposes of the present study are:

- To compare the recurrence and progression rates with the scores obtained from the EORTC and CUETO scoring systems to speculate on the utility of these tools.
- To characterize the genetic and epigenetic anomalies of some candidate genes in biopsies and exfoliated cells in urine to develop new biomarkers for the diagnosis, prognosis and therapeutic management of bladder cancer in Morocco.

I. Background

I.1 Function, Anatomy and Histology of bladder

The bladder is an integral part of the urinary system and an elastic sac serving as a temporary reservoir for urine and allowing urination to be infrequent and controlled. It forms a part of the pelvic organs and is located just posterior to the pubic symphysis. In adulthood, the average volume that a normal bladder can store is between 300 and 400 mL (Lukacz et al., 2011). Aging changes bladder capacity, the bladder wall becomes less stretchy, and the organ cannot carry as much urine as before (Siroky, 2004).

Anatomically, the bladder is connected to the ureters above; these tubular structures connect the kidneys to the urinary bladder. The urethra is situated at the bottom of the bladder that carries urine to the outside. It is divided into four anatomical sections: the dome is directed forward towards the anterosuperior part of the bladder. The fundus is the base part of the bladder. The body is the main part of the bladder located between the apex and the fundus. The bladder neck is a group of muscles that connects the bladder to the urethra (Shermadou et al., 2022).

Histologically, the urinary bladder wall is formed of four distinct layers. From the inside to the outside, they are: Lining epithelium, the inside of the bladder is lined by a layer of cells called urothelial cells. The urothelium comprises an apical layer, an intermediate layer, and a basal layer. Then, a suburothelial layer of connective tissue also known as the basement membrane called the chorion or laminae propria which separates the urothelium and muscularis propria (detrusor muscle). It is composed of an extracellular matrix with capillaries, elastic fibers, immune cells, lymphatics, afferent nerve endings, myofibroblasts, fibroblasts, adipocytes, an indistinct smooth muscle layer and the muscularis mucosae. The bladder's third wall comprises smooth muscle cells called detrusors covering the chorion, also known as the muscularis propria. It consists of three layers arranged in a longitudinal, circular and spiral. These layers are well defined around the neck of the urinary bladder and are mainly used to facilitate micturition. Finally, a thin and loose connective tissue layer covers the outside of the bladder; it also contains variably sized blood vessels that may be close to variable adipose, epithelium and lymphatics tissue (Bolla et al., 2022).

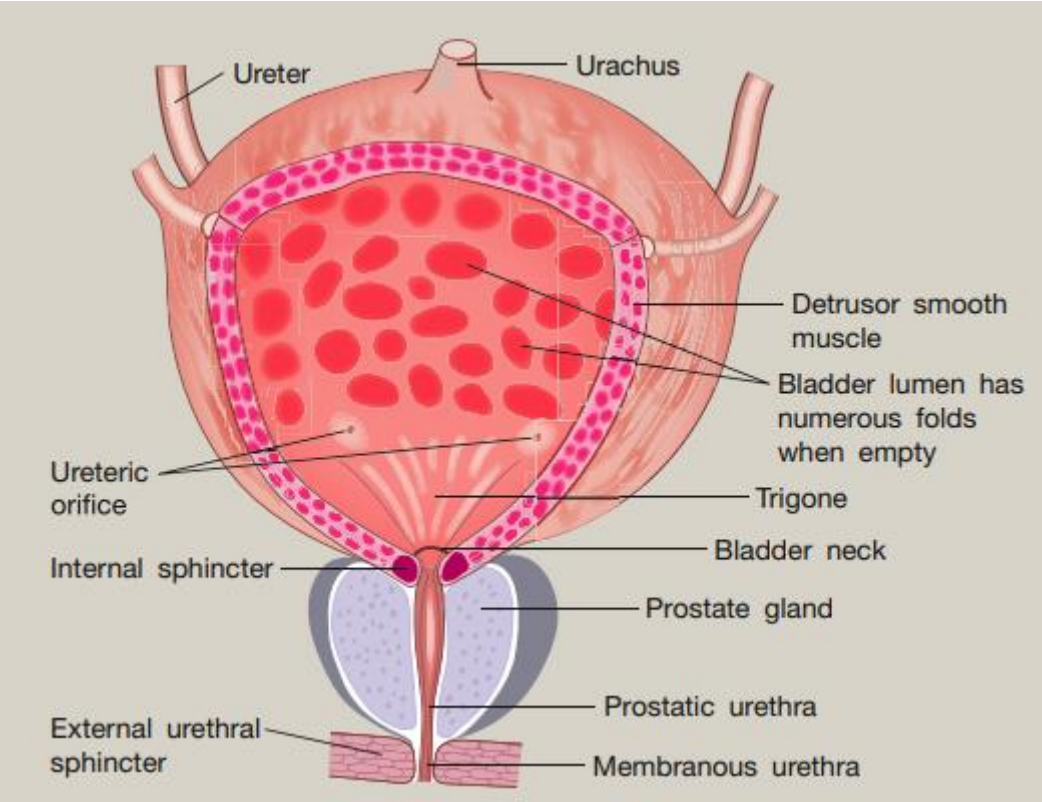

Figure 1: Human anatomy of the bladder (Alleemudder et al, 2016)

I.2 Bladder diseases

Various factors may influence bladder function, and disorders of the urinary bladder may cause lower urinary tract symptoms, such as poor and intermittent stream, prolonged micturition, the components of the overactive bladder syndrome, and feeling of incomplete bladder emptying… (Barry & O'Leary, 1995). These symptoms are more common in the elderly, and women are at higher risk of urinary tract infections than men due to their shorter urethras:

- Cystitis is a bladder inflammation caused by a bacterial infection of the urinary bladder. *Escherichia coli* is the common agent which generates approximately 75-95% of uncomplicated urinary tract infections in women (Raymund & Leslie, 2022).
- Urine retention is the inability to urinate normally or completely. The origin of retention is different by gender and can be an obstruction of the urethra, a benign prostatic enlargement in men, or secondary to non-obstructive causes (Brasure et al., 2014).
- Urinary incontinence is defined as any involuntary loss (or leakage) of urine. This disease is prevalent in the elderly and affects their quality of life, but younger adults can also be affected (Tran & Puckett, 2022).

- Overactive bladder is a chronic disease characterized by urinary urgency due to involuntarily contracts of the detrusor and may affect both women and men (Wallace & Drake, 2015).
- Hematuria is characterized by the presence of blood in the urine; this can be caused by an infection or bladder cancer (Saleem & Hamawy, 2022).
- Bladder cancer is the most prevalent neoplasm of the urinary tract. It is usually diagnosed by the presence of the blood in the urine. Chemical exposures and smoking are the main risk factors for BC (Lenis et al., 2020).

II. Bladder cancer

II.1 The natural history of bladder cancer

Intraepithelial neoplasia is the initial step that leads to a non-invasive lesion in the development of bladder cancer. This non-invasive lesion may result from genotoxic carcinogens or from the occurrence of spontaneous genetic changes that lead to genetic instability and carcinogenesis. The proliferation of transformed cells may promote the growth of transitional cells, representing more than 90% of bladder cancer cases. 80% of patients with this histological type have tumors confined to the mucosa or submucosa initially, called non-muscle invasive bladder cancer (NMIBC). This localization is probably dependent on their biochemical capabilities (Friedell et al., 1980).

The proliferation of neoplastic cells progresses with the formation of several layers of cancer cells forming papillary surrounding a central fibrovascular core. The generated papillary tumors are generally of low and moderate grade. These tumors remain locally confined to the epithelial layers and express little propensity to become progressive (<5%). The proliferation of cells continues and produces a thickened epithelial layer that extends along the plane of the bladder wall, maintaining the flattened contour of the urothelial lining. This "flat" Carcinoma *in situ* (CIS) generally comprises higher-grade cells than those found in papillary and mucosally confined tumors. High-grade superficial papillary tumors and high-grade muscle-invasive cancers are often associated with this form of CIS. Most papillary tumors may penetrate the basement membrane and infiltrate the lamina propria (Lee & Droller, 2000).

The natural history of superficial bladder tumors is difficult to predict, because it represents an extremely heterogeneous group of tumors that include:

- Ta (Papillary in nature and limited to the mucosa) which is associated with a high rate of recurrence (50 to 75%), but with low risk of progression
- Tis (high grade and flat and confined to the epithelium)
- T1 (Invasive into the submucosa or lamina propria)

Tis and T1 forms represent a higher likelihood of progression (30% to 50%) (Lee & Droller, 2000).

Invasive tumors are already present in approximately 15-20% of patients with newly diagnosed Transitional cell carcinoma (TCC). The vast majority of these cancers (84–90%) are high-grade malignant carcinomas and are frequently associated with carcinoma *in situ* lesions. Although about 10% of patients with low- and moderate-grade superficial tumors and 25–45% with high-grade superficial one's progress to muscle-invasive disease. The tendency of invasive TCC to be present at the initial tumor diagnosis is especially disturbing since nearly half of all patients with the muscle-invasive disease have concomitant occult distant metastases. Many of these patients eventually experience systematic progression. Consequently, the ultimate prognosis is worse (Foresman & Messing, 1997).

II.2 Bladder cancer's risk factors

A Risk factor is any characteristic, attribute or condition that may increase the chance of developing a disease. BCcan occur in anyone, and its exact cause is not known at the time. Still, some major risk factors related to this disease include tobacco smoking, demographic, environmental and occupational exposure, inflammation due to parasitic infection, hereditary and genetic susceptibility (Zhang & Zhang, 2015).

- **Smoking**

The most notable risk factor for developing BC is tobacco smoking, representing approximately 50-65% of cases in men and 20-30% of cases in women (Freedman, 2011). Previous observations reported that cigarette is strongly associated with lung cancer and is the most significant cause of cancer incidence and deaths worldwide (Thun et al., 1997). Statistically, cigarette smokers are twice likely to develop BC compared to non-smokers; a dose-response relationship was reported between smoking intensity, smoking duration and BC disease risk (Zhao et al., 2022).

Tobacco contains more than 7000 chemicals, including at least 70 carcinogens, especially beta-naphthylamine and polycyclic aromatic hydrocarbons. These substances promote inflammation

of the bladder and other organs at risk, causing DNA-adduct formation and permanent genetic mutations. These genetic alterations may activate oncogenes and suppress tumor suppressor genes, thus promoting carcinogenesis (Centers for Disease Control and Prevention (US) et al., 2010).

- **Environmental and Occupational Exposure**

The second important preventable risk factor is occupational exposure to carcinogens, particularly polycyclic aromatic hydrocarbons, aromatic amines and chlorinated hydrocarbons (Zeegers, 2001). Indeed, workers in the industrial production of paint, dyes, metals, rubber or petroleum products are the most exposed to the risk of developing BC (Burger et al., 2013). Exposure to aromatic amines such as naphthalen-2-amine, benzidine and 4-aminobiphenyl and polycyclic aromatic hydrocarbons, which are frequently used in some industries, may also increase the risk of BC (Zeegers et al, 2001).

- **Demographic**

Around the world, BC is diagnosed four times more frequently in men than in women, with a mortality rate three times higher in men (GLOBOCAN, 2020). This variation in incidence rates may be related to higher cigarette consumption among men than women (Freedman, 2011; Zhao et al., 2022). In some countries, the incidence of BC is higher among women. In Lebanon, for example, women are more likely to smoke than men (Bray et al., 2018).

Bladder cancer affects mainly people older than 60 years, with an average age of 65-70 years. This indicates that disease evolution and progression require decades after exposure to carcinogenic agents. Although, low grade and non-invasive BCs can be diagnosed in young adults and children (Linn et al., 1998).

- **Infectious factor**

Urogenital schistosomiasis (UGS) is a *Schistosoma haematobium* infection that commonly occurs in Sub-Saharan Africa and the Middle East (Rambau et al., 2013). A link between UGS and bladder cancer was first published in 1905 by Goebel (Goebel, 1905). before being further clarified in 1911 by Ferguson (Ferguson, 1911). This parasite affects the mucosa and submucosa layers of the bladder, causing a chronic granulomatous inflammation caused by producing schistosome eggs in the bladder wall, which may lead to bladder fibrosis that promotes the development of squamous cell carcinoma (SCC) of the bladder (Rambau et al., 2013).

- **Hereditary and Genetic Factors**

Bladder cancer is an excellent disease model to investigate genetic susceptibility and gene-environment interaction contributing to cancer etiology (Gu & Wu, 2011). It was previously reported that hereditary non-polyposis colorectal cancer could significantly increase a person's chance of developing bladder cancer (Lenz & Harpster, 2003). Another study reported a direct association between bladder cancer risk and a family history of bladder cancer (Turati et al, 2017).

In addition, genetic changes in common candidate genes or pathways that cause activation of oncogenes and inactivation of tumor suppressor genes have been widely studied. They may lead to the development and progression of bladder tumors (Sitong et al., 2021). Activation of oncogenes, such as Fibroblast Growth Factor Receptor 3 (*FGFR3),* Retinoblastoma 1 *(RB1),* Harvey Rat Sarcoma viral oncogene homolog *(HRAS),* Tumor Protein 53 *(TP53),* Telomerase Reverse Transcriptase *(TERT)* and others, by point mutations, represents an excellent diagnostic and/or prognostic biomarker of BC. Considering that, in normal cells, these genes play a key role in the control of cell division (Kompier et al., 2010).

- **Epigenetics alterations**

The molecular factors change depending on the disease's stage, and DNA modification via methylation and acetylation also plays a crucial role in the pathogenesis of BC (Porten, 2018).

These epigenetic mechanisms may be examined in various stages and grades of BC. High-grade invasive tumors showed a higher intensity of methylation than low- and intermediate-grade tumors (Kitchen et al, 2016). Environmental exposures may induce epigenetic variations in DNA sequence.

Chromatin and histone modifications have been recognized as an interesting therapeutic target in many cancer types, including bladder cancer (Porten, 2018). Indeed, inhibition of histone deacetylase (HDAC) and DNA methyltransferase (DNMT) enzymes can restore the expression of tumor suppressor genes. Combining epigenetic drugs with chemotherapy has moved into clinical trials and revealed that HDAC and DNMT inhibitors, alone or in association with chemotherapy, can have synergistic effects in inhibiting cell division and inducing tumor cell apoptosis in bladder cancer cell lines (Shang et al., 2008; Faleiro et al., 2017).

II.3 Tumor staging

Urothelial carcinoma (UC) is among the most frequent histologic type involving the urinary system. However, about 75% of these patients are diagnosed with NMIBC and have a 5-year survival of over 80%. While the rest are diagnosed as MIBC with a 5-year survival of under 50%, this type of cancer spreads into the bladder's detrusor muscle (Sweeney et al., 1992; Rizzo et al., 2021) Therapeutic decisions and prognosis are oriented according to the Tumour, Node, Metastasis (TNM) classification system developed and maintained by the American Joint Committee on Cancer (AJCC); this classification concerns only the urothelial carcinomas (Table 1):

- T tells how far the main (primary) tumor has grown through the bladder wall and nearby tissues.
- N indicates the evidence of metastases and refers to the number of nearby lymph nodes. Lymph nodes are bean-sized collections of lymphocytes and other immune system cells, to which cancers often spread first.
- M designates if cancer has spread (metastasized) to distant sites, such as other organs, including the lungs or liver, or lymph nodes that are not near the bladder (Kaseb & Aeddula, 2022).

Then a number, that indicates the gravity of cancer, is added to each category. A higher number means that cancer is spreading further. Most bladder cancers develop in the urothelium and grow in the different bladder layers and become more advanced.

Table 1: Tumor, Node, Metastasis (TNM) classification system for bladder cancer (Arianayagam et al., 2011)

Stage	Stage grouping	Stage description
0a	**Ta N0 M0**	The cancer is a non-invasive papillary carcinoma (Ta). It has grown toward the hollow center of the bladder but has not grown into the connective tissue or muscle of the bladder wall. It has not spread to nearby lymph nodes (N0) or distant sites (M0).
0is	**Tis N0 M0**	The cancer is a flat, non-invasive carcinoma (Tis), also known as *flat carcinoma in situ (CIS)*. The cancer is growing in the inner lining layer of the bladder only. It has not grown inward toward the hollow part of the bladder, nor has it invaded the connective tissue or muscle of the bladder wall. It has not spread to nearby lymph nodes (N0) or distant sites (M0).
I	**T1 N0 M0**	The cancer has grown into the layer of connective tissue under the lining layer of the bladder, but has not reached the layer of muscle in the bladder wall (T1).

		The cancer has not spread to nearby lymph nodes (N0) or to distant sites (M0).
II	**T2a or T2b N0 M0**	The cancer has grown into the inner (T2a) or outer (T2b) muscle layer of the bladder wall, but it has not passed completely through the muscle to reach the layer of fatty tissue that surrounds the bladder. The cancer has not spread to nearby lymph nodes (N0) or to distant sites (M0).
IIIA	**T3a, T3b or T4a N0 M0**	The cancer has grown through the muscle layer of the bladder and into the layer of fatty tissue that surrounds the bladder (T3a or T3b). It might have spread into the prostate, seminal vesicles, uterus, or vagina, but it's not growing into the pelvic or abdominal wall (T4a). The cancer has not spread to nearby lymph nodes (N0) or to distant sites (M0).
	OR	
	T1-4a N1 M0	The cancer has grown: • into the layer of connective tissue under the lining of the bladder wall (T1), OR • into the muscle layer of the bladder wall (T2), OR • into the layer of fatty tissue that surrounds the bladder, (T3a or T3b) OR • it might have spread into the prostate, seminal vesicles, uterus, or vagina, but it's not growing into the pelvic or abdominal wall (T4a). And the cancer has spread to 1 nearby lymph node in the true pelvis (N1). It has not spread to distant sites (M0).
IIIB	**T1-T4a N2 or N3 M0**	The cancer has: • grown into the layer of connective tissue under the lining of the bladder wall (T1), OR • into the muscle layer of the bladder wall (T2), OR • into the layer of fatty tissue that surrounds the bladder (T3a or T3b), OR • it might have spread into the prostate, seminal vesicles, uterus, or vagina, but it's not growing into the pelvic or abdominal wall (T4a). AND the cancer has spread to 2 or more lymph nodes in the true pelvis (N2) or to lymph nodes along the common iliac arteries (N3). It has not spread to distant sites (M0).
IVA	**T4b Any N M0**	The cancer has grown through the bladder wall into the pelvic or abdominal wall (T4b). It might or might not have spread to nearby lymph nodes (Any N). It has not spread to distant sites (M0).
	OR	
	Any T Any N M1a	The cancer might or might not have grown through the wall of the bladder into nearby organs (Any T). It might or might not have spread to nearby lymph nodes (Any N). It has spread to distant lymph nodes (M1a).

IVB	**Any T** **Any N** **M1b**	The cancer might or might not have grown through the wall of the bladder into nearby organs (Any T). It might or might not have spread to nearby lymph nodes (Any N). It has spread to 1 or more distant organs, such as the bones, liver, or lungs (M1b).

II.4 Tumor grading

The grade is described by examining the morphologic differentiation of the cancer cells compared to normal cells. Grading is a critical parameter used to determine the management approach and treatment plan for patients with NMIBC (Mostofi et al., 1999).

During the last 50 years, grading systems for urothelial carcinoma (UC) have changed. In 1973, World Health Organisation (WHO) adopted the first grading system, which divides urothelial cell carcinoma (UCC) into three grades (G1, G2 and G3) (Sobin, 1974). In 2004, the International Society of Urological Pathology (ISUP) proposed a new version of bladder cancer grading, convened with the world health organization (WHO), to replace the WHO 1973 grading system. This classification divides tumors named G1 into papillary urothelial neoplasia of low malignant potential (PUNLMP) and low grade (LG) tumors; G2 tumors were classified as LG or high grade (HG), while all G3 tumors were known as HG (Miyamoto et al., 2010).

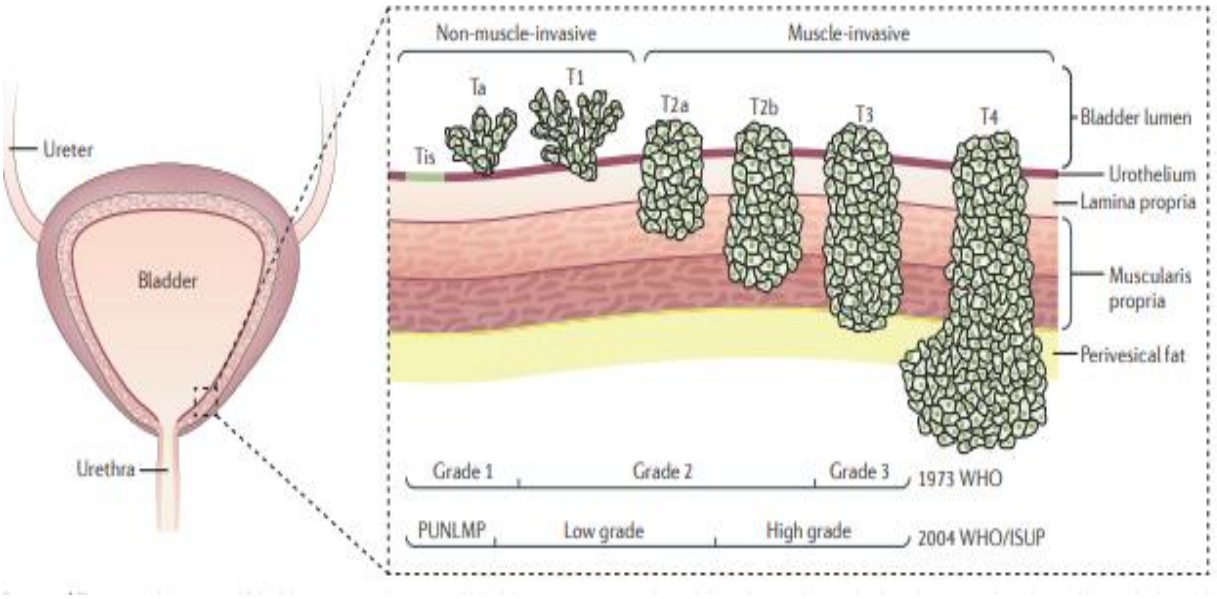

Figure 2: Bladder cancer WHO 1993 and 2004 Staging of urothelial tumors (Knowles & Hurst, 2015)

The 2016 WHO classification of bladder tumors is based on a combination of the WHO/ISUP 1998 classification and the WHO 2004 classification. According to this system, NMIBCs is

classified as LG and HG, and all MIBCs are considered HG tumors (Figure 1) (Compérat et al., 2019).

However, a recent study was performed to compare the prognostic performance and reproducibility of the World Health Organization (WHO) 1973, 2004 classification systems and 2016 grading systems and concluded that both grading systems predict progression more than recurrence, but the prognostic value of WHO 1993 was higher than that of WHO 2004/2016 classification systems (Figure 2) (Rhijn et al., 2021).

According to the European Association of Urology (EAU), bladder cancer is divided into two subtypes: NMIBC and MIBC (Babjuk et al., 2022). The earliest stage cancers, namely Ta, T1 and Carcinoma *in situ*, are classified as NMIBC. Ta tumors are confined to the urothelium; T1 tumors invade the lamina propria without the muscularis propria and represent 5% to 20% of NMIBC, Tis tumors recur frequently. But, Ta, T1 and CIS are believed to be a precursor of muscle-invasive tumors. According to the invasion depth, T2, T3 and T4 tumors are classified as MIBC. T2, T3 and T4 tumors invade the superficial muscle, perivesical fat, and surrounding organs, respectively (Sjödahl et al., 2012).

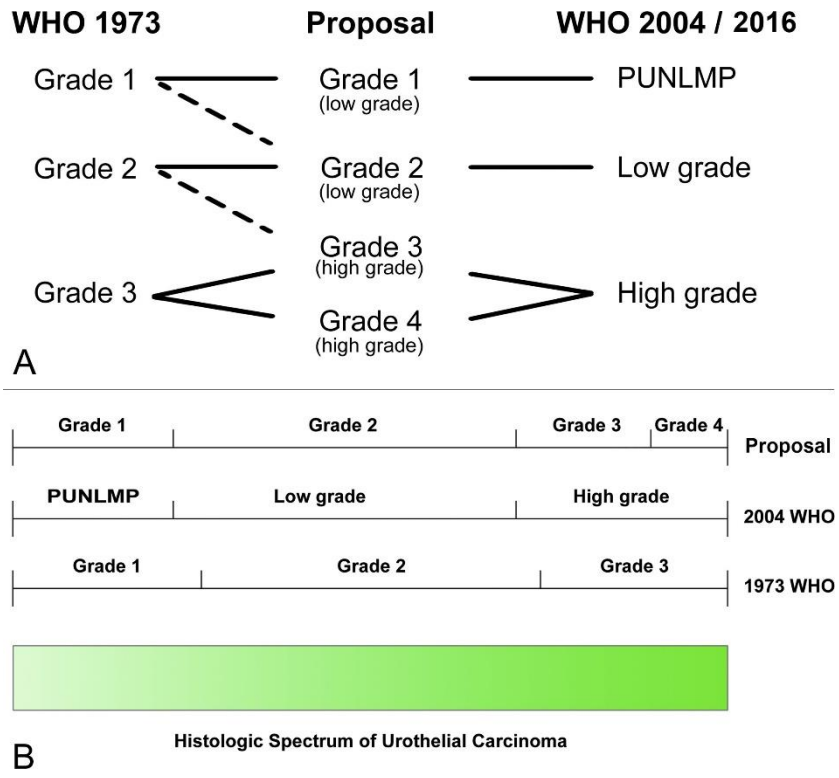

Figure 3: Comparison between WHO 1973 and WHO 2004/2016 grading systems (Jones & Cheng, 2021)

II.5 Histological classification of Bladder Cancer

A wide range of histopathological forms characterizes bladder cancer. There are different classifications based on histological features and molecular phenotypes; It is essential for the clinician to understand the characteristics of each type of bladder cancer and improve the diagnosis, prognosis and the appropriate treatment for each patient (Matau & Roy, 2014).

BC can be classified into two broad categories:
Epithelium tumors: represent 95% of all bladder tumors. These cancers arise from the epithelium and are subdivided into three subtypes urothelial carcinoma, Squamous cell carcinoma (SCC) and Adenocarcinoma (ADC) (Kaseb & Aeddula, 2022).

- Urothelial carcinoma or transitional cell carcinoma: The most common subtype is urothelial carcinoma which accounts for over 90% of all urinary bladder carcinomas. They start from the superficial layer of the bladder wall, without invasion neither into the lamina propria nor into the muscularis propria, except for some tumors due to different clinical and histological features. In addition, there are two subtypes of UC, papillary and flat, based on their growth pattern. CIS or flat tumors can present a higher risk of progression, while papillary carcinoma has a low-risk of progression but a high-risk of recurrence (Sylvester et al., 2006; Rhijn et al., 2009).
- Squamous cell carcinoma is the second most common cell type, constituting around 2%–15%. Two forms have been identified depending on the causal factor: the bilharzian form, which is the most prevalent parasitosis with the highest incidence rates of 35 to 75% in endemic countries, such as Egypt and Sudan, and is caused by infection with the parasite *Schistosoma haematobium*. The origin of the other form, non-bilharzian, varies from case to case. Usually, this type occurs at sites of chronic inflammation and irritation or chronic infection (Matau & Roy, 2014; Lobo et al., 2020).
- Adenocarcinoma is a rare type of bladder cancer, representing less than 2%. It is composed of two types of cuboidal and columnar cells. Adenocarcinomas are subdivided into three types of tumors:
    - Primary bladder adenocarcinoma of glandular metaplastic origin, which is divided from the bladder urothelium but has a pure histological glandularphenotype. The origin of the Secondary adenocarcinomas is unknown but can be a result of direct extension from another site or metastasis from distant

organs. Most patients with secondary adenocarcinomas represent hematuria and other symptoms such as pelvic pain and weight loss.

- Finally, metastatic adenocarcinomas of the unknown primary site are frequently derived from the prostate, ovary and colon. There are other histological variants, but stratification remains the most accurate factor in terms of prognosis (Bates & Baithun, 2000).

Non-epithelial tumors are uncommon cancers of all the vesical tumors that represent less than 5% of the urinary bladder, including melanoma, paraganglioma, sarcoma, carcinosarcoma and lymphoma that have the same origin without crossing the mucosa (Ravi et al., 2013).

II.6 Molecular Classification of Bladder cancer

Pathological assessment of bladder cancer is one of the major problems in daily clinical practice. Therefore, efforts have been made to standardize a pathological assessment tool with molecular biomarkers. Molecular classification of bladder tumors based on gene expression signature and mutational status has distinct morphological and clinical characteristics. This tool may guide cancer patients' therapy choices and evaluate new cancer therapies (Kaushal & Khandakar, 2021).

Concerning bladder cancers, the Cancer Genome Atlas (TCGA) project has identified more than 302 mutations that include 32 control cell cycle regulations, 204 copy number variations and 22 rearrangements (Cancer Genome Atlas Research Network, 2014). Several chromosomal alterations, including those affecting Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*), *RB1* and *E2F* Transcription Factor 3 (*E2F3*), and a large number of mutations, including those of *TP53, FGFR3* and *PIK3CA* and others involved in several signalling pathways, may facilitate bladder cancer classification into molecular subtypes for more precise patient stratification to guide treatment options (Jemal et al., 2011; Forbes et al., 2011).The molecular subtyping of bladder cancer can be divided into 5 major subtypes with distinct biological and clinical properties:

- **Urobasal tumors A** were mostly found to be low-grade papillary, NMIBC. They are characterized by high expression of Fibroblast Growth Factor Receptor 3 (*FGFR3)*, Cyclin D1 (*CCND1*) and Tumor Protein P63 (*TP63*), Tumor Protein *p53 (TP53)* and Keratin 5 (*KRT5*). Most type A urobasal tumors were found to be NMIBC with

FGFR3 mutations, infrequent *p53* mutations, and low-grade papillary, thus presenting a good prognosis (Aine et al., 2015).

- **The genomically unstable subtype** is characterized by frequent *TP53* mutations, overexpression of cyclin E1 (*CCNE*) and Erb-B2 Receptor Tyrosine Kinase 2 (*ERBB2*) and low expression of cytokeratin Phosphatase and TENsin homolog (*PTEN*). Genomic instability was equally distributed between MIBC and those representing a high-grade NMIBC and is associated with a poor prognosis (Aine et al., 2015).
- **The SCC-like subtype,** characterized by a high expression of basal keratins: *KRT4, KRT6A, KRT6B, KRT6C, KRT14* and *KRT16,* is not normally expressed in the urothelium. This subtype expresses squamous differentiation markers and is associated with a poor prognosis (Satyal et al., 2019).
- **Urobasal B tumors** had frequent mutations of *TP53*; they are characterized by several similarities with urobasal A tumors, such as a high frequency of *FGFR3*, *CCND1* and *TP63* mutations. Urobasal B tumors are characterized by the expression of several keratins specific to the SCC-like subtype. Urobasal B and SCC-like subtypes have a worse prognosis. (Aine et al., 2015; Satyal et al., 2019).
- **highly infiltrated by non-tumor cells subtype** has a significant level of biomarkers that characterize immune and stromal cells with different mutation and expression patterns in *FGFR3.* Therefore, this group of tumors was not regarded as an intrinsic subtype of UC, with immunohistochemical evaluation revealing that malignant cells in tumors from this group expressed markers characteristic of the other subtypes (Aine et al., 2015).

The previously mentioned studies based their findings on epigenetic, genetic, gene expression, and proteomics analyses. Differences in results within the established subtypes are due to targets, methods and study interpretation variations. All studies confirm the need for biomarkers that can be used for treatment selection in clinical practice and decrease the toxicities in resistant BC patients. It is also essential to consider molecular classification in parallel with traditional histopathology in therapeutic management (El AHANIDI et al., 2019).

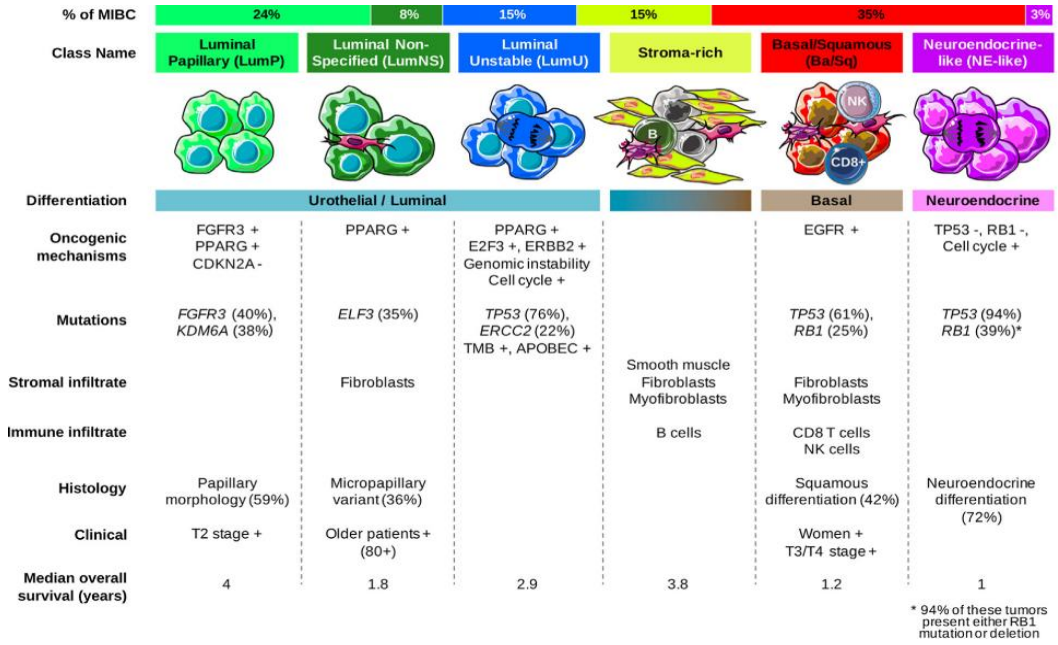

Figure 4:Overview on current taxonomy on molecular subtypes of urothelial bladder cancer and expected response to chemotherapy (Kamoun et al; 2020)

Ba/Sq = basal/squamous; LumNS = luminal nonspecified; LumP = luminal papillary; LumU = luminal unstable; MIBC = muscle-invasive bladder cancer; NE = neuroendocrine; NK = natural killer.

II.7 Bladder Cancer diagnosis

Hematuria, or blood in the urine, is often the first detectable sign of bladder cancer. Hematuria can be macroscopic or microscopic. Gross hematuria is a visible blood in the urine and may be correlated with an advanced disease stage. Hematuria is not a definite symptom of bladder cancer because it can be a sign of other urological disorders such as an infection or a renal parenchyma disease (Saleem & Hamawy, 2022).

To confirm the diagnosis, several tests are used, including cystoscopy, urinary cytology, imaging methods, fluorescence *in situ* hybridization and urine protein detection.

1. Magnetic Resonance Imaging (MRI)

It is a pre-therapeutic evaluation that provides information on the prognosis and the tumor evolution. It allows a precise diagnosis of the structure of tissues, with a sensitivity of 83% and a specificity of 80% than cystoscopy. The clinical utility of MRI helps in the choice of appropriate treatment and prognosis by identifying the size and location of the tumor; However this imaging method is unable to detect the Carcinoma *in situ* histological type of bladder cancer (Matau & Roy, 2014).

2. Endoscopy: Flexible Cystoscopy

It is a current diagnostic tool for bladder cancer and is used in men and women using a small flexible telescope inserted into the bladder. Cystoscopy is an invasive examination that causes complications such as urinary tract infections, pain and bleeding…(Raitanen et al., 2001). The sensitivity and specificity of this diagnosis method range from 88 to 100% and 77.1 to 97%, respectively, depending on the stage and grade of the tumor (Zhu et al., 2019). Moreover, this tool has a low sensitivity for detecting CIS, and it is operator-dependent, especially the difficulty for detection of recurrence or small tumors such as CIS (Raitanen et al., 2001).

3. Urinary Cytology

It is a non-invasive diagnostic method performed on fresh or fixed urine and aims to detect tumor cells. Normal or tumoral urothelial cells can be naturally exfoliated in urine and then identified by histological methods (Têtu, 2009). The overall sensitivity of urine cytology ranges from 28 to 100%, with a median of 44% and a specificity of 91%. This method is particularly useful for identifying high-grade and high-stage cancers but is less sensitive for low-grade tumors (Lotan & Roehrborn, 2003). A positive cytologic does not necessarily mean the presence of a tumor in the urinary tract. However, negative cytology does not exclude its presence (Raitanen et al., 2002).

4. Transurethral resection of bladder tumor (TURBT)

The patient should undergo Transurethral resection of the bladder tumor (TURBT) if cystoscopy and urine cytology are positive in order to perform an anatomopathological test to know the exact stage/grade of the tumor and to adopt an effective therapeutic modality. NMIBC should be managed based on risk stratification. In contrast, the management of MIBC should be based on the extent and stage of the disease (Murez et al., 2020). This procedure is used for diagnosis and treatment and successfully removes the entire lesion (Russell et al., 2016).

II.8 Bladder cancer treatment

Treatment options and management of patients with bladder cancer depend on several factors, including the size, number and stage/grade of the tumor. The standard first-line treatment is an endoscopic treatment with TURBT, which consists in the removal of a visible lesion. However, many efforts have been undertaken to improve survival rates and patients' quality of life. Surgery can be combined with other types of treatments, including chemotherapy,

immunotherapy and radiotherapy, depending on the type of cancer, the stage, the patient's general health…

1. **Treatment of superficial urothelial tumors**

- **Endoscopic resection of bladder tumor**

Transurethral resection remains the gold standard treatment option for 75% to 85% of NMIBC (Cassell et al., 2019). This procedure is both a diagnostic and therapeutic approach. However, about 50 to 70% of NMIBC tumors will recur, and 10 to 20 % progress to MIBC (Sylvester et al., 2006; Kim & Patel, 2020). The eradication of tumors with TURBT needs to be complete; if the tumor is larger than 3 cm in diameter, multifocal and BC relapse rates are highest, then neoadjuvant therapy is associated with surgical resection (Kim & Patel, 2020).

- **Adjuvant treatment: Intravesical instillations**

After TURBT, the majority of patients with NMIBC will require additional treatment with intravesical instillations, which may be necessary to minimize the risk of recurrence, using mitomycin C (MMC) chemotherapy or Bacillus Calmette Guérin (BCG) immunotherapy (Rouprêt et al., 2020).

Intravesical instillation of MMC is an antitumor antibiotic chemotherapeutic agent isolated from *Streptomyces caespitosus*. Its mechanism of action is based on the inhibition of DNA synthesis by DNA alkylation (Song et al., 2018). Standard treatment with MMC is 8 weekly instillations of 40 mg in 40 ml (weekly instillation), followed or not by monthly instillations for up to 1 year (maintenance therapy) (Rouprêt et al., 2020).

Instillation with Bacillus Calmette Guérin: It is a Mycobacterium that adheres to bladder mucus and the urothelium. BCG therapy activates the immune system to eliminate the tumor cells (Neuzillet & Lebret, 2010). BCG therapy is initiated only after the bladder has healed from TURBT, usually 2 to 4 weeks after resection, at the latest 6 weeks, and in the absence of any residual tumor. The induction treatment consists of 6 intravesical instillations per week, ideally of 2 hours each. Maintenance treatment is recommended in all cases and consists of 3 instillations at 3, 6, and 12 months after resection for intermediate-risk tumors (one-year maintenance) and continued every 6 months until the 36^{th} month for high-risk tumors (3-years maintenance) (Rouprêt et al., 2020).

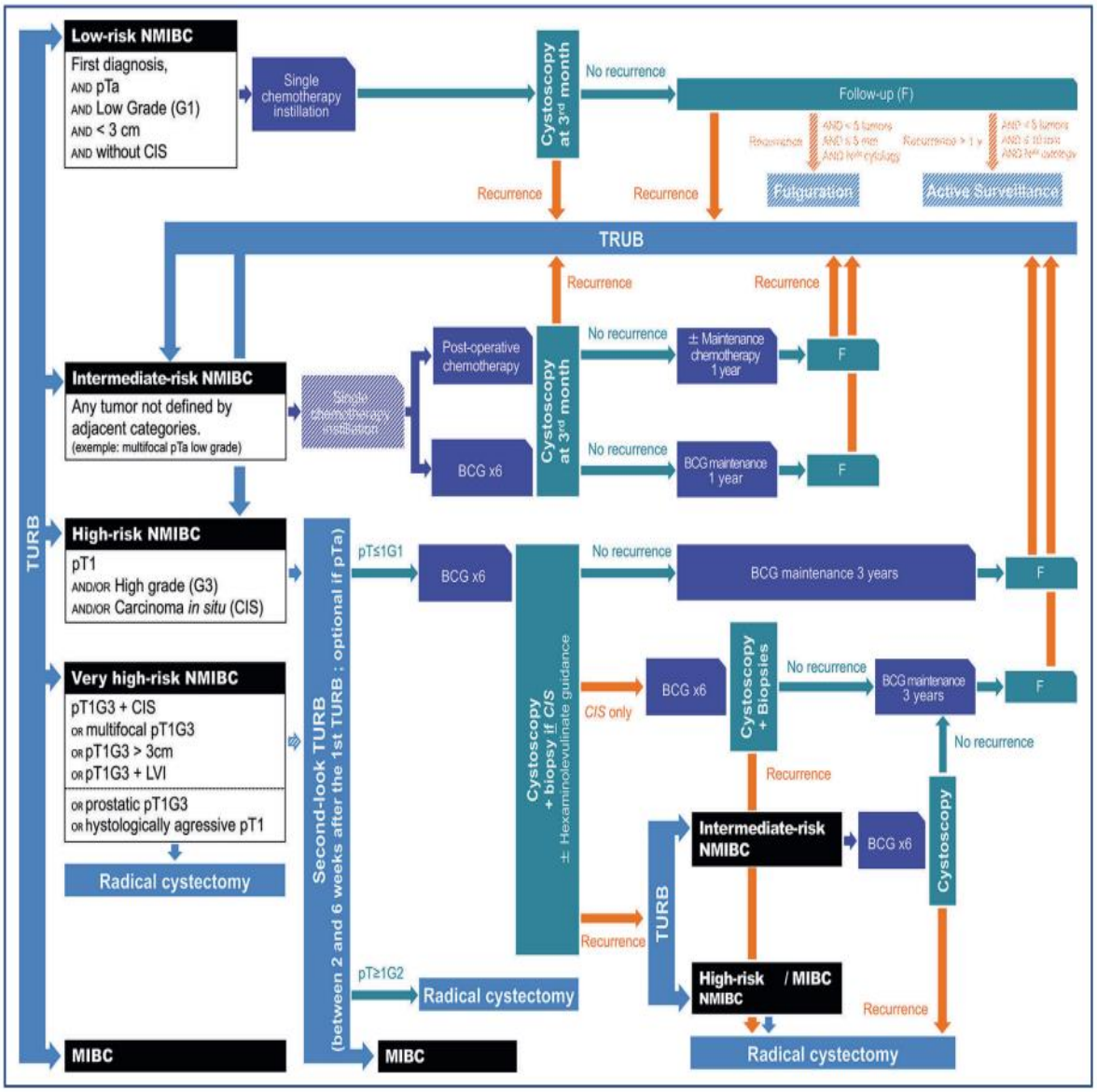

Figure 5: Algorithm for NMIBC management (Rouprêt et al., 2020).

2. Treatment of infiltrating urothelial tumors

Surgical treatment "cystectomy"

Cystectomy is a treatment based on the removal of the bladder and is considered the standard gold treatment for non-metastatic MIBC and for NMIBC that does not respond to non-invasive treatments (Rouprêt et al., 2020). In men, Radical cystectomy includes the removal of the prostate and seminal vesicles. In women, this procedure involves the removal of the uterus and ovaries, fallopian tubes and part of the vagina (Beauval et al., 2015).

If radical cystectomy is contraindicated or declined by the patient, alternative therapies are available such as transurethral resection, open partial cystectomy, radiotherapy, and chemotherapy (Niedworok & Gratzke, 2015).

- Transurethral resection of bladder tumor alone

TURB may be proposed to patients who are not eligible for cystectomy or who refuse open surgery or trimodal therapy and have a short life expectancy. This procedure is possible as a therapeutic option only if tumor growth is limited to the superficial muscle layer and re-staging biopsies are negative for residual (invasive) tumors. It can be offered only in the following cases: unifocal tumor, stage pT2, a size ≤ 3 cm or in the absence of carcinoma *in situ* (Rouprêt et al., 2020; Hertfordshire and Bedfordshire Urological Cancer Centre et al., 2021).

- Partial cystectomy

The partial cystectomy involves the removal of the segment of the diseased bladder; it can be proposed in case of unifocal primary lesion on a mobile portion of the bladder more than 2cm from the cervix and trigone, absence of CIS, size ≤ 4 cm, stage T3a maximum; intradiverticular tumor or urachal adenocarcinoma. It should be associated with lymphadenectomy in the same manner as radical cystectomy (Rouprêt et al., 2020).

- Chemotherapy

Chemotherapy is used for advanced bladder tumors, specifically when there are lymph nodes or distant metastases. Chemotherapeutic agents for MIBC are Methotrexate, Vinblastine, Adriamycin and Cisplatin (MVAC regimen) or Cisplatin and Gemcitabine (CG regimen), which may give an excellent prognosis . In some patients, the combination of TURBT and chemotherapy may extend survival (Hertfordshire and Bedfordshire Urological Cancer Centre et al., 2021).

- Radiotherapy

Radiotherapy (RT) is performed in patients with muscle-invasive bladder cancer who cannot tolerate bladder removal surgery because of their advanced age or in patients who refuse surgery (Rödel, 2004). It has been reported that no difference in survival was seen between patients with MIBC treated with RT and those who underwent cystectomy (Hayter et al., 1999).

Concomitant chemoradiotherapy therapy with fluorouracil and mitomycin C combined with radiotherapy is more effective than radiotherapy alone for patients with muscle-invasive bladder cancer (James et al., 2012).

- Immunotherapy

Immune checkpoint inhibitors (ICIs) for the treatment of bladder cancer

Immune checkpoint inhibitors are monoclonal antibodies that block co-inhibitory signaling pathways between tumor cells or Antigen-presenting cells (APC) and T cells, thereby promoting immune-mediated elimination of tumor cells. The genetic instability of BC remains a challenge for targeted therapies. Indeed, tumor cells have developed several mechanisms for evading immune surveillance, which rely on the role of various molecules (checkpoints) that inhibit the immune response, namely Cytotoxic T- lymphocyte-associated protein 4 (CTLA-4) and Programmed Cell Death 1 (PD-1) / PD-L1 Programmed Cell Death-Ligand 1 (PD-L1) (Kostine et al., 2019). It has been reported that high PD-L1 expression was associated with higher stage and grade, higher recurrence, poor survival and worse outcome in bladder cancer (Zhu et al., 2019); hence the key role of monoclonal antibodies anti-CTLA-4 and anti-PD-A/PD-L1 in cancer therapeutics. But side effects are caused by immune checkpoint inhibitors that can attack many normal body parts in addition to cancer cells; the more common side effects include diarrhea, nausea, skin rash, and poor appetite. Therefore, it is essential to control immunologic toxicity without compromising anti-tumor efficacy (Choi & Lee, 2020). The efficacy of checkpoint inhibitors depends on PD-L1 expression; patients with high PD-L1 expression do not respond to anti-PD-L1 therapy. Thus, there is an urgent need to better understand and enhance the body's response to ICIs (Lu et al., 2019).

Chemotherapy (CT) is still the best strategy to treat many solid tumors, especially for patients with advanced BC or metastatic disease. However, BC is characterized by chemoresistance, and the toxicity caused by these regimens limits their use in the highest-risk patients (Drayton & Catto, 2012). ICIs such as: Atezolizumab, Durvalumab, and Avelumab antibodies targeting PD-L1 remain an alternative first-line treatment for patients categorized as cisplatin-resistant and second-line therapy for patients with metastatic urothelial carcinoma (mUC) of the bladder. Indeed, the use of Anti-PD-L1/PD-1 and Anti-CTL4 drug combinations appears important for metastatic tumors and is currently included in several ongoing clinical trials (Lopez-Beltran et al., 2021).

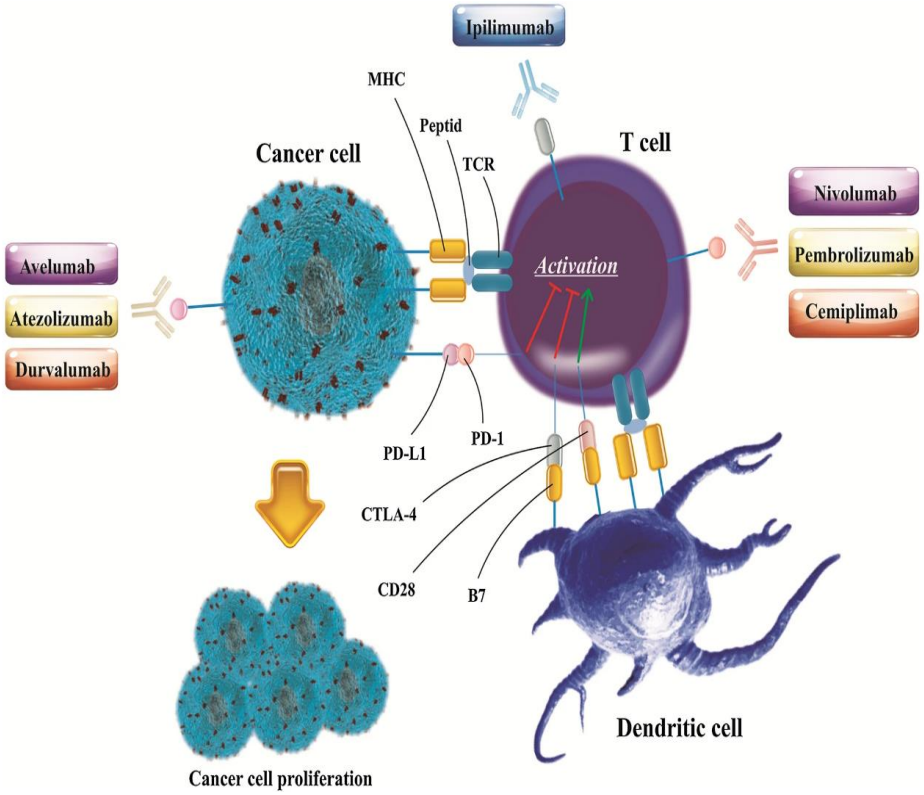

Figure 6: Combination therapy with immune checkpoint inhibitors (ICIs) (Vafaei et al., 2022)

As defined below, Atezolizumad and Pembrolizumab were proved to be successful therapeutic strategies for tumors with high expression of PD-L1. Erdafitinib is a Tyrosine Kinase inhibitor (TKI) that inactivates the fibroblast growth factor receptor (FGFR) in order to inhibit the tyrosine kinase activities and progress on platinum-Based CT (Perera et al., 2017). TKIs have achieved great success in numerous tumor types in recent years; however, due to the excessive use of FGFR inhibitors undergoing clinical or pre-clinical trials, resistance to FGFR-TKI has become a concern (Yue et al., 2021).

Since its discovery over 20 years ago, the role of telomerase in the immortalization of cancer cells via telomere extension and its use as a biomarker of tumor progression and prognosis has been considered an interesting target for cancer therapy. Previous studies have focused on immortalizing cancer cells through telomere maintenance mechanisms as a target for immunotherapy (Kim et al., 1994). These efforts have led to the development of peptide vaccines that target *hTERT* and have been tested in various cancer types, showing that these vaccines are highly specific to tumors (Vonderheide, 2002). Vaccines based on hTERT-derived epitopes and including several drugs, such as GV1001, GX301, GRNVAC1 and VX-001, have been described to date (Mizukoshi & Kaneko, 2019).

GRNVAC1 and TAPCells are Dendritic Cells- based cancer vaccines that have been investigated to develop hTERT-targeted immunotherapy. Both vaccines have demonstrated an effective immune response, and clinical studies have proven them to be safe and well-tolerated (Mizukoshi & Kaneko, 2019).

II.9 Bladder cancer epidemiology

In the world

Bladder cancer is the tenth most common cancer affecting men and women worldwide. In 2020, bladder cancer was diagnosed in more than 573 278 new cases worldwide for both sexes (Sung et al., 2021). According to the latest GLOBOCAN data, bladder cancers account for approximately 3% of diagnosed cancers s in the world. A total of 90% of diagnosed bladder cancer occurred in individuals aged 55 years and older (Saginala et al., 2020).
The geographic representation in shows estimated incidence rates by country (Figure 3). European and North American countries reported the highest rates of BC. These Variations in incidence worldwide can be attributed to global changes in exposure to bladder cancer risk factors. The incidence also varies by gender; the majority of bladder cancer patients are male (GLOBOCAN, 2020).

The type of BC depends on the geographical location and is related to the predominant risk factors in each country. About 90% of all bladder cancers are classified as urothelial carcinomas (UC), and less than 5% of bladder cancers are squamous cell cancers (SCC), small-cell carcinoma, or adenocarcinoma (ADC) (Kaseb & Aeddula, 2022).

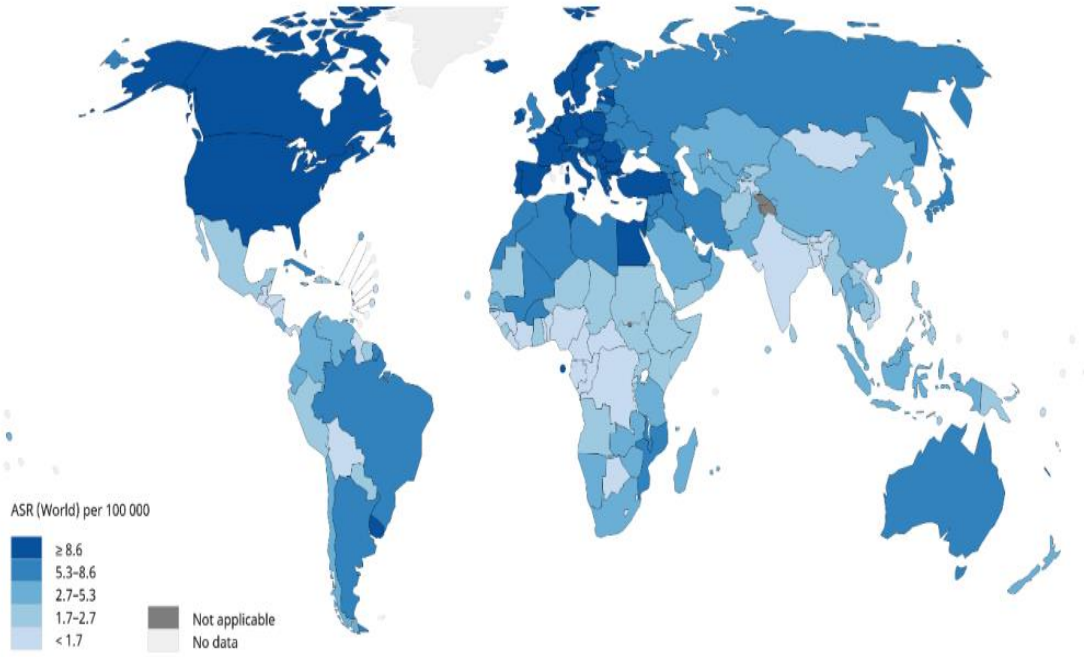

Figure 7: Estimated age-standardized incidence rate of bladder cancer worldwide in 2020 (GLOBOCAN, 2020).

In Africa

Among the main types of cancer in Africa, BC is the eighth most common cause of cancer death (Figure 4). There were an estimated 33 196 new cases and 18 747 deaths from bladder cancer in 2020 (GLOBOCAN, 2022). Smoking and parasitic infection with *Schistosoma haematobium* are two major factors responsible for bladder cancer disease in Africa (Mantica et al., 2021). It is the fifth most frequently diagnosed cancer in women and the ninth most frequently diagnosed cancer in men (Figure 5) (GLOBOCAN, 2022).

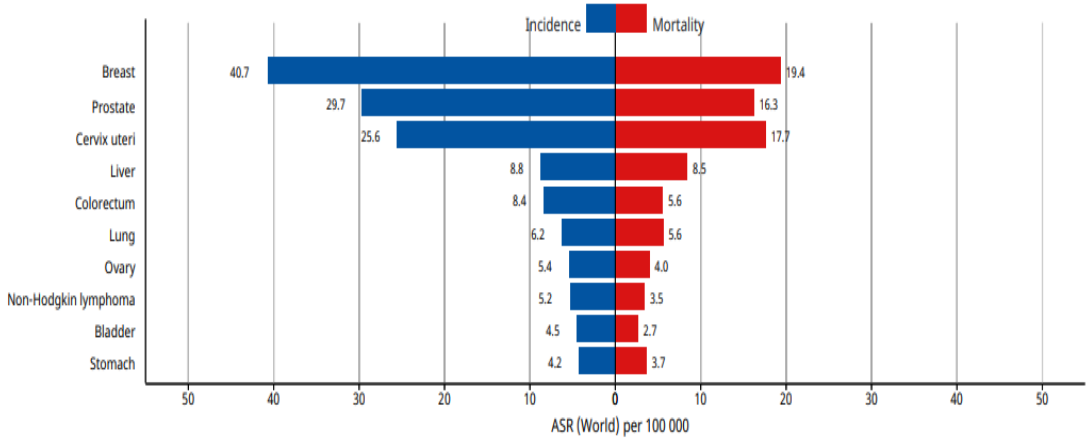

Figure 8: Incidence and mortality rates of the top 10 cancers in Africa (GLOBOCAN, 2022)

Bladder cancer incidence varies according to the countries; Egypt has a high rate of 19.0/100.000 in males, followed by Malawi with an incidence rate of 9.2/100.000 in females. Concerning mortality, Egypt has the highest recorded mortality rate 5.6/100.000 (Antoni et al., 2017).

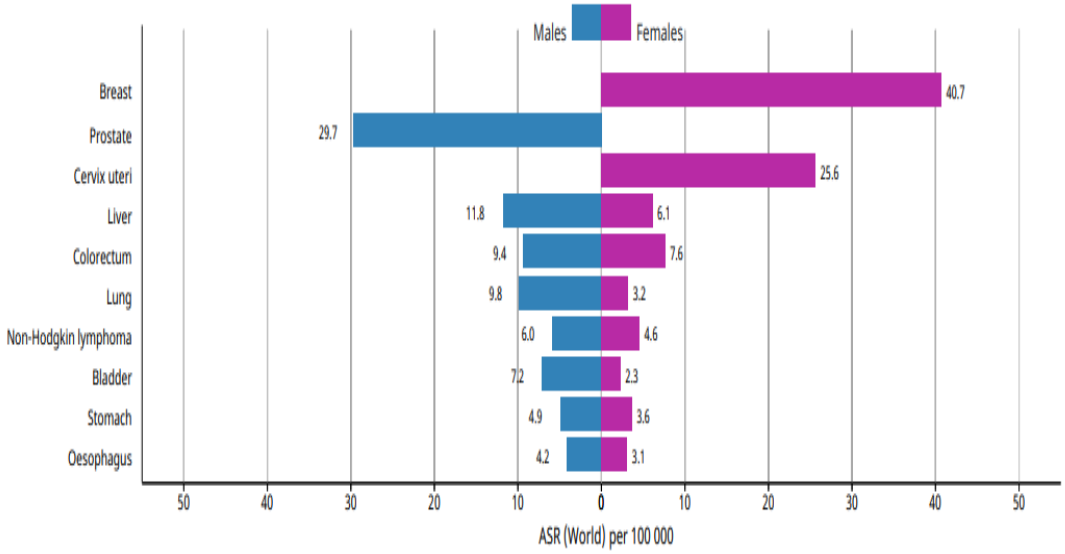

Figure 9: Incidence rates per sex of the top 10 cancers in Africa (GLOBOCAN, 2022).

In Morocco

Bladder cancer is one of the most frequently diagnosed malignancies in males. According to International Agency for Research on Cancer (IARC) report in 2020, BC is ranked the eighth most common cancer in both sexes in Morocco, with an incidence rate of 10.2/100.000 in males and 1.5/100.000 in females (Figure 6). However, BC is considered a serious health problem, fatal and very high cost treatment. It is the eighth leading cause of death in Morocco. In men, BC was ranked fourth, with an incidence of 9.7% and a mortality rate of 5%. While in women, it was ranked ninth in the list of the ten most frequent cancers (GLOBOCAN, 2022).

Referring to the latest version of the regional register of Casablanca, which hosted 12% of the total population of the country and conducted during the period 2008-2012, BC represents 3.1% of global cancer diagnoses, the male subjects present 85.3% compared with female subjects 14.7% (Figure 7) (Benider et al., 2016).

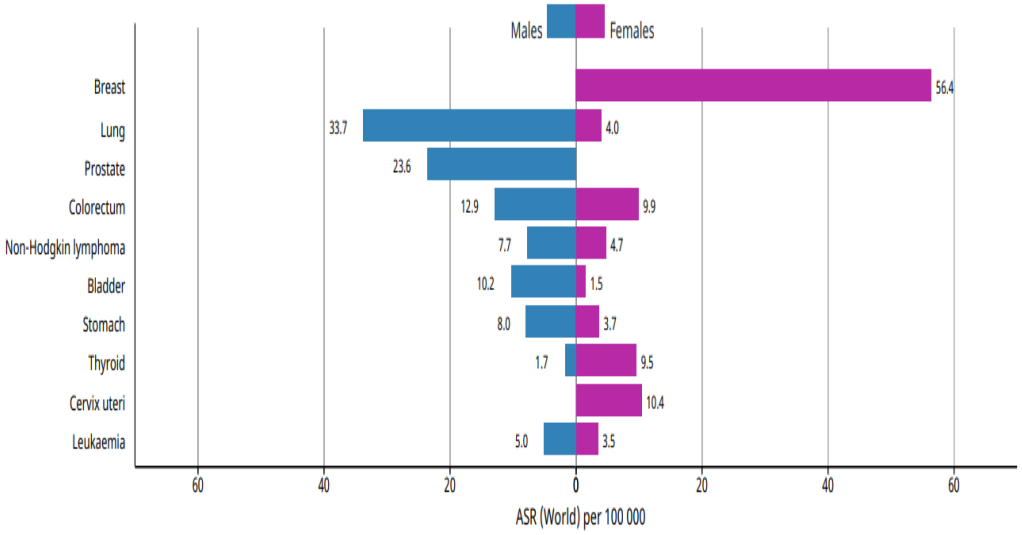

Figure 10: incidence rates per sex of the top 10 cancers in Morocco (GLOBOCAN, 2022).

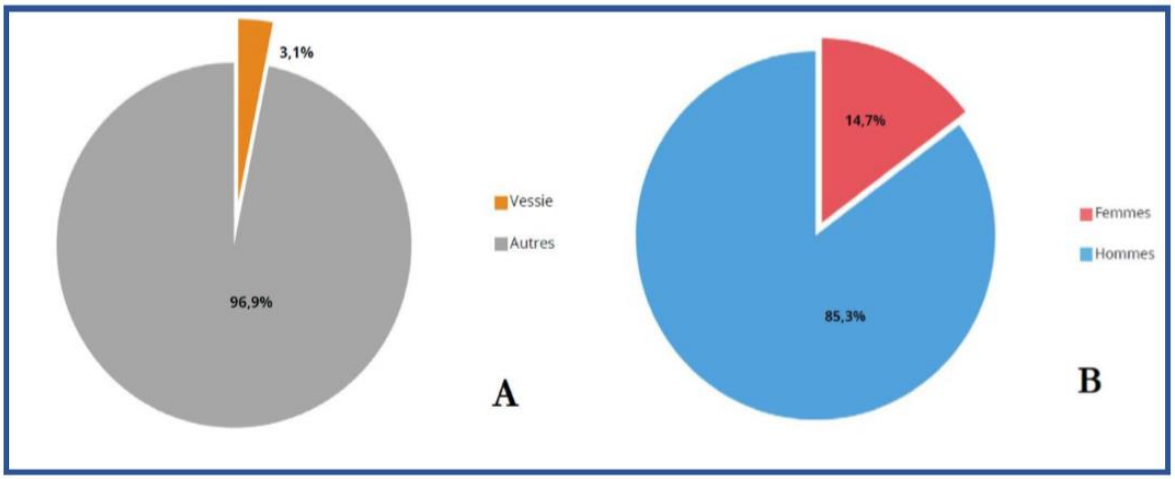

Figure 11: Distribution of bladder cancer in the Grand Casablanca region (%) in 2008-2012 (Benider et al., 2016).

According to the latest version of the regional register of Rabat, published in June 2012, BC was the 3rd most common cancer diagnosed among men 6.9% (Figure 8), and the occurrence of BC increases steadily with age from 45 years to 65 years (Tazi et al., 2013).

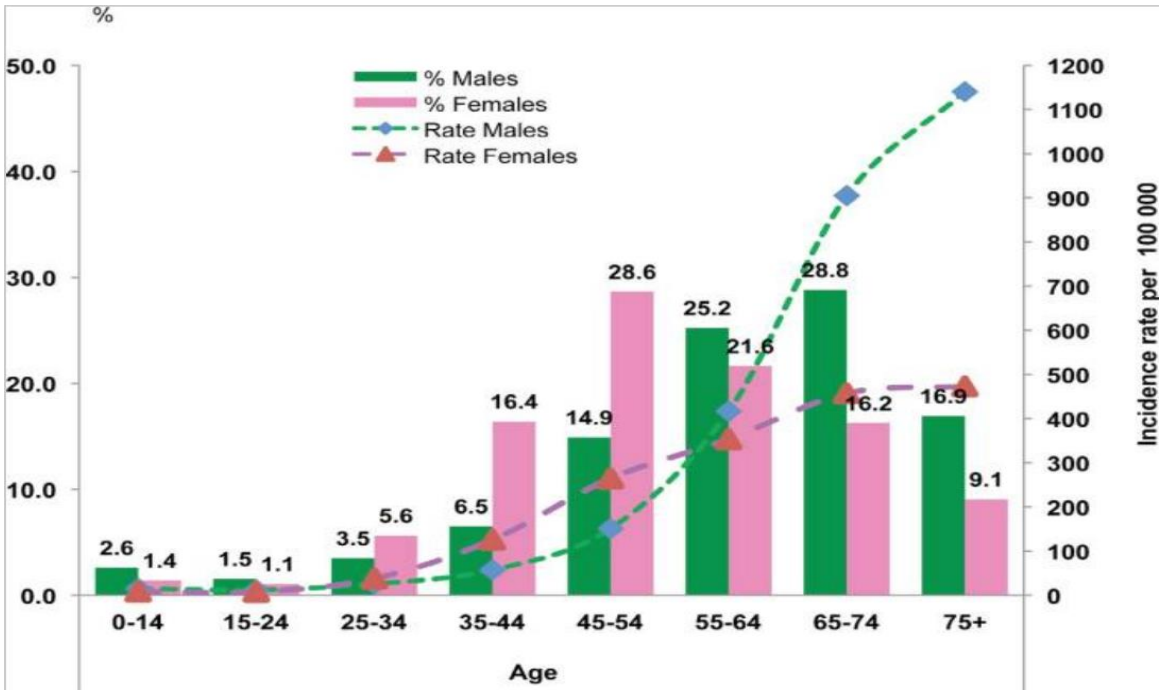

Figure 12: Distribution of cancer incidence in both sex, Rabat-Morocco 2006-2008 (Tazi et al., 2013).

III. Risk prediction models for bladder cancer

Non-muscle invasive bladder cancer (NMIBC) represents 70-80% of bladder cancers; NMIBC is characterized by large biological heterogeneity and a significant probability of disease recurrence and progression to muscle-invasive bladder cancer (Matulewicz & Steinberg, 2020). The American Urological Association (AUA), the American Society of Clinical Oncology (ASCO), the Amercian Society for Radiation Oncology (ASTRO), and the Society of Urologic Oncology (SUO) formulated an evidence-based guideline in 2020 to provide a clinical framework for the management of NMIBC.

On the other hand, the European Association of Urology (EAU) has issued guidelines, which were released in 2021, to provide practical recommendations for clinical management of NMIBC, with a focus on clinical presentation and recommendations (Babjuk et al., 2022). Risk stratification for NMIBC by these guidelines or by using cancer prediction tools nomograms/risks calculators can help inform the probability of recurrence and progression and guide the treatment decision, but may not be applicable to all patients (Babjuk et al., 2022).

The probability rates of recurrence and progression depend on several clinical-pathological factors (Sylvester et al., 2021). The most significant clinical and pathological factors selected

to predict the recurrence and progression probability rates of Ta and T1 tumors at one and five years are: the number of tumors, tumor size, prior recurrence rate, concomitant CIS, T category and grade, age, gender. The main prognostic factors contributing to recurrence are the number of tumors, size of tumors and the prior recurrence rate, while the main prognostic factors contributing to progression are stage and grade and the presence of CIS (Sylvester et al., 2006).

To be able to facilitate treatment recommendations, the EAU guidelines suggest stratifying patients into four risk groups. This new stratification is based on their probability of progression to MIBC, based on the analysis of patients' Individual Participant Data (IPD) and the calculation of their disease progression rate using (2021 EAU NMIBC scoring model) at 1, 5 and 10 years (Table 2), available at www.nmibc.net (Babjuk et al., 2022).

Table 2: Probabilities of disease progression in 1, 5 and 10 year(s) for the new EAU NMIBC risk (Babjuk et al., 2022).

Risk group	**Probability of Progression and 95% Confidence Interval (CI)**		
	1 Year	**5 Years**	**10 Years**
New Risk Groups with WHO 2004/2016			
low	0.06%(CI: 0.01%−0.43%)	0.93% (CI: 0.49%−1.7%)	3.7% (CI: 2.3%−5.9%)
Intermediate	1.0% (CI: 0.50%−2.0%)	4.9% (CI: 3.4%−7.0%)	8.5% (CI: 5.6%−13%)
High	3.5% (CI: 2.4%−5.2%)	9.6% (CI: 7.4%−12%)	14% (CI: 11%−18%)
Very high	16% (CI: 10%−26%)	40% (CI: 29%−54%)	53% (CI: 36%−73%)
New Risk Groups with WHO 1973			
Low	0.12% (CI: 0.02%−0.82%)	0.57% (CI: 0.21%−1.5%)	3.0% (CI: 1.5%−6.3%)
Intermediate	0.65% (CI: 0.36%−1.2%)	3.6% (CI: 2.7%−4.9%)	7.4% (CI: 5.5%−10%)
High	3.8% (CI: 2.6%−5.7%)	11% (CI: 8.1%−14%)	14% (CI: 10%−19%)
Very high	20% (CI: 12%−32%)	44% (CI: 30%−61%)	59% (CI: 39%−79%)

The EAU guidelines also recommend the use of the European Organisation for Research and Treatment of Cancer (EORTC) or Club Urologico Espanol de Tratamiento Oncologico (CUETO) scoring models to predict the risk of tumour recurrence and progression in individual patients treated with intravesical BCG immunotherapy (Babjuk et al., 2022).

Predicting the risk stratification of recurrence and progression in patients with NMIBC can be performed using nomograms tools/ risks calculator generated by EORTC and CUETO. Both the EORTC and CUETO groups' nomograms and risk models recognize that there are the same and unique factors associated with an increased risk of recurrence and/or progression in NMIBC. These scoring models was validated to stratify patients into high-risk groups. However, there are certain limiting factors to their use that are important to understand and consider (Sylvester et al., 2006; Fernandez-Gomez et al., 2009).

The EORTC bladder cancer calculator is developed to estimate the probabilities of an NMIBC patient's recurrence and progression; this tool is available at: www.eortc.be/tools/bladdercalculator (Figure 9). Risk estimates of early and later recurrence and progression are given based mainly on the following pathological parameters: the number of tumours (1, 2–7, 8 or more), tumour diameter (<3 cm, ≥3 cm), prior recurrence rate (primary, recurrence ≤ 1 per year, recurrence > 1 per year), category T (Ta, T1), concomitant CIS (yes/no), and WHO 1973 tumour grade (grade 1, 2, 3) (Sylvester et al., 2006). For each patient, short and long-term scores recurrences and progression rates could be calculated based on prognostic factors, from 0% (best prognosis) to 17/23% (worst prognosis). Patients can then be stratified into four risk groups according to their scores (Sylvester et al., 2006).

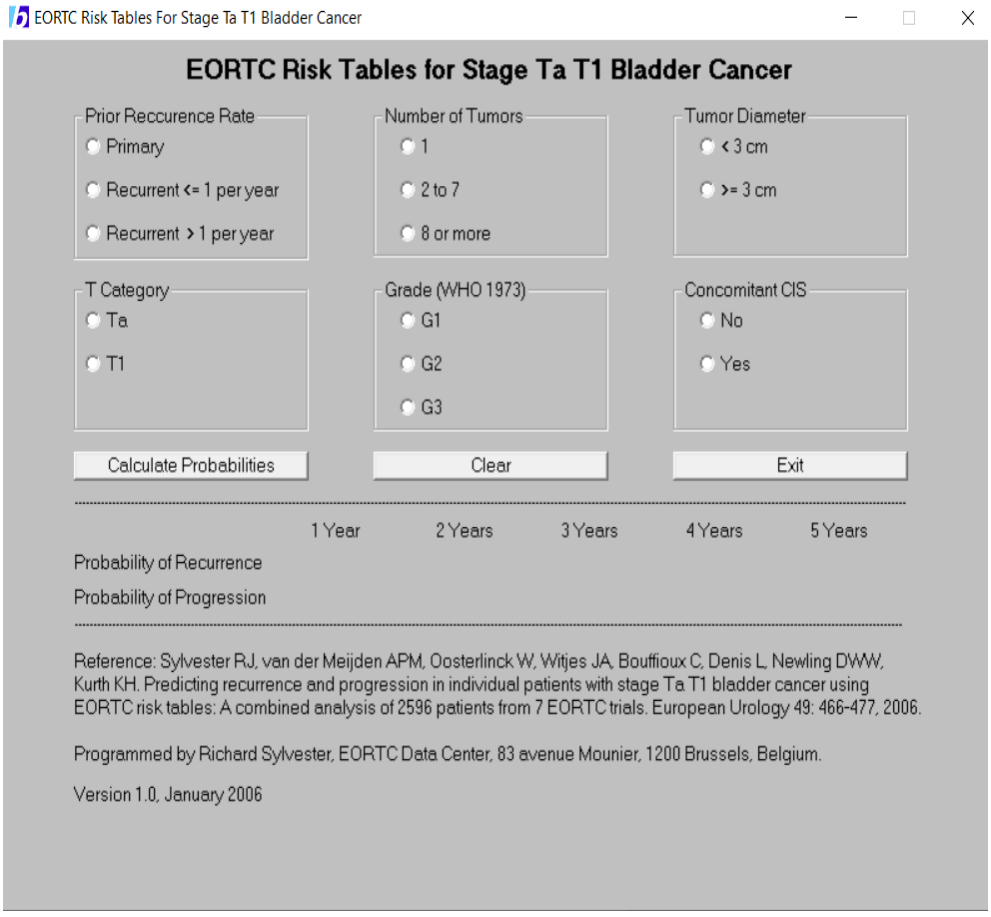

Figure 13: European Organization for Research and Treatment of cancer.
The risk tables allow estimation of the probability of recurrence bladder cancer based on risk factors (Sylvester et al., 2006)

Sylvester et al. (2006) provided an EORTC scoring system that allows urologists to easily calculate NMIBC patients' short and long-term probability of recurrence and progression after TURBT. This model was based on the analysis of 2596 patients from seven EORTC trials However, Sylvester et al. Mentioned that the recurrence and progression rates reported in their

results might be overestimated compared with those found in current clinical practice, especially in high-risk patients who received BCG therapy, which reduces the percentage of patients who recur and progress by about 15% and 4%, respectively (Sylvester et al., 2006).

Therefore, The EORTC prediction model requires external retrospective and prospective validation to compare the results obtained with those reported by Sylvester et al. (Table 3) to aid the therapeutic decision process (Sylvester et al., 2006).

Table 3: Probability of recurrence and progression according to total score (Sylvester et al., 2006).

Recurrence score	**Prob recurrence 1 year (95% CI)**	**Prob recurrence 5 years (95% CI)**
0	15% (10%, 19%)	31% (24%, 37%)
1-4	24% (21%, 26%)	46% (42%, 49%)
5-9	38% (35%, 41%)	62% (58%, 65%)
10-17	61% (55%, 67%)	78% (73%, 84%)
Progression score	Prob progression 1 year (95% CI)	Prob progression 5 years (95% CI
0	0.2% (0%, 0.7%)	0.8% (0%, 1.7%)
2-6	1.0% (.4%, 1.6%)	6% (5%, 8%)
7-13	5% (4%, 7%)	17% (14%, 20%)
14-23	17% (10%, 24%)	45% (35%, 55%)

CUETO has developed a scoring model for patients treated with BCG therapy that predicts the risk of recurrence and progression in the short and long term. This model was based on the analysis of 1,062 patients from four CUETO trials to compare different intravesical BCG treatments. Using the CUETO model, recurrence and progression rates can be calculated with a score of 0 to 16 and 0 to 14, respectively. Fernandez-Gomez et al. reported that the estimated risk of recurrence is lower than that obtained by the EORTC tables. The lower risks in the CUETO tables (Table 4) may be attributed to the use of BCG in their cohorts, which is an effective endovesical treatment (Fernandez-Gomez et al., 2009)**.**

Table 4: Recurrence and progression probabilities at 1, 2 and 5 years by total score (Fernandez-Gomez et al., 2009)

Score	% 1 Yr (95% CI)		% 2 Yrs (95% CI)		% 5 Yrs (95% CI)	
	Recurrence	**Progression**	**Recurrence**	**Progression**	**Recurrence**	**Progression**
0-4	8.24 (5.91–10.57)	1.17 (0.15–2.19)	12.6 (9.76–15.44)	2.16 (0.77–3.55)	20.98 (17.33–24.63)	3.76 (1.9–5.62)
5-6	12.07 (7.95–16.19)	3 (0.82–5.18)	22.28 (16.93–27.63)	4.97 (2.34–7.6)	35.57 (29.18–41.96)	11.69 (7.57–15.81)
7-9	25.36 (19.56–31.16)	5.55 (2.73–8.37)	39.61 (32.93–46.29)	11.95 (7.93–15.97)	47.65 (40.55–54.75)	21.26 (15.85–26.67)
10 or Greater	41.79 (28.05–55.53)	13.97 (6.64–21.3)	52.55 (38.48–66.62)	24.81 (15.6–34.02)	67.61 (53.67–81.55)	33.57 (23.06–44.08)

This model calculates the probability of recurrence and progression of patients after intravesical adjuvant BCG immunotherapy with similar maintenance, at 1, 2 and 5 years, based on the analysis of seven prognostic factors such as gender, age, prior recurrence, number of tumors, stage, grade, Carcinoma in situ CIS. This model is available at: https://www.aeu.es/Cueto.html (Figure 10) (Fernandez-Gomez et al., 2009).

Predicción de Recurrencia y Progresión de Tumor Vesical No Musculo-Invasivo Tratado con Bacilo de Calmette-Guerin: El Modelo CUETO de Puntuación English

Factor		**Puntuación**	
		Recurrencia	**Progresión**
Sexo	◉ Hombre	0	0
	○ Mujer	3	0
Edad (años)	◉ Menor que 60	0	0
	○ De 60 a 70	1	0
	○ Mayor que 70	2	2
Tumor recurrente	◉ No	0	0
	○ Sí	4	2
Nº tumores	◉ Menor o igual a 3	0	0
	○ Mayor que 3	2	1
Categoría T	◉ Ta	0	0
	○ T1	0	2
Tis asociado	◉ No	0	0
	○ Sí	2	1
Grado	◉ G1	0	0
	○ G2	1	2
	○ G3	3	6
Puntuaciones totales		**0**	**0**

Probabilidad de recurrencia y progresion a 1, 2 y 5 años por puntuación total

Tiempo	**Recurrencia (0-4)**	**Progresión (0-4)**
	Prob. (%) I.C. 95% (L.Inf-L.Sup)	**Prob. (%) I.C. 95% (L.Inf-L.Sup)**
1 año	**8.24 (5.91-10.57)**	**1.17 (0.15- 2.19)**
2 años	**12.60 (9.76-15.44)**	**2.16 (0.77- 3.55)**
5 años	**20.98 (17.33-24.63)**	**3.76 (1.90- 5.62)**

Figure 14: Club Urologico Espanol de Tratamiento Oncologico. The scoring system allow estimation of the probability of recurrence and progression bladder cancer treated with BCG, based on risk factors (Fernandez-Gomez et al., 2009)

Both the EORTC and CUETO risk tables stratified patients into four risk groups based on an analysis of clinical trial data. Some multi-institutional validation studies have reported some limitations in their predictive abilities (Lammers et al., 2013; Xylinas et al., 2013).

Lammers et al reported that these models underestimate the progression rate, specifically for high-grade tumors (EORTC =21%, CUETO =24%) (Lammers et al., 2013). Xylinas et al. have carried out an external validation of the usefulness of these models by performing a retrospective analysis of data of 4689 patients with NMIBC. They reported that the EORTC risk tables and the CUETO scoring model showed poor discrimination for recurrence and progression and both models overestimated the risk of progression, particularly in high-risk NMIBC patients (Xylinas et al., 2013).

IV. Molecular markers for bladder cancer management

Cystoscopy and urine cytology remain the mainstay of both diagnosis and surveillance of bladder cancer. The main limitation of cystoscopy is an invasive procedure, time-consuming and expensive. Moreover, the overall sensitivity of urine cytology is low for the detection of low-grade tumors and recurrence (Raitanen et al., 2001; Lotan & Roehrborn, 2003). To overcome the limitations of these two procedures, an additional, more cost-effective, sensitive and specific non-invasive diagnostic test is needed. Urinary biomarkers have been evaluated as alternative tests for their ability to detect urothelial carcinoma by analysing protein and nucleic acid (Lee & Kim, 2020). Indeed, molecular urine tests can replace cystoscopy during the follow-up of patients after TURBT (Schmitz-Dräger et al., 2016). Also, urinary biomarkers are sufficiently accurate to help assess response to neoadjuvant chemotherapy, adjuvant chemotherapy, or intravesical instillation (Kamat et al., 2012).

What is a biomarker?

A biomarker is an objectively measured characteristic that can be evaluated as an indicator of normal biological; It can also be used to detect and confirm the presence of various diseases or pharmacological responses to a therapeutic intervention. Biomarkers can be detected and measured in target tissues (tissue biomarkers) or body fluids (soluble biomarkers). An ideal biomarker should be: Specific to a particular disease; safe, rapid, easy to measure; able to give accurate results (Harris et al., 2007).

IV.1 Biomarkers approved for clinical use

Many novel urinary biomarkers tests have been identified and approved by the United States Food and drug administration (FDA) for clinical use in conjunction with cystoscopy, as potential diagnosis and surveillance tools (Sugeeta et al., 2021), such as:

- The Nuclear Matrix Proteins NMP22 kit (Qantitative) or NMP22 BladderChek (Qualitative), that targets the NMP22 protein, which is more prevalent in BC, is the most investigated biomarker as an assay in both diagnosis and recurrence of BC (Sugeeta et al., 2021). Quantitative NMP22, which is used for surveillance, has a sensitivity of 52-59% and specificity of 87-89% and qualitative NMP22, which is used for diagnosis, has a sensitivity of 62-75% and 70-83% in specificity (Chou et al., 2015; Chakraborty et al., 2019).
- Bladder Tumor Antigen (BTA) test for BC surveillance in conjunction with cystoscopy only which is approved by FDA. This test detects human complement factor H and complement factor H-related proteins involved in cancer cell growth (Sugeeta et al., 2021). The specificity and sensitivity of the BTA Stat (Qualitative test) are 67% and 75%, respectively (Guo et al., 2014), while those of BTA TRAK (Quantitative test) are 74% and 65%, respectively (Chou et al., 2015).
- ImmunoCyt/uCyt+ assay is the only commercially available test for therapeutic follow-up. Exfoliated BC cells are captured using the carcinoembryonic antigen and mucins (LDQ10 and M344) (Sugeeta et al., 2021). Lokeshwar et al. found an overall specificity ranging from 62% to 84% and a sensitivity ranging from 67% to 100% (Lokeshwar & Soloway, 2001).
- The UroVysion assay is a molecular test using a multi-target fluorescence *in situ* hybridization assay to detect aneuploidy in chromosome copy numbers 3, 7, 17, and 9p21 and to identify loss of the P16 tumor-suppressor gene, which are genetic abnormalities seen in BC (Sugeeta et al., 2021). UroVysion showed the sensitivity and specificity of 57.1-84% and 78-92%, respectively. The sensitivity of these tests is superior to that of urine cytology, especially for low-grade BC (Shariat et al., 2004; Mowatt et al., 2010; Schmitz-Dräger et al., 2016).

IV.2 Promising protein biomarkers

ADXBLADDER assay is performed to detect mini-chromosome maintenance protein 5, which is overexpressed in hematuric BC patients (Mukhtar & Perry, 2011). Dudderidge et al calculated

an overall sensitivity and specificity of 76% and 69%, respectively, although the sensitivity increases up to 95–97% for high-risk and muscle-invasive BC (Dudderidge et al., 2020).

Uro17 is an immunocytochemical test using Keratin 17 (K17). This test is able to capture low- and high-grade cancers in patients with haematuria. It is the most sensitive and specific urine test for BC, with a sensitivity and specificity of (100%) from urine samples (Babu et al., 2019).
UroSEEK, a DNA biomarker assay, is more sensitive than cytology in both surveillance (71% vs. 25%) and diagnostics (95% vs. 43%). This new test is designed to detect genetic alterations in *TERT, FGFR3, PIK3CA, TP53, HRAS, KRAS, ERBB2, CDKN2A, MET, MLL,* and *VHL* that include the most common genetic alterations in BC (Springer et al., 2018; Rodriguez Pena et al., 2020).

The Assure MDX panel assay is a non-invasive, urine-based test that may detect methylation and mutation biomarkers. It is a DNA methylation biomarker test using next-generation PCR sequencing for *OTX1, ONECUT2* and *TWIST1 genes* (Kessel et al., 2017). It measures the mutational load of the *FGFR3, TERT,* and *HRAS* genes panel with a sensitivity ranging from 93 to 97% and a specificity of 83 to 86% (Beukers et al., 2017).

Telomerase plays a protective role in cancer cell chromosomes. This biomarker has recently been proposed as a reliable marker of bladder malignancies because it is associated with the NMIBC recurrence (Masutomi & Hahn, 2003). The telomerase Repeat Amplification Protocol assay is a popular method, which includes an Internal Telomerase Assay Standard for determining telomerase activity. The combination of telomerase activity measurement and cytology produces an increased sensitivity of 60–87% and a specificity of around 65–90% compared to urine cytology alone (Brems-Eskildsen et al., 2010).

IV.3 Future urinary biomarkers

In recent years, Advances in the next generation sequencing technologies have identified a variety of somatic mutations associated with urothelial BC and have significantly changed therapeutic guidelines for the selection of molecular target therapies. These Include the *FGFR3-TACC3* fusion that acts as an oncogene in pathways such as the mitogene-activated protein Kinase (*MAPK*) pathway and the phosphoinositide 3-Kinase (*PI3K*) pathway in solid tumors (Costa et al., 2016), and the receptor tyrosine kinase-*RAS* pathway including *FGFR3* and *Erb-B2* Receptor Tyrosine Kinase 3 (*ERBB3*), and in the *PI3K-RACα* serine/threonine-protein kinase

(*AKT*) mechanistic target of rapamycin (*mTOR*) pathway, including *PIK3CA* and *AKT3* (Costa et al., 2016).

Urine-based DNA-methylated genetic biomarkers have shown potential in BC detection and monitoring. Detection of abnormal methylation of urinary Twist Family BHLH Transcription Factor 1 (*TWIST*) and Nidogen 2 (*NID2*) using methylation specifique pcr (MSP) confers a high sensitivity (90%) and specificity (93%) for the detection of urothelial carcinoma (Renard et al., 2010). A previous study reported that urine cytology with the addition of the *TWIST1* and *NID2* assay enhances the ability to detect BC, and the test is sensitive to the classification of equivocal cytology (Fantony et al., 2017).

The Zinc Finger Protein 154 (*ZNF154*) and POU Class 4 Homeobox 2 (*POU4F2)* genes have yielded the highest sensitivity in early diagnosis or recurrence surveillance of BC (>80%) (Koch et al., 2018). The *VIM* gene is a promising diagnostic biomarker for BC, achieving a sensitivity of 73%-82% and a specificity of 61%-100%. Its combination with Growth Differentiation Factor 15 (*GDF15*) reached a sensitivity of 91% and a specificity of 100% (Larsen et al., 2019).

There are numerous limitations that must be considered when assessing urinary biomarkers to avoid a poor quantity and quality of cell and DNA molecules, including time between sampling and processing and specialized detection tools like sedimentation PCR methodology. The urine samples should be stored at temperatures between -20 and −80 °C for four weeks, which is the recommended temperature for storage of DNA material (Kessel et al., 2017).

IV.4 Genetic Biomarkers evaluated in this study

Due to the genetic instability and molecular heterogeneity of BC, the search for different treatments and diagnosis methods is ongoing. Research has been carried out to identify the hotspot mutations in various types of genes, including *TERT*, *P53* and *PIK3AC/AKT* genes, which have been shown to occur frequently in urothelial bladder carcinoma and can serve as potential biomarkers for BC diagnosis and disease monitoring (Muller & Vousden, 2013; Vinagre et al., 2013; Kachrilas et al., 2019). On the other hand, alterations in DNA methylation were the first and most significant epigenetic abnormalities and may contribute greatly to the pathogenesis and progression of BC. Hypermethylation of the promoter region of the *HTERT, VIM2, TWIST1* and *NID2* genes have been reported in several studies and highlight their major role as regulator in the transduction of epithelial-mesenchymal transition (EMT) signaling that

is involved in the invasion and metastasis of BC (Satelli & Li, 2011; Sui et al., 2013; Yegin et al., 2013; Brabletz et al., 2021).

IV.4.1 Telomere and Telomerase

DNA (deoxyribonucleic acid) is the cell's genetic material arranged in chromosomes, located in the nucleus in the middle of the cells. Telomeres are DNA segments located at the ends of the chromosomes in eukaryotic cells. These telomeres play a protective role by shielding the natural ends of the chromosomes from inappropriate recombination events. They also play an important role in ensuring that our DNA is copied correctly when cells divide, thus preventing the loss of genetic information. In addition to this protective function, they also participate in the nuclear organization and correct segregation of chromosomes during meiosis (Oganesian & Karlseder, 2009). Human telomeres lose approximately 50 to 200 base pairs (bp) with each cell division. This loss of telomeric DNA is due in part to the fact that DNA polymerase cannot fully replicate the ends of chromosomes (Muraki et al., 2012). In normal somatic cells, this incomplete replication of the DNA end leads to a progressive shortening of telomeres, which induces cell cycle arrest by senescence and apoptosis (Harley et al., 1990).

1. Telomere structure

In human cells, telomeres are formed by a six-nucleotide repeat sequence "5′-TTAGGG-3". The length of telomeres changes between 5 and 15 kb for the double-strand region and between 200 and 300 bases for the single-strand region, named the "3'G-overhang". To protect these ends of chromosomes from being recognized by DNA repair machinery as double-strand DNA breaks, telomeres may adopt specific unusual conformations: The telomeric "G-quadruplex" and the "T-loop (T-loop/D-loop)" thus protecting chromosome ends from nucleolytic degradation by nucleases and from uncontrolled recombination by DNA repair enzymes and checkpoint signaling (Lange, 2002) (Figure 11).

G-quadruplexes are a non-canonical nucleic acid structure; a telomeric DNA repeat is looped out, forming a "hairpin" structure in which four guanines assemble in a planar arrangement to form a G-quadruplex by hydrogen bonds (Sundquist & Klug, 1989). These G-quadruplex structures induce the formation and stabilization of the T-loop, thus representing a stabilized displacement loop (D-loop) at the end of the chromosome (Yan et al., 2007).

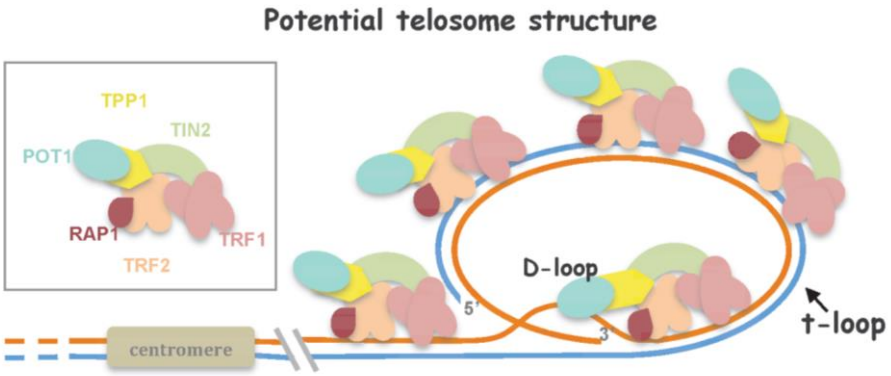

Figure 15: Structure of the complex "G-quadruplex" and the "T-loop (T-loop/D-loop)" The G-quadruplexes in the figure are composed of three stacked G-quartets (Boutou et al., 2013).

2. Telomere-associated proteins

Telomeric structures comprise telomeric DNA, histone octamers and many proteins that bind to telomeric DNA. Six proteins assemble to form the shelterin complex that contains a protective "cap" and has a crucial role in regulating telomere length (McEachern et al., 2000).

The shelterin complex is composed of 6 proteins in human cells: TRF1 (Telomeric Repeat-Binding Factor 1), TRF2 (Telomeric Repeat-Binding Factor 2), TIN2 (TRF1-Interacting Nuclear Factor 2), POT1 (Protection of Telomere 1), TPP1 (TIN2 and POT1 interacting Protein) and RAP1 (Repressor Activator Protein 1). The proteins of the shelterin complex are abundant at the chromosome ends, and are present at telomeres throughout the cell cycle, providing more stability to the telomere loop (Figure 12) and protecting the telomere ends (Martínez & Blasco, 2011).

- TRF1 and TRF2 make high-affinity contact with the double-stranded part of DNA and play a crucial role in protecting telomeres and regulating their lengthening. Indeed, TRF1 is proposed to act as a negative regulator of telomere length by inhibiting telomerase activity at the chromosome ends. At the same time, TRF2 plays an important role by promoting T-loop stabilization that protects chromosome ends (Martínez & Blasco, 2011).
- POT1 specifically binds to the single-stranded array of TTAGGG repeats by interacting with other shelterin proteins (TPP1, TIN2, TRF1 and TRF2). Its overexpression induces telomere elongation in cells with inhibited telomerase activity, whereas in telomerase-positive cells, POT1 prevents telomerase access to the end of the telomere while inhibiting its extension (Martínez & Blasco, 2011).

- TIN2 binds between TRF1 and TRF2 on the one hand and TPP1 on the other. This protein plays a regulatory role by promoting TRF1 interaction with DNA. TIN2 stimulates telomerase recruitment into telomeres by stabilizing the TPP1-POT1 interaction with telomerase, as it relies on TPP1-TERT interaction (Pike et al., 2019).
- TPP1 contains an N-terminal OB-fold and is a previously identified binding partner of POT1. The TIN2-TPP1-POT1 complex is a telomerase processivity factor that regulates telomerase access to the chromosome end (Martínez & Blasco, 2011).
- RAP1 is one of the most conserved Shelterin proteins; this protein is recruited to telomeres via direct interaction with the TRF2 subunit and plays an important role in protecting telomeres from chromosome fusions and degradation by inhibiting DNA break repair mechanisms (Martínez & Blasco, 2011).

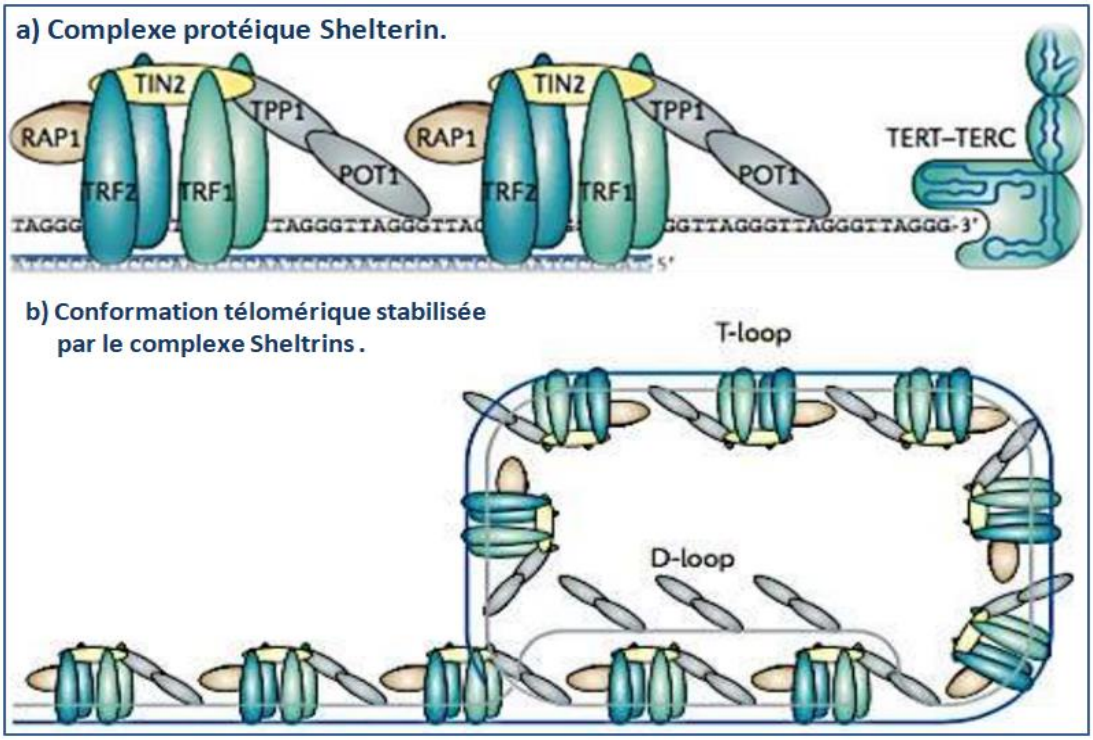

Figure 16: Schematic representation of the Sheltrins protein complex at the telomere. a) Distribution of the 6 proteins of the complex on the telomeric DNA. b) protein interactions between the Shelterin complex (Martínez & Blasco, 2011).

3. Telomerase complex

Telomerase, a ribonucleoprotein reverse transcriptase, stabilizes telomere length by adding TTAGG repeats to the telomeric ends. Human telomerase is expressed only in some types of stem cells and germ cells and is inactive in normal somatic cells. It is composed of a catalytic reverse transcriptase subunit hTERT (human Telomerase Reverse Transcriptase) and a

ribonucleic subunit TERC or hTR (human Telomerase RNA component) and other associated proteins (Weinrich et al., 1997).

- **TERC or hTR ribonucleic subunit**

The *TERC* gene is mapped on the long arm (q) of chromosome 3 (3q26.2.) (Soder et al., 1997). containing a short sequence of 11 bp (5'-CUAACCCUAAC-3') complementary to the 3'-single-stranded end of telomeres that encodes for telomeric repeat TTAGG (Zhang et al., 2011). The synthesis of telomeric DNA TAGGG depends on the association between the two telomerase subunits "hTR" and "hTERT" by the intervention of a protein complex that is bound to the catalytic subunit hTERT. Indeed, this protein complex contains the TEP1 protein subunit, an RNA-binding protein associated with the TERC and TERT subunits, and plays an essential role in the assembly and regulation of telomerase activity (Liu et al., 2000). Dyskerin is a nucleolar protein that participates in the folding and processing of the maturation of TERC, which is necessary to allow stability and proper localization of the TERT-TERC complex (Penzo et al., 2015). p23 and Hsp90 are proteins that bind to hTERT and promote active telomerase assembly (Boltze et al., 2003).

- **Catalytic subunit hTERT**

***hTERT* gene**

In humans, the *hTERT* gene, which encodes the catalytic subunit of telomerase, is situated on the short arm of chromosome 5 (5p15.33) and is subdivided into 16 exons and 15 introns. The *hTERT* transcript encodes an active 1132 amino acids (127 kDa) protein (Wick et al., 1999). TERT has four conserved functional domains, including the TEN domain with the GQ motif, the TRBD domain with the CP, QFP and T motifs, and the RT domain with the 1, 2, A, B′, C, D and E motifs, the N-terminal linker and the CTE region (Autexier & Lue, 2006). *TERT* is the determinant of telomerase activity. It is highly expressed in germ cells and stem cells but not in most normal somatic cells. *hTERT* expression/telomerase is reactivated in up to 90 % of cancers (Masutomi & Hahn, 2003).

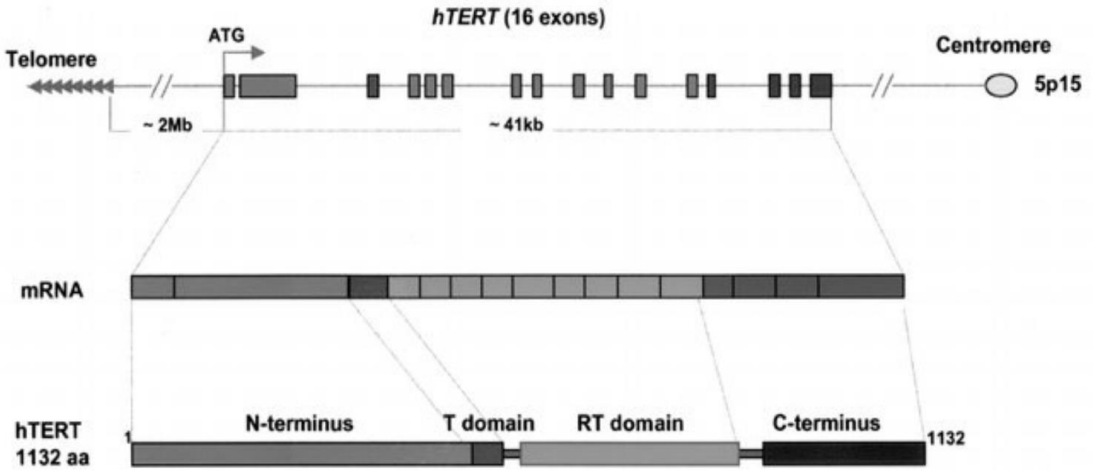

Figure 17: Gene organisation of the hTERT gene (Cong et al., 2002)

4. Genetic regulation of *hTERT*

The transcriptional control of the *hTERT* gene represents the major mechanism to regulate telomerase activity in human cells. Several transcription factors may be involved in *hTERT* expression; the majority is the binding of activating: c-myc, Sp1, STAT, Ets or ER and NFκB receptors (Pestana et al., 2017) or repressing (WT1, p53 and CTCF) transcription factors in the promotor region (Yao et al., 2012).

hTERT gene expression is regulated by Myelocytomatosis viral oncogene (c-Myc) protein. The formation of the Myc/Max complex favors its binding to the E-box sequence (CACGTG), which is an indispensable element for the binding of upstream stimulating factors (USF1/2), leading to TERT transcription (Pestana et al., 2017).

Estrogens can also activate telomerase via direct and indirect effects on the *hTERT* promoter. Indeed, c-myc is indirectly stimulated by estrogen, which is largely modulated by the Estrogen receptors (ER) subtypes, promoting activation of the *PI3 Kinase / AKT* pathway and consequently leading to increased *c-myc* expression. The activating role of *NF-κB* on the *hTERT* gene is mediated by binding to the *TERT* promoter, which contains an *NF-κB* binding site, it is not yet clear, but it is able to enhance *c-Myc* expression driving *TERT* transcription. The *STAT5* is activated by phosphorylation via stimulation of interleukin 2 (IL-2), that can form the high-affinity to IL2 receptor and then leads to a phosphorylation cascade, which allows *TERT* transcription (Pestana et al., 2017).

Concerning inhibitors of hTERT transcription, Mad1 (MAX dimerization protein 1) was the first protein identified as a transcriptional repressor during a screening for hTERT regulators.

Mad1 forms a complex with the Max protein, which replaced c-myc/Max at the E-box of the promoter. C-Myc and Mad1 are involved differently in the regulation of TERT transcription because they bind to the same promotor sites (E-box) (Yao et al., 2012). Other proteins such as p53, p63, p73, E2F-1, WT-1 (Wilms' Tumor 1), CTCF (CCCTC binding factor), SIP1 (SMN-Interacting Protein 1), MZF2 (Myeloid specific Zinc Finger protein 2) and MEN1 (Multiple Endocrine Neoplasia type 1) have also been shown to play a role in *TERT* transcriptional repression (Yao et al., 2012).

The different transcription factors and gene activators, and inhibitors at the *hTERT* promoter are shown in Figure 13.

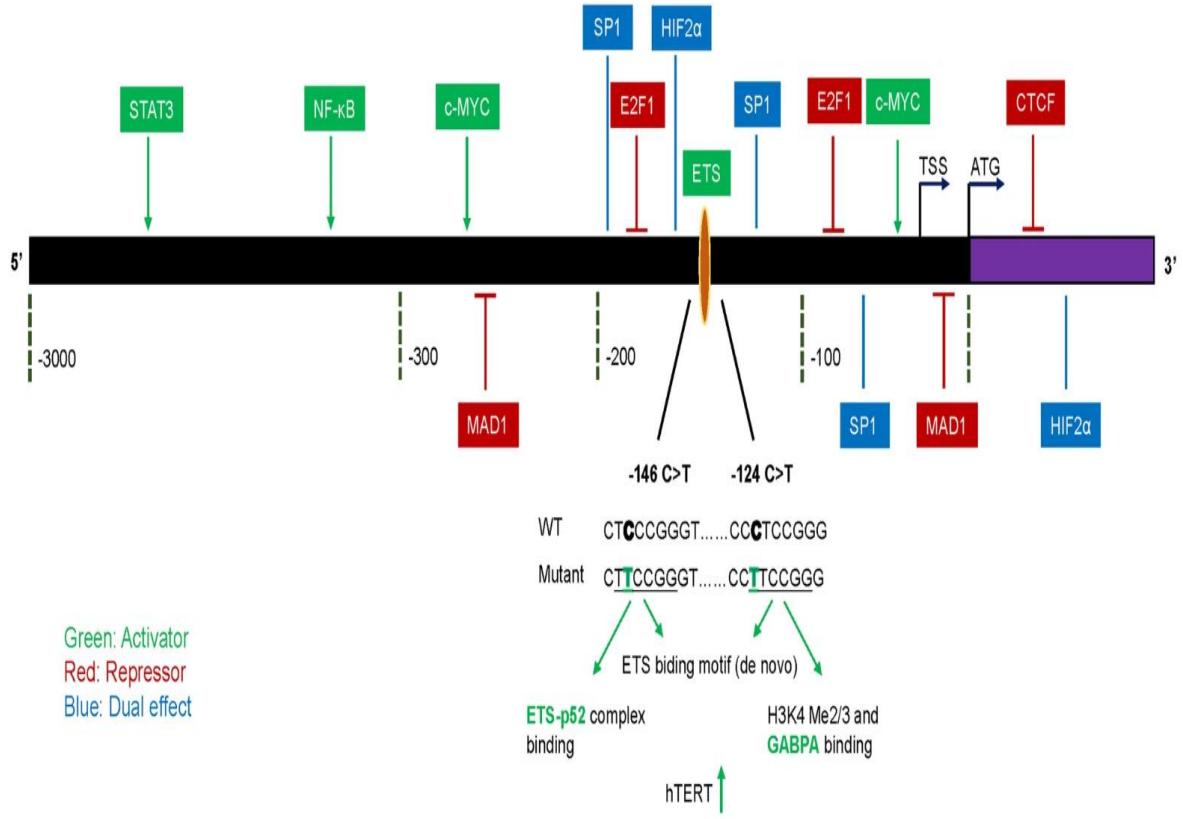

Figure 18: Transcription factors that regulate the expression of hTERT (Jie et al., 2019).

5. TERT reactivation mechanisms

Telomere maintenance mechanisms in cancer cells are numerous and diverse. *TERT* is regulated through various transcriptional, post-transcriptional and epigenetic regulations, but the exact mechanism that reactivates telomerase during carcinogenesis is unclear.

- Genetic induction

TERT somatic mutations are the most common non-coding mutations in many cancer cells in two key positions (C-228T and C-250T). In contrast, both mutations in *TERT* promoter generate a new ETS1 binding site and several native ETS/GABP consensus motifs. They are associated with increased *TERT* mRNA, causing transcriptional hyperactivation of telomerase (Akincilar et al., 2016). These *TERT* mutations are very common in glioblastoma and BC, being detected in up to 80% and 63%, respectively. (Griewank et al., 2013). In ovarian cancer (15%) and head and neck cancers (16%) (Vinagre et al., 2013). Therefore, point mutations in the *TERT* promoter could unravel a novel mechanism for *TERT* reactivation in cancer cells. Recurrent mutations have been identified in Chr.5:1,295,228(C>T) or (CC>TT), Chr.5:1,295,250 C>T, Chr.5:1,295,242_1,295,243CC>TT in 19 % of cancers (Vinagre et al., 2013).

- Epigenetic induction

DNA methylation, histone methylation, and histone acetylation are basic epigenetic mechanisms involved in the expression of *hTERT* (Moore et al., 2013; Sui et al., 2013).

Histone modifications: Acetylation leads to an upregulation of *hTERT* transcription accumulation and telomerase activity by allowing access to the transcriptional machinery via a local chromatin decondensation. On the other hand, histone methylation and hypermethylation may result in up- or down-regulation of *hTERT* gene transcription, depending on the methylated residues and the degree of methylation (Sui et al., 2013).

DNA methylation: Is one of several epigenetic mechanisms that participate in gene expression by recruiting proteins involved in gene regulation, chromatin formation and maintenance, and other fundamental processes. Concurrently, this chemical modification occurs via a specific enzyme: DNA methyltransferases (DNMT), which catalyzes the transfer of a methyl-CH3 group from an S-adenyl methionine (SAM) to the C5 position of cytosine residue to form 5-methylcytosine by the formation of S-adenosylhomocysteine (SAH) (Figure 14) (Moore et al., 2013).

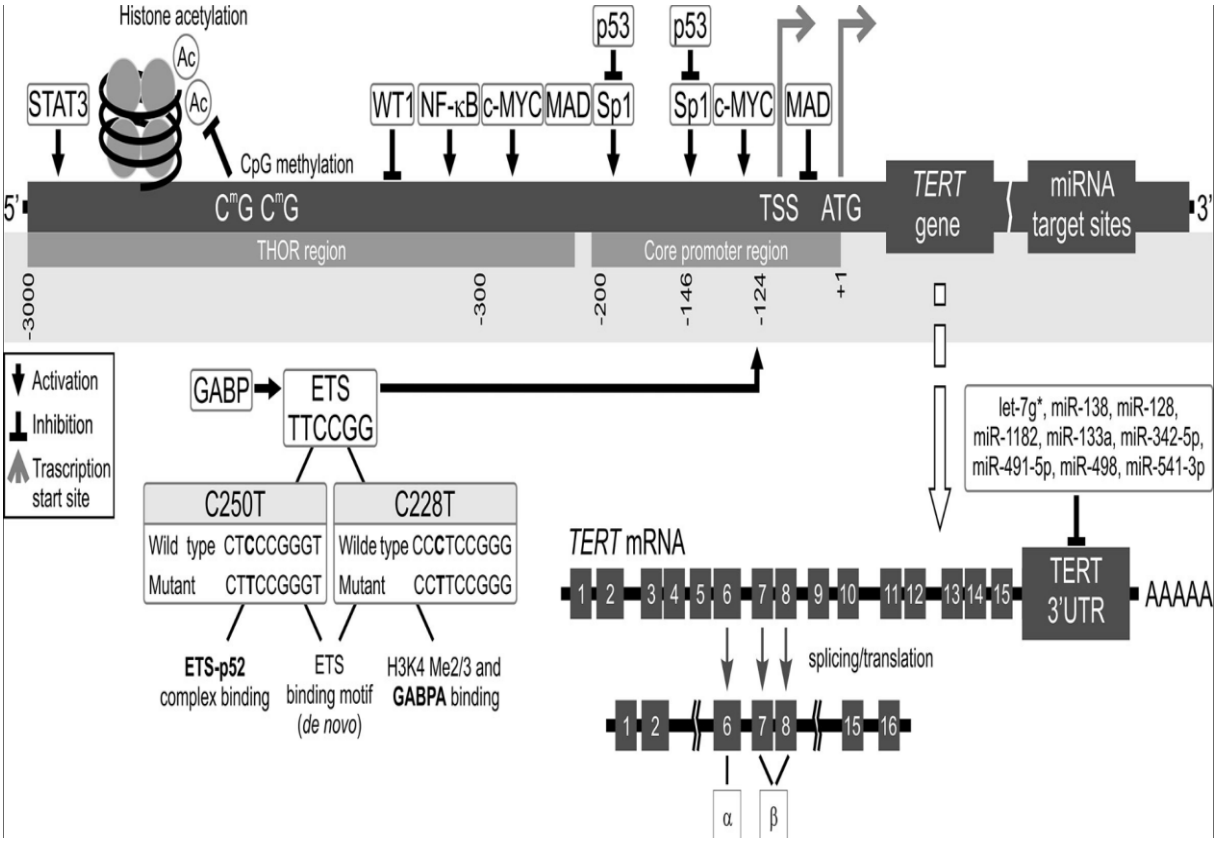

Figure 19: Mechanisms of TERT transcription regulation (Dratwa et al., 2020)

Modulation of the methylation profile of certain genes can cause their inhibition (Tumor suppressor genes) or their activation (proto-oncogenes). DNA methylation frequently occurs in the promoter regions of genes with a high concentration of Cytosine - Guanine dinucleotides constituting CpG islands. Usually, these CpG islands are demethylated and can activate the gene transcription. However, during tumorigenesis, hypermethylation of gene promoters occurs and might prevent the induction of transcription (Herman & Baylin, 2003).

Methylation of the CpG island in *hTERT* promoter region plays a crucial role in gene expression regulation. Some studies have demonstrated that DNA methylation on the *hTERT* promoter represses the expression of the *TERT* gene (Deaton & Bird, 2011).

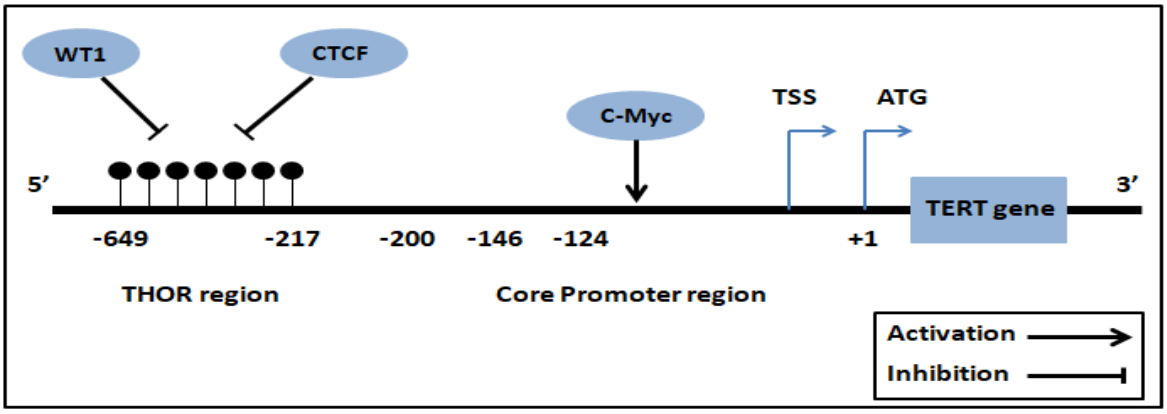

Figure 20: Mechanisms of hTERT regulation in cancer (Leão et al., 2018).

Hypermethylation of CpG sites prevents the binding of transcriptional repressors (WT1, CTCF) specifically to the THOR region. This induces activation of telomerase in malignant cells after binding some appropriate activating transcriptional factors such as Sp1 or estrogen and C-Myc (Figure 15) (Leão et al., 2018).

DNA methylation can control the binding of transcription factors (c-Myc) and *hTERT* repressor (WT1 and CTCF) to the *hTERT* promoter, in which methylated CpGs prevent their binding to target sites, leading to *hTERT* activation (THOR region) (Leão et al., 2018).

IV.4.2 Tumor Suppressor *p53*

The *Tp53* gene was discovered by Michel Kress in 1979, who identified the middle T of the SV40 virus with a molecular weight of approximately 53kDa, and was first classified as an oncogene (Kress et al., 1979). One year later, Michel Kress reported that the SV40 virus does not code the protein; he also found that this protein accumulates in the nucleus of tumour cells and binds the SV40 T antigen (Kress et al., 1979).

Since *Tp53* was initially considered as an oncogene, the year 1989 was a turning point in the history of *Tp53*. The demonstration of its inactivation in several types of human cancers and its capacity to control the cell cycle and induce apoptosis after DNA damage changed the status of *Tp53* to a tumour suppressor gene (Finlay et al., 1989; Baker et al., 1989).

1. *Tp53* gene

The human *Tp53* gene encoding for the P53 protein is located more precisely in the distal band of the short arm of the chromosome 17 (17p13) (McBride et al., 1986). It is composed of 11 exons, the first and second exons being separated by a very large 10 kb intron. In humans, the first exon is non-coding and exons 2-11 are coding exons (McBride et al., 1986). Two promoters have been identified in the *p53* gene, the first one, p53P1, is located 100 to 250 bp upstream of exon 1 and regulates transcription of FSp53 (transcript contains 11 exons) and p53l2 variants. The second one, p53p2, is located within the first intron. This p53p2 is a stronger promoter than p53p1 and maps within the 10.739-bp of the first intron (Figure 16) (Reisman et al., 1988).

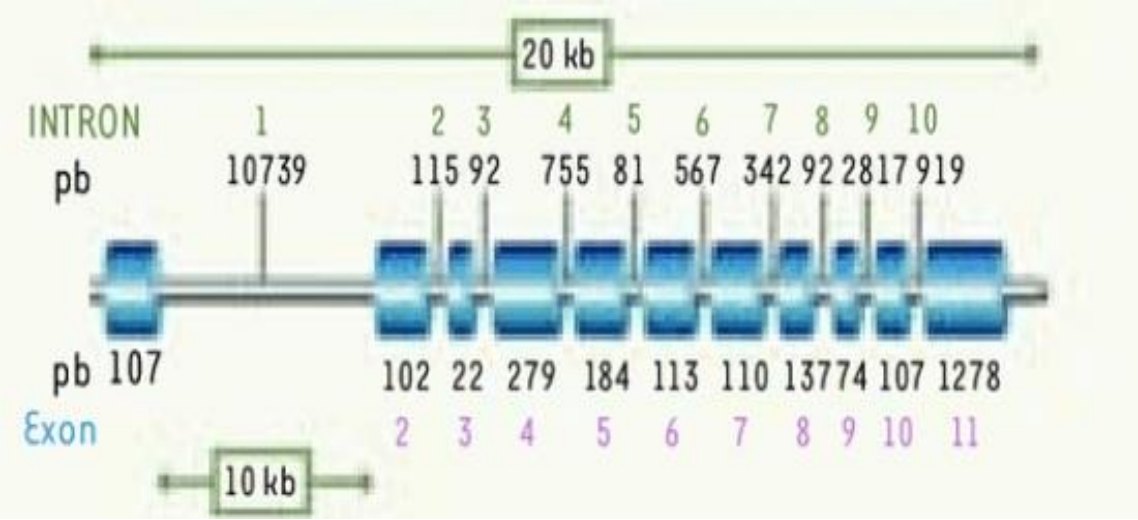

Figure 21 : Structure of the human p53 gene (Dridi et al., 2006).

2. P53 protein

The human P53 is a phosphoprotein consisting of 393 amino acids, which plays an essential role in several signaling pathways controlling various functions to maintain genomic integrity. It is an homotetramer transcription factor comprising 4 × 393 amino acid residues surrounded by a distinct amino group (NH2) and a carboxyl group (COOH). The P53 protein is composed of 5 different functional domains (Figure 17), including:

- The N-terminal domain: Contains its transactivation domain and allows the ability of p53 to bind to other target molecules in response to genotoxic stress by enhancing p53 activity, which is tightly linked to its tumor suppressive function. The N-terminus (1-67 residues) can be further subdivided into two linked Tandem Activation Domains (TAD): TAD I (residues 1-40) and TAD II (residues 41-67) (Joerger & Fersht, 2008). TAD I is a common transactivation domain that recruits general transcriptional coactivators;

TAD II appears to modulate the transactivation of specific target genes subsets mediated by TAD I (Raj & Attardi, 2017).

- The proline-rich region (residues 67-98) appears to have an important structural role and is conserved in the majority of P53. This region favors a polyproline II helix structure and serves as a rigid linker to release the TAD domain away from the DNA-binding domain to facilitate the binding of coactivators for transcriptional activation (Joerger & Fersht, 2010). The selective stabilization of p53 is impacted by mouse double minute 2 (MDM2), that inhibits the transcriptional activity of p53 and promotes its degradation (Batinac et al., 2003).
- The central core domain or DNA-binding domain (DBD) (residues 98-303): according to crystallographic studies, the DNA-binding domain contains an essential Zinc 2+ to form the Loop-sheet-helix motif that influences p53 DNA recognition and stability of DBD. Mutations mapped in this domain are thought to cause a conformational change in this domain, suppressing the role of p53 as a transcriptional activator (Okorokov & Orlova, 2009). Other studies have shown that the majority of mutations that occur in this region are often associated with an oncogenic function of p53 (Hafner et al., 2019).
- The oligomerization domain (residues 326-356) triggers a tetramerization mechanism essential for the activation of the p53 pathway (Kamaraj & Bogaerts, 2015). P53 tetramerization is crucial for its function to lead transcriptional activity and to upregulate arrest and apoptosis genes (Joerger & Fersht, 2010).
- The C-terminal domain: This end (residues 363-393) which is a flexible region, may act as a negative regulatory domain (Kamaraj & Bogaerts, 2015) and may also be involved in several other post-translational mechanisms, including acetylation, methylation, ubiquitination, phosphorylation. These modifications may modulate p53 function and control its apoptotic activity (Joerger & Fersht, 2010).

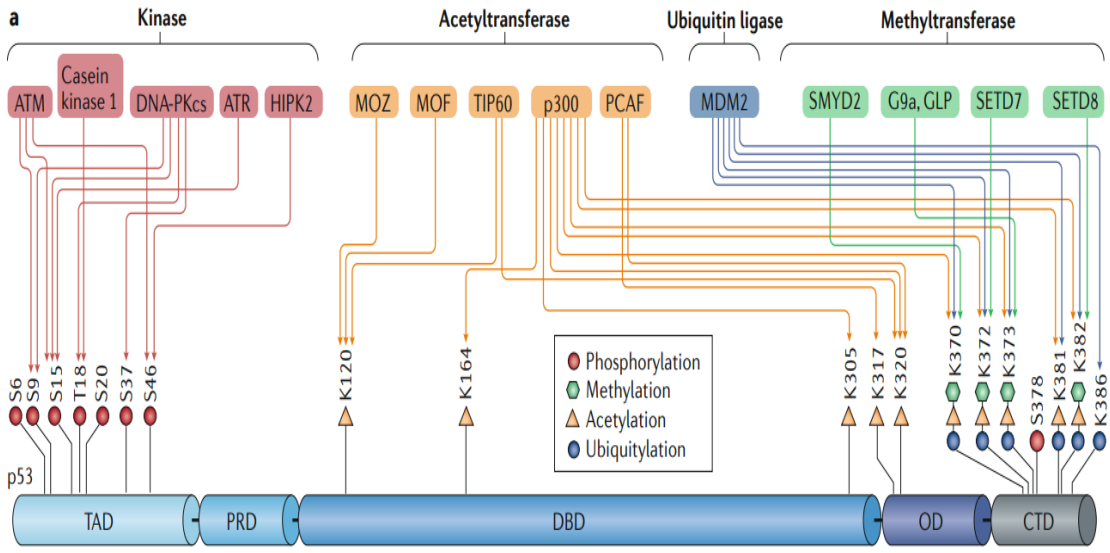

Figure 22: The different functional domains of the p53 protein (Hafner et al., 2019)

3. P53 function

P53 is commonly referred to as "the guardian of the genome" because of its crucial role in genomic stability. In normal cells, MDM2 participates in the regulation of p53 by promoting its ubiquitination by acting as an E3 ubiquitin ligase that induces its degradation. However, following cellular stress, including DNA damage, replicative stress induced by an oncogenic activation, phosphorylation can lead to the activation of p53 as a transcription factor. Consequently, MDM2 is inactivated. Indeed, p53 is able to halt cell proliferation in response to aberrant oncogene expression, suggesting the involvement of this tumor suppressor in inhibiting the accumulation of mutations in cancer-related genes (Kastenhuber & Lowe, 2017). Through this regulation, p53 has a variety of fundamental physiological roles, such as cell cycle arrest, DNA repair, cellular senescence and cellular metabolism.

4. *P53* mutations and cancer

In more than 50% of human malignancies, somatic changes involving the *p53* gene give rise to a stable mutant protein whose accumulation is considered a "hallmark of cancer cells". In fact, the majority of *p53* mutations in human cancer are missense mutations (up to 75% mutations) and are grouped in the DNA-binding-domain (4-9 exons) of *p53* genes (Liu et al., 2014). Consequently, these missense *p53* mutations are able to modify their ordinary role as a transcriptional factor. Previous studies have showed that mutant *p53* has oncogenic functions in the absence of wild-type *p53* in tissue culture systems. More specifically, mutant *p53* can form aberrant protein complexes with several interacting partners, which normally associate

with its wild type. This association provides their oncogenic functions, including the ability to promote invasion, angiogenesis, migration, stem cell expansion, survival, scattering, proliferation and tissue remodelling (Muller & Vousden, 2013).

There are three main categories of molecular mechanisms to explain the effects of mutant *p53* in tumor biology. These mechanisms reflect the alterations in DNA-binding capacity or changes in the binding abilities of mutant *p53* with other proteins, including other transcription factors or proteins not directly related to the regulation of gene expression (Muller & Vousden, 2013).

The great majority of these missense mutations are clustered within the central most conserved DNA binding region of *p53*; mutation can occur at almost any amino acid in this domain. The International Agency for Research on Cancer (IARC) is a popular source that contains occurrence and phenotype data of *TP53* germline and somatic variations in the DNA-binding domain of the protein. A list of the most 50 common missense mutations ranked by frequency in multiple human cancer derived in the IARC database shows that there are preferential mutations hotspots found in different cancer cells, indicating that the nature and sites of these mutation offer several clues as to how p53 function is affected (Bouaoun et al., 2016). Indeed, a number of hotspots (including R175, G245, R248, R249, R273 and R282) have been reported. Mutants altered the DNA-binding surface can be defined as contact mutations (R273H, R248Q and R248W). These mutations cause conformational instability of the protein are characterized as a structural mutation (R175H, G245S, R249S and R282H) (Joerger & Fersht, 2010).

Altered *p53* status represents one of the most common genetic events in bladder cancer, occurring in ∼50% of transitional cell carcinomas. Previous studies have shown that alterations in *p53* were higher in deep invasive tumors (stage T2 and high grade) than in NMIBC (low grade and stage Ta-T1) (Sidransky et al., 1991; Fujimoto et al., 1992). The disrupted pathways in association with *TP53* mutation status could serve as driver mutations in BC, which accelerate metastatic tumor cell proliferation, promote invasion ability and influences cancer prognosis and therapeutic strategy (Lang et al., 2004; The Oxford-Illumina WGS500 Consortium et al., 2014).

5. *Tp53* signaling pathway

In response to a wide variety of cellular stresses, such as DNA damage, hypoxia, or the presence of activated oncogenes, *Tp53* is reported to directly regulate a set of target genes involved in cell death, apoptosis, senescence, or growth arrest (Jan & Chaudhry, 2019).

- Apoptosis

The mechanism of apoptosis occurs through two core pathways, the intrinsic and extrinsic pathway. The intrinsic pathway (also known as the mitochondrial pathway) is regulated by BCL-2 proteins. TP53 stabilizes and can activate transcription of pro-apoptotic proteins of the Bcl-2 family and transcriptional repression of anti-apoptotic Bcl2 proteins that can inhibit this pathway. P53 can directly target both PUMA, PMAIP1 and Bax that successively stimulate the release of cyt C via MOMP, which uniquely encodes pro-apoptotic BH3 proteins. The expression of these genes is directly upregulated by p53 and may trigger rapid apoptosis in tumor cell lines (Chi, 2014). P53 is also involved in the extrinsic apoptotic pathway by inducing the transcription of genes encoding three transmembrane proteins: FAS, DR4, and DR5. Regulation of these death receptors is mediated by p53, which may increase their sensitivity to their cognate ligands, such as FASL and TNF (Chi, 2014).

- Cell cycle arrest

This mechanism is mainly mediated by the p21/WAF1 transcriptional activation complex. When p53 is activated by phosphorylation, it binds to two sites of size 2.4Kb and 1.4Kb, respectively, located upstream of the p21 promoter. Then, p21 binds to cyclin E/Cdk2 and cyclin D/Cdk4, causing inhibition of pRb, which promotes E2F/RB binding and prevents the G1/S transition (Kim et al., 2014).

In the G2/M phase, p21 can also contribute to block transcription of cyclin B/Cdc2, thus preventing activation of the cyclin B/Cdk1 complex necessary for the G1/M transition (Dash & El-Deiry, 2005). the ATM/CHK2/cdc25 or ATR/CHK1/cdc25 pathways are activated by p53 and can also inhibit this complex and thus cause cell cycle arrest (Thanasoula et al., 2012).

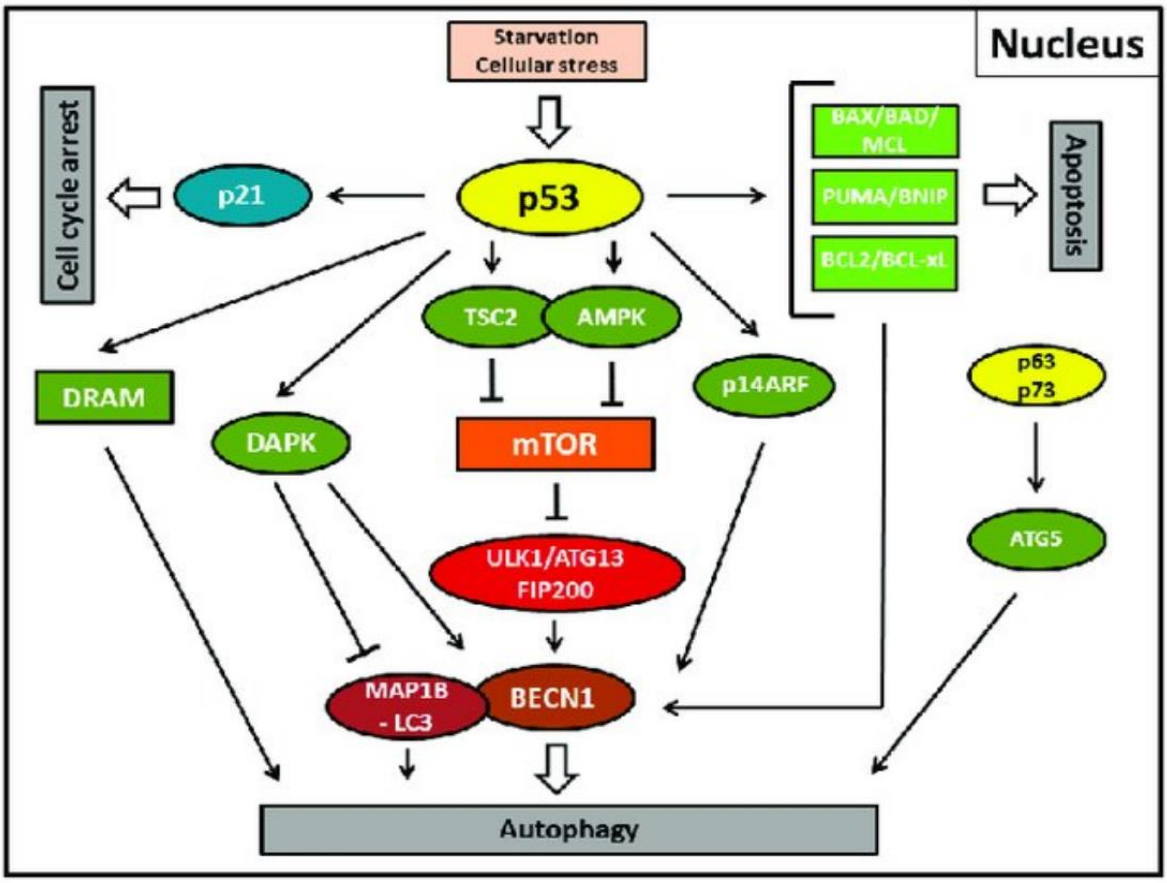

Figure 23: Autophagy, apoptosis, and cell cycle arrest mediated by the activity of nuclear p53 protein (Mrakovcic et al., 2018).

6. p53 in the DNA-Damage-Repair Process

Various DNA repair mechanisms are usually able to keep up with endogenous and exogenous genotoxic assaults to preserve the genetic material of cells. As a central tumor suppressor, p53 involves in a variety of repair machinery, according to each type of repair:

- Cell cycle checkpoint activation

DNA double-strand breaks by ionizing irradiation activate DNA-damage checkpoints that can induce both cell cycle arrest and apoptosis, through stabilization and activation of the p53 gene that induces the transactivation of the growth inhibitory p21. P21 binds and inhibits different CDK/ cyclin complexes, depending on different cell cycle transitions (el-Deiry et al., 1993)

- Transcriptional program activation by mismatch repair (MMR)

This mechanism is activated for nucleotides that have been erroneously inserted during replication. The relationship between p53 and MMR is centered on the MSH2 protein, a core component of MMR that is involved in other repair systems. P53 is involved in these DNA

repair pathways to modulate the sensitivity of cells to genotoxic agents. However, MSH2 in complex with MSH6 can enhance the binding between p53 with DNA substrates, depending on the phosphorylation status of P53. Thus, it is able to promote cell arrest and apoptosis (Zink et al., 2002).

- Transcriptional program activation by base excision repair (BER)

BER is a multi-enzymatic pathway with apurinic/apyrimidinic AP sites, and it is initiated by a DNA glycosylase that recognizes and removes the damaged bases. Several studies have demonstrated the relationship between p53 and AP endonuclease. The tumor suppressor promotes the BER pathway via its direct interaction with APE1/Ref1, that modulates the conformation of p53 and increases DNA binding. This pathway occurs during the G0/G1 transition of the cell cycle and inhibits the mutagenic effect caused by genotoxic agents (Offer et al., 1999).

- Transcriptional program activation by nucleotide excision repair (NER)

The NER system is involved to correct the most abundant DNA damage by the removal of an entire oligonucleotide of about 30 base pairs using a multiprotein complex, such as PCNA, DNA polymerase ε and δ, XPE, GADD45. These molecules are regulated by p53, which GADD45 provides a binding role to UV damaged chromatin and may affect lesion accessibility. This protein interacts with PCNA and also p21 to participate in DNA repair (Marteijn et al., 2014).

- Homologous recombination (HR)

HR begins by the activation of the Mre11/Rad50/Nbs1 protein complex, coordinates DNA repair, and promotes signaling events at double-strand breaks. Although p53 may play a crucial role in the fidelity of homologous recombination by specific mismatch recognition in heteroduplex intermediates. P53 can inhibit this recombination through interactions with Rad51. Inhibition of p53 activity promotes spontaneous and radiation-induced homologous recombination between direct and inverted repeats (Menon & Povirk, 2014).

- Non- Homologous End Joining (NHEJ)

The NHEJ pathway is a predominant repair system for the repair of DNA and can be activated in all phases of the cell cycle. However, this system can lead to errors due to the joining of incorrect ends. The exact regulatory role of p53 in NHEJ has not been fully understood, but it

has been shown to regulate NHEJ (Menon & Povirk, 2014). A previous study showed that inhibiting NHEJ by repression of XRCC4 and Ligase IV factors, DNA DSBs are not repaired, inducing apoptosis in a p53-dependent manner. The interactions between p53 and the different molecules that constitute this repair system influence the cell cycle (Gao et al., 1998). Genetic interactions between p53 and the other molecules that constitute this repair system and p53 downstream effects cell cycle progression and proliferation by inhibiting exonucleolytic proofreading or by inhibiting NHEJ (Menon & Povirk, 2014).

IV.4.3 PIK3CA/AKT1 pathway

1. PI3K protein

Phosphatidylinositol 3-kinase (PI3K) is a heterodimer composed of two subunits: a regulatory subunit (p85, PIK3R1) with an SH2 domains for recognition of phosphotyrosines of receptor tyrosine kinase and a catalytic subunit (p110, PIK3CA) with lipid kinase activity (Shull et al., 2012).

The 85 kD regulatory subunit of PI3K is encoded by the phosphoinositide-3-kinase regulatory subunit (PIK3R1) gene. The human PIK3R1 gene is found on chromosome 5, encompasses 86102 bp of DNA and contains 16 exons beginning with a noncoding exon 1. The catalytic subunit of PI3K is encoded by the *PIK3CA* gene. The human *PIK3CA* gene is located on chromosome 3q26.3; it is approximately 34 kb in length and consists of 20 exons encoding for 1068 amino acids and yielding a protein of 124 KDa. *PIK3CA* mutations are clustered across exons 9 and 20 (Figure 18), which encode for the helical domain of p110alpha and the kinase domain, respectively (Jiang & Liu, 2009).

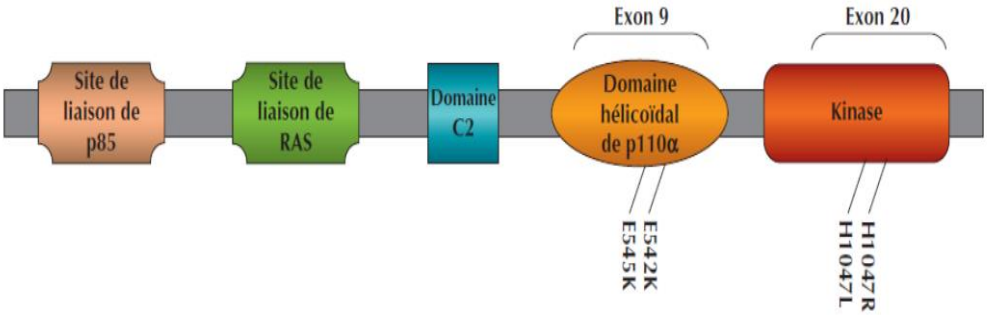

Figure 24: Structure of PIK3CA gene (Harlé et al., 2013).

2. *AKT1* gene

AKT serine/threonine Kinase 1 (*AKT1*) gene is found on chromosome 14, band q32, contains 14 exons encoding for 480 amino acid protein, and covers a genomic region of approximately 26.4 Kb; the open reading frame of the coding region is 1443 bp (Staal et al., 1988).

Protein Kinase B (PKB) is a member of a family of protein kinases that comprises three mammalian isoforms of PKB, PKBα (AKT1), PKBβ (AKT2), and PKBγ (AKT3). The AKT1 gene encodes the kinase B alpha (PKBα) protein of 480 amino acids and 55686 Daltons. PKB*α* possesses an amino-terminal pleckstrin homology (PH) domain that binds strongly to 3-phosphoinositides, a short *α*-helical linker (hinge region), a central catalytic domain (a kinase domain KD), and a C-terminal regulatory motif (Zhou et al., 2006).

3. PIK3CA/AKT signaling pathways

Protein kinase B (PKB, or Akt) plays a key role in a wide variety of cellular processes, including cell metabolism, growth, proliferation, survival, and inhibition of apoptosis. Its activation is modulated by a multi-step process implicating the phosphoinositide-3-kinase (PI3K) (Hemmings & Restuccia, 2012). The PIK3/AKT signaling pathway consists of numerous activators, inhibitors, effectors and second messengers. This pathway begins with the activation of phosphatidylinositol 3 kinase via its binding to insulin receptor substrate proteins. This activation of PIK3 leads to the formation of phosphatidylinositol (3,4)-bisphosphate (PIP2), which is a regulatory component of the membrane, PIP2 lipids are converted into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PKB/AKT binds to PIP3, leading to the activation of PKB/Akt. In normal cells, PKB/AKT is a proto-oncogene regulated or inhibited by the caspase cascade that leads to apoptosis (Hemmings & Restuccia, 2012).

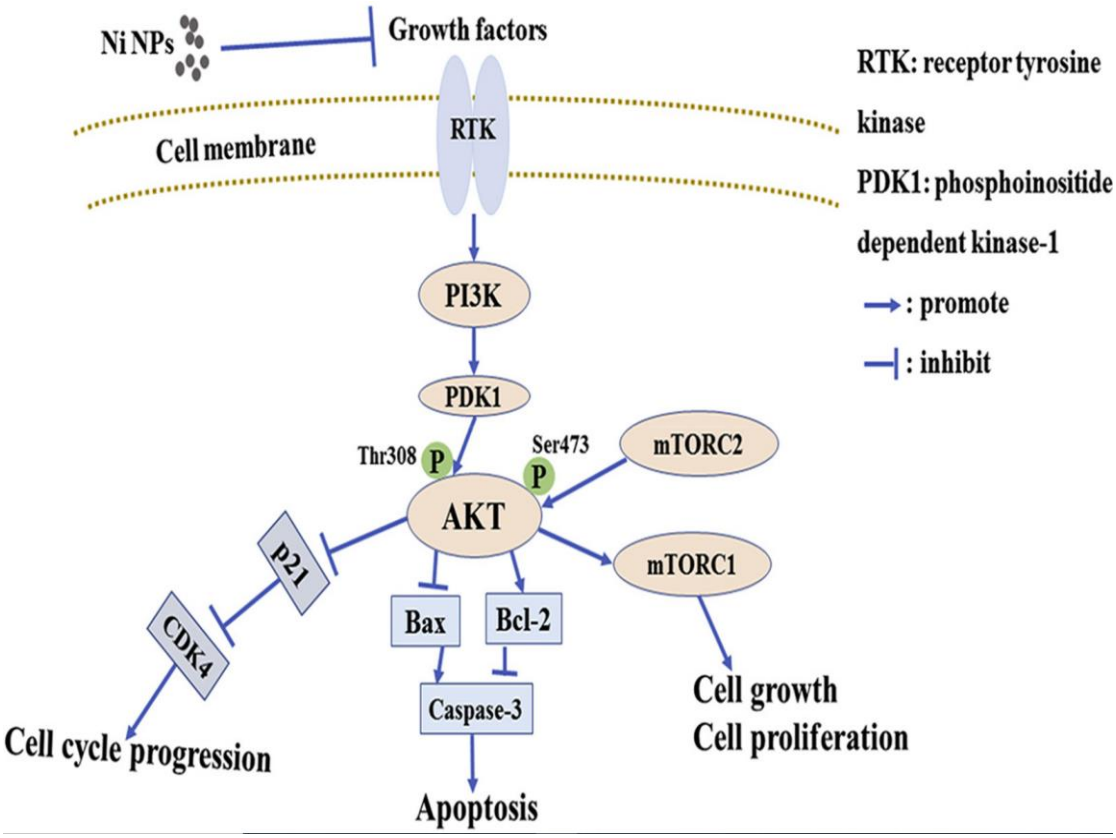

Figure 25: The PI3K/AKT/mTOR signaling pathway (Wu et al., 2020)

The PIK3/AKT pathway can be aberrantly activated via various mechanisms and is commonly observed in many human cancers, including bladder, lung, and prostate cancers. Hyperactivation of this pathway is closely related to tumor progression and resistance to cancer therapies (Morgan et al., 2009; Sarris et al., 2012; Kachrilas et al., 2019). In malignant cells, alterations of *PIK3*, *PKB/AKT*, or *mTOR* can induce their hyperactivation. Therefore, a multi-step protein cascade activates protein synthesis or translation. The cascade begins with the activation of the RHEB protein, which activates mTOR, the latter interacts and activates S6K1, which in turn phosphorylates the ribosomal protein S6 (S6/RPS6), promoting protein synthesis and cell proliferation (Hemmings & Restuccia, 2012).

IV.5 Epigenetic biomarkers evaluated in this study

IV.5.1 TWIST1

1. *Twist* gene and protein structure

The human *TWIST1* gene is located on the short arm of chromosome 7 (7p21.2). TWIST1 comprises two exons and one intron of 536 bp (Ghouzzi et al., 1997). The *TWIST1* gene encodes TWIST1 protein that consists of 202 amino acids, which are members of the basic helix-loop-

helix and zinc finger protein family. The TWIST1 protein is a transcription factor that is involved in cell growth, containing a basic helix-loop-helix (bHLH) portion and a tryptophan-arginine dipeptide (WR motif), a highly conserved part during development, located in the COOH terminal region (Spring et al., 2000).

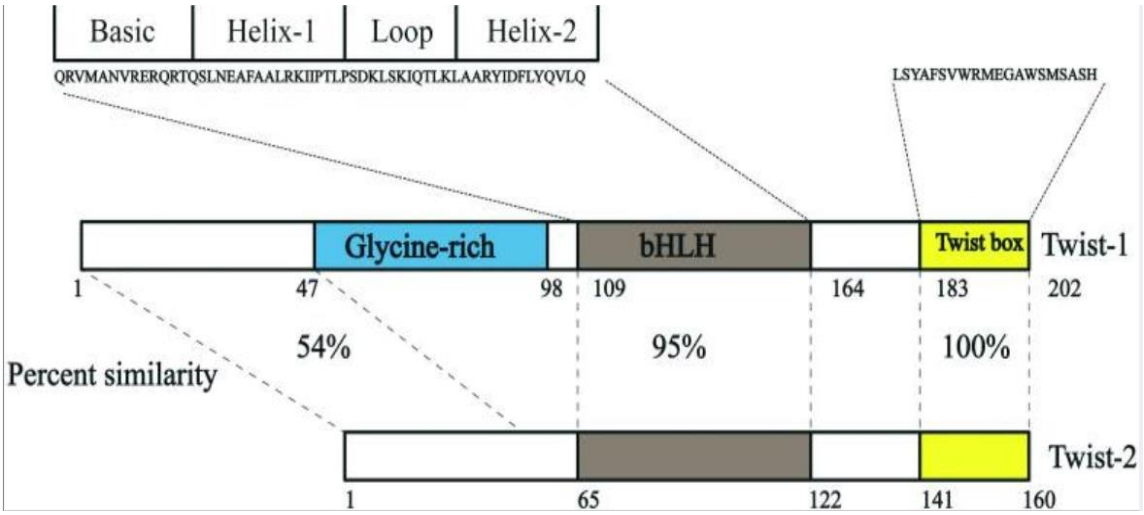

Figure 26: Functional domains of human Twist-1 and Twist-2 proteins (Pei et al., 2017)

This transcription factor comprises a DNA binding domain followed by a basic helix-loop-helix (HLH) motif. A loop domain separating the helices is important for the tertiary structure of a protein (Spring et al., 2000). The TWIST1 protein can form functional homodimer or heterodimer complexes with E12, Hand1, and Hand2, which allows it to play its role as a transcription factor by binding to the E-box consensus region (CANNTG) of the promoter (Qin et al., 2012).

The Twist1 protein includes the bHLH conserved domains in humans that consists of two α-helices dissociated by an inter-helical loop and an interaction domain called the "Twist box". The bHLH has an important role, illustrated by the fact that point mutation in this domain reduces TWIST1 DNA-binding capability (El Ghouzzi et al., 2001). TWIST1 also interacts with MyoD, a bHLH transcription factor that regulates muscle differentiation. TWIST1 inhibits MyoD and MEF2 functions through interaction with these genes, resulting in inhibition of muscle differentiation (Hamamori et al., 1997). The WR motif, also known as the twist box, is a highly conserved interaction domain in vertebrates; it is hypothesized that it is required for TWIST1 protein the folding and activity (Figure 19) (Gripp et al., 2000).

2. Twist and cancer

The TWIST1 protein is overexpressed in numerous cancers such as breast cancer, prostate cancer, bladder cancer, melanoma, colorectal cancer, hepatocellular carcinoma, and neck carcinoma. At the same time, *TWIST1* expression levels remain low in the normal cells (Puisieux et al., 2006). Furthermore, *TWIST1* expression level was associated with advanced stage, high grade and poor prognosis in bladder cancer patients (Yu et al., 2010). In bladder carcinomas, *TWIST1* expression is higher than in a normal urothelial cell. *TWIST1* is not very expressed in the low grades T1G1-G2 but is much more present in high-grade superficial T1G3. On the other hand, overexpression of this protein in advanced stages of bladder cancer may be a potential biomarker for disease aggressiveness (Zhang et al., 2007).

3. Genetic induction

TWIST1 expression is regulated to exert multiple biological effects by an array of different upstream and downstream regulators *via* multiple pathways depending on the type of cancer.

Stimulation of these pathways leads to T*WIST1* transcription by interacting with a variety of transcription factors in the nucleus. Signal transducer and activator of transcription 3 (STAT3), for example, is involved in the regulation of *TWIST1* via its binding to the second proximal STAT-3-binding site of *TWIST1* promoter and promotes its transcriptional activity (Ling & Arlinghaus, 2005). STAT3 can also indirectly modulate *TWIST1* transcription through HIF1 by directly binding to the Hypoxia-response element HRE of the *TWIST1* promoter (Cho et al., 2013). Other intracellular mediators in the nucleus and transmembrane receptors regulate the expression of *TWIST1* at the transcriptional level or *TWIST1* stability at the protein level (Zhao et al., 2017).

TWIST1 can also exert several biological effects through multiple downstream pathways, acting as transcription factors to regulate the expression of a set of target genes at the gene level in the nucleus (such as PDGFRα, YB1, MDR1, AKT2, N-cadherin, E-cadherin, ARF, p53, and CD24) or modulate effector function and stability (e.g. Jagged1, VEGF, mTOR, Bcl2, p53 and Bmi1) at the protein level in the cytoplasm (Zhao et al., 2017).

4. Hypermethylation and over-expression of TWIST1

Twist1 promoter hypermethylation is a prevalent event, mediating epigenetic reprogramming of Twist1 in many cancers of different origins, such as breast, bladder, gastric, colon and rectum, lung, ovary, and uterine cervix cancer. Hypermethylation of this gene plays an

important role in epithelial-mesenchymal transition (EMT) pathways (Khan et al., 2013). Yegin et al. (2013) reviewed that hypermethylation of *TWIST1* is a good predictor of human bladder cancer presence and was detected in 98.2% of bladder tumors (Yegin et al., 2013). Moreover, DNA methylation of *TWIST1* has been studied as a diagnosis and surveillance biomarker with a high sensitivity ranging between 87.5 and 90% for the detection of urothelial carcinoma. Still, its functional significance remains unknown (Renard et al., 2010; Yegin et al., 2013). Hypermethylation of the *TWIST1* gene Promoter may also suggest a role in silencing and repression of TWIS1 expression. Meanwhile, it is noteworthy that there was no correlation between hypermethylation of *TWIST1* promoter and *TWIST1* mRNA or protein expression (Gort et al., 2008; Auwera et al., 2010).

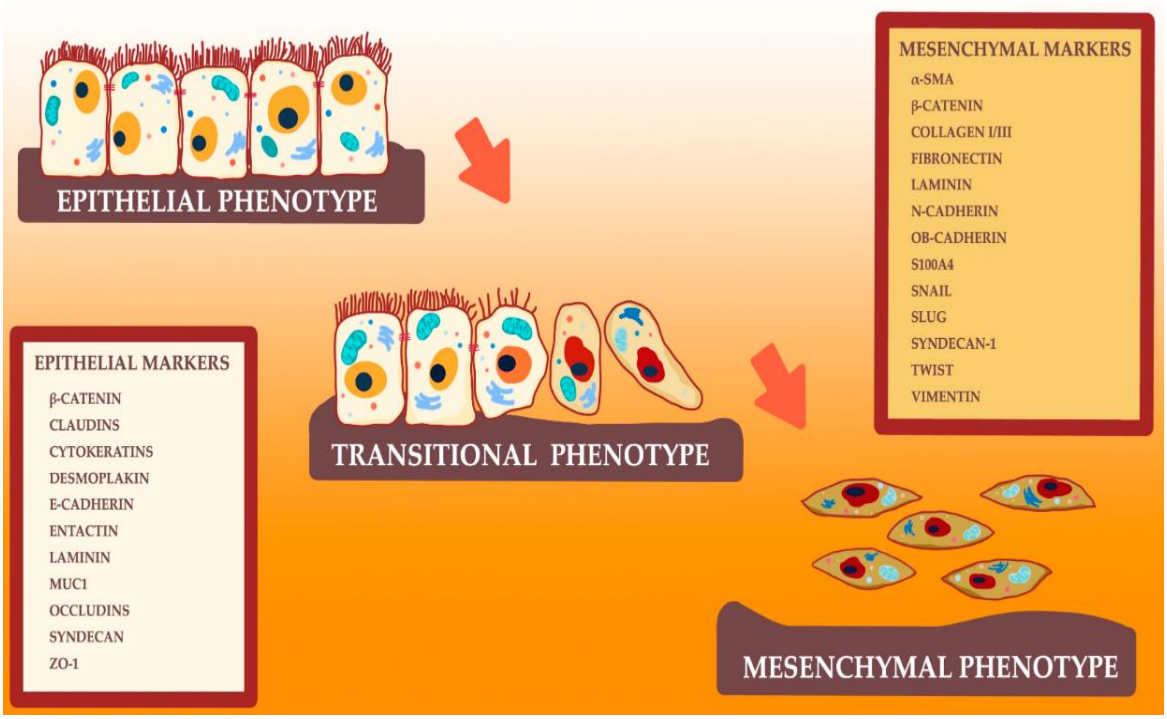

Figure 27: Schematic of the epithelial-mesenchymal transition and chosen epithelial and mesenchymal markers (Baj et al., 2020).

IV.5.2 Nidogene 2

1. Nidogene 2 gene

The human nidogen gene (*NID*), also named entactin, encodes a member of the nidogen family of basement membrane glycoproteins, it is a single copy gene located on chromosome 1q43. The entire gene is more than 90 kb in length and comprises 20 exons. The promoter regions of NID contain a dense CpG island spanning about 2kb around the first exon. All introns interrupt

protein coding sequences. The size of individual introns varies significantly, ranging from 0.6 to 18 kb (Zimmermann et al., 1995).

2. Nidogen 2 protein

In humans, two nidogen proteins have been identified; Nidogen 1 (Nid1) (150-kDa protein) is shorter than the 200-kDa Nidogen-2 (Nid2). *Nid2* encodes an active 1375 amino acid and has a molecular mass of 127 kDa for the mature protein. This sequence contains 49 residues, many of them are located in the central region, 5 putative N-linked glycosylation sites (Asn at positions 417, 658, 693, 703 and 1124), and 2 Tyr residues (at positions 310 and 317) situated in a consensus sequence for O-sulfation. Nid2 has a modular structure with three globular domains (G1 to G3) separated by a short flexible binding region between G1 and G2 and by a rod-like element G2 and G3 (Figure 20) (Kohfeldt et al., 1998). Nid2 comprises several typical extracellular protein modules, which include five epidermal growth factor-like (EG) modules, two of which have a consensus sequence for calcium-binding, two thyroglobulin type-I (TY) modules and five LDL receptor YWTD (LY) modules (Bork et al., 1996).

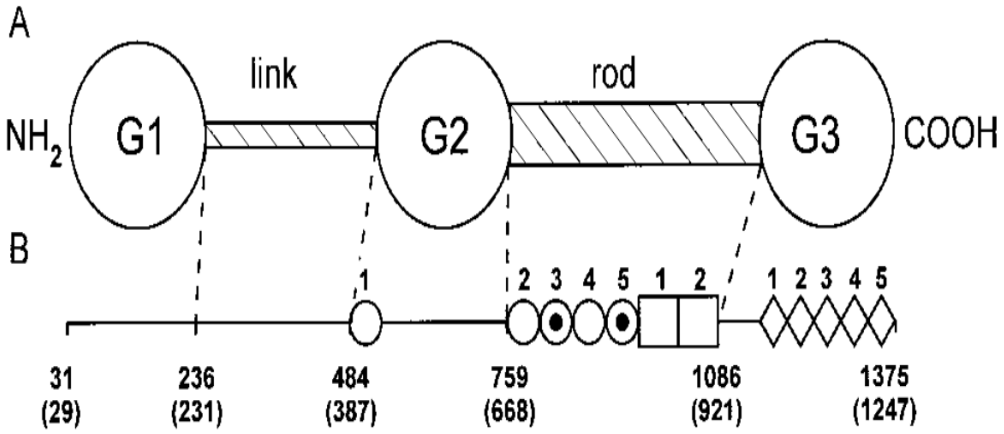

Figure 28: Structure of human nidogen-2 protein, predicted domain (A) and modular (B) (Kohfeldt et al., 1998)

3. Hypermethylation of Nidogen 2 and cancer

The nidogen family includes NID1 and NID2, ubiquitously present in the basement membrane. Both connect laminin and collagen IV networks in the extracellular matrix (ECM) and serve to maintain the integrity and stability of the basement membrane (Kohfeldt et al., 1998). Silencing NID2 expression has been reported in several cancer types, and the aberrant promoter hypermethylation might be a critical event detected in different malignancies, in particular gastric (Ulazzi et al., 2007), bladder (Renard et al., 2010) and invasive cervical cancers (Yu et

al., 2019). Detection of NID2 methylation was suggested as a biomarker for the early diagnosis of many cancers, including non-small cell carcinoma of the lung (Esteller et al., 1999), urothelial carcinoma (Yegin et al., 2013) and squamous cell carcinoma of oral cavity (Guerrero-Preston et al., 2011). A further study showed that NID2-deficient mice injected with melanoma cells into the tail vein had higher lung metastasis. They imply an important role of NID2 in suppressing the metastatic potential of cancer cells (Mokkapati et al., 2012). The latest research on the pathogenesis of nasopharyngeal carcinoma (NPC) and esophageal squamous cell carcinoma (ESCC) showed that promoter methylation of *NID2* was notably enhanced in NPC and ESCC samples than in their adjacent non-tumor tissue. Consistently, down-regulation of *NID2* expression was noted in both NPC and ESCC samples and cell lines. Re-expression of NID2 significantly inhibits liver metastasis and migration abilities of transduced NPC and ESCC cells. Indeed, NID2 suppresses the EGFR/Akt and integrin/FAK/PLCγ metastasis-related pathways, which highlights the crucial role of NID2 in the suppression of tumor metastasis (Chai et al., 2016).

In previous studies, hypermethylation of NID2 genes revealed potential diagnostic value in BC with high sensitivity ranging from 90% and 95.8% and specificity from 93% to 100% and was significantly better than cytology (48% and 96%, respectively); making it a more effective biomarker in advanced BC (Renard et al., 2010; Yegin et al., 2013). Another study has demonstrated that a urine-based methylation assay combining *TWIST1* and *NID2* genes may be useful for screening or monitoring NMIBC (Abern et al., 2014).

IV.5.3 Vimentin

1. Vimentin gene

The human vimentin gene (VIM) is localised in the short arm of chromosome 10 at 10p13 (Ferrari et al., 1987); this gene comprises about 10kb and consists of nine exons with a total length of 1848 nucleotides that compose the unique sequences of the Vimentin mRNA, separated by eight introns. The first exon is preceded by a TTATAAA sequence homologous to the consensus promoter for RNA transcription in eukaryotic genes. The last exon ends with the sequence AATAAA, which is considered a signal for the addition of poly (A) (Quax et al., 1983).

2. Vimentin protein

Vimentin protein is a 57kDa classified as type III intermediate filament protein, which is encoded in humans by the VIM gene and has 466 animo-acids. The vimentin protein consists of a central rod domain, with approximately 310 amino-acid residues, which comprises 116 charged animo acids (70 acidic and 46 basic amino acids) (Perreau et al., 1988). It is capped at each end by the nonhelical amino domain (N-terminal part of this rod domain), which is organized into sequences of seven amino acid residues, and the carboxyl domain (C-terminal part of the rod domain), which has a hydrophobic pattern with a different periodicity, 11-residue-long repeats, where the first, fourth and eighth residues create a hydrophobic core (Figure 21) (Parry, 2006).

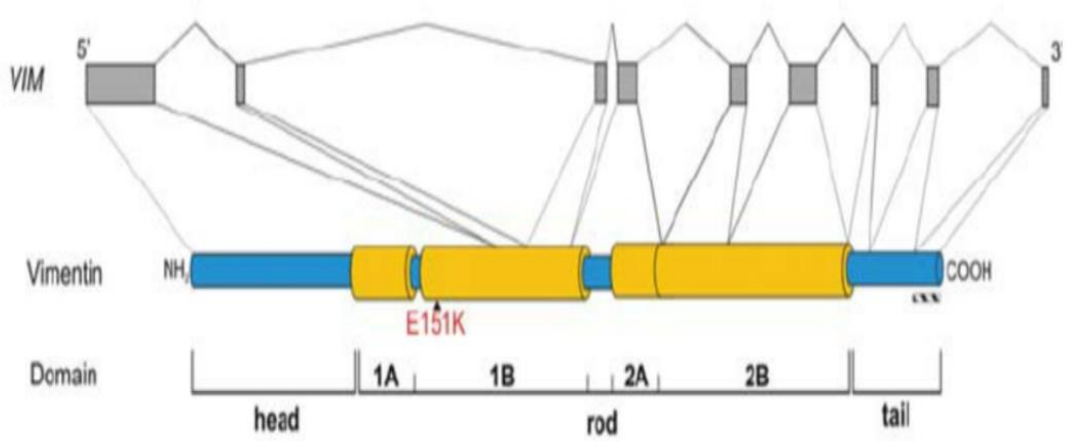

Figure 29 : Schematic representation of vimentin gene and protein domain organization (Muller et al., 2009)

3. Vim and cancer

Vimentin is a cytoskeletal protein that is ubiquitously expressed in normal mesenchymal cells and plays a crucial role in various cellular processes such as cell migration, adhesion and mortality and also promotes resistance to various cell stressors (Ivaska et al., 2007). Vimentin has been shown to be implicated in apoptotic progression; it is preferentially cleaved by multiple caspases -3, -7 and -6, resulting in disruption of intermediate filaments and promoting apoptosis (Byun et al., 2001). AKT1 Binding to Vim results in its direct phosphorylation, provides its protection from caspase mediated cleavage, and delays or blocks apoptotic progression, thus leading to cancer (Zhu et al., 2011). In solid cancers, in addition to the TWIST1 and Nid2 genes, Vim also *act*s as a major regulator in the transduction of epithelial-mesenchymal transition (EMT) signaling that promotes and stabilizes pro-EMT pathways. Therefore, high V*im* expression has been shown to be associated with more aggressive disease and worse outcomes in patients with *a* solid cancers (Thiery, 2002). Indeed, Vim

overexpression has been reported in a wide range in cancers, such as pancreatic precursor cells, sertoli cells, neuronal precursor cells, trophoblastic giant cells, fibroblasts, endothelial cells ling blood vessels, renal tubular cells, macrophages, neutrophils, mesangial cells, leukocytes and renal stromal cells (Satelli & Li, 2011).

4. Methylation of vimentin and cancer

The *Vim* promoter region is rich in cytosine-guanine dinucleotides (CpG island). Methylation of the CpG sites of the *Vim* promoter has been commonly described in invasive carcinoma cells, while they were rarely methylated in normal cells (Jung et al., 2011). This epigenetic alteration in VIM promoter has been considered as a powerful biomarker for the early diagnosis and prevention of many cancers (Shirahata et al., 2009). In fact, aberrant methylation of this gene has been reported in advanced colorectal carcinoma, in the primary tumors of colon cancer, in gastric carcinomas (Jung et al., 2011). Also hypermethylation has been observed in BC tissues and can be detected in urine specimens, with high sensitivity in the range 82% to 89% and specificity ranging from 94% to 100%, making it a promising biomarker for diagnosis and surveillance of BC recurrence (Reinert et al., 2012).

It is well known that hypermethylation of the VIM promoter is responsible for the repression and inhibition of gene expression in cervical cancer cells. Therefore, reactivation of VIM gene expression in cancer cells by exogenous regulations may be responsible for the activation of cell proliferation and migration. (Jung et al., 2011).

V. Problem statement

Although bladder cancer was widely studied and a lot of advances in the diagnostic tools and treatment of BC have been developing in the past decade, 573 278 new cases of BC are diagnosed each year worldwide (Globocan, 2020). Tumor heterogeneity of MIBC leads to rapid progression with metastatic, and NMIBC presents a high risk of recurrence and progression to MIBC. Indeed, BC is the most costly cancer to treat over the lifetime of patients because of a high (50-75%) recurrence rate (Lavallee et al., 2021).

Mechanisms driving bladder tumor heterogeneity can arise from genetic alterations and epigenetic changes due to genomic instability. A dynamic process that leads to the acquisition of therapeutic resistance, which poses major difficulties for drug development and clinical trial design (Drayton & Catto, 2012; Lavallee et al., 2021).

Early detection of BC is pivotal for successful patient treatment and management. Diagnosis of BC is mainly restricted to cystoscopy (invasive procedure) and urine cytology, which is somewhat limited in effectiveness (Arianayagam et al., 2011) . The development of molecular methods for accurate diagnosis, or that can augment routine diagnostics, would be a considerable advance. Many commercial molecular tests were discovered as a potential technique for diagnosing and prognosis of bladder tumors from body fluids. Due to the genomic heterogeneity of this disease, the search for different treatments and diagnostic tools is ongoing to develop biomarkers panel tests based on an optimal linear combination of multiple targets (Sugeeta et al., 2021).

In this context, we proposed in this work to evaluate the utility of two scoring models in the prediction of risk recurrence and progression and their usefulness in stratifying patients into risk groups. We are also interested in studying genetic and epigenetic changes in some genes involved in BC development. This would augment the delivery of patient care and better management of Moroccan BC patients.

Objectives of the thesis

VI. The aim of this thesis

This research work was conducted as a part of a research project at the National Center for Energy Sciences and Nuclear Techniques (CNESTEN); in order to contribute to a better understanding, from both genetic and epigenetic alterations, of how bladder cancer arises and progresses and to improve the quality of life of bladder cancer patients.

The first purpose of my Ph.D. thesis was to assess, in a preliminary study, the reliability of EORTC and CUETO scoring models for predicting recurrence and progression in Moroccan BC patients with non-muscle invasive bladder cancer.

The second aim of this thesis was to establish Moroccan population-based biobanks comprising samples (Biopsies and urines), as well as epidemiological/clinical data collected in the urology and anatomical pathology department at Mohammed V Military Teaching Hospital, to study the role of individual genetic and epigenetic factors in the development and progression of bladder cancer.

The third objective was to evaluate and appraise the sensitivity and specificity of multiple urine-based biomarkers and to evaluate their potential role as a non-invasive test for BC diagnosis, follow-up and treatment response monitoring to complement cystoscopy and urine cytology methods.

The specific aims of the papers presented in this thesis were to:

- Evaluate the utility of EORTC and CUETO scoring models in estimating recurrence and progression of Moroccan patients with NMIBC.
- Evaluate the mutational status of the *TERT* promoter in tumor biopsies and urine sediments from Moroccan bladder cancer patients.
- Determine the genetic alterations namely hotspots mutations in *PI3K* and *AKT1* genes in bladder cancer biopsies and matched urines.
- Investigate the mutational profile of the *Tp53* gene (exons 4-9) in 70 bladder cancer tumors.
- Evaluate DNA methylation status in promoter regions of *hTERT*, *TWIST1*, *VIM* and *NID2* genes in bladder cancer patients.
- Explore urine sediments as a non-invasive matrix for the detection of DNA methylation in bladder cancer.

Papers presentation

First part:

Utility of EORTC and CUETO scoring models in management of Moroccan bladder cancer patients

Introduction

NMIBCs constitute three-quarters of all primary bladder tumors diagnosed. About 75% of this tumor type recurs, and 20% progress to MIBC during the lifetime of patients (Babjuk et al., 2022). For risk-adapted surveillance and treatment of NMIBC patients, various risk group tools and predictive models were developed. EORTC and CUETO scoring system was developed to predict the short-term and long-term probabilities of BC recurrence and progression and to stratify patients into four risk groups. The proposed models score the most significant clinical and pathological factors in patients treated by chemotherapy and BCG therapy (Sylvester et al., 2006; Fernandez-Gomez et al., 2009).

In Morocco, the management of patients with NMIBC is based on the practical recommendations of the ccAFU and EAU guidelines. However, these guidelines provide a classification of patients into risk groups without calculating probabilities of recurrence and progression. EORTC and CUETO scoring models are not used in daily clinical practice due to overestimating recurrence and progression rates worldwide; thus, several studies were conducted to validate the utility of these tools (Rouprêt et al., 2020; Rhijn et al., 2021).

This part of the thesis aims to assess the usefulness and validate the risk stratification schemes of EORTC and CUETO scoring models on Moroccan patients with NMIBC, and to highlight the opportunity to introduce these tools in clinical practice in order to improve the management of Moroccan BC patients

Utility of EORTC and CUETO Scoring Models in the Estimation of Recurrence and Progression of Non-Muscle-Invasive Bladder Cancer

VI.1 Utility of EORTC and CUETO Scoring Models in the Estimation of Recurrence and Progression of Non-Muscle-Invasive Bladder Cancer

Bladder cancers are subdivided into non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), based on how far they have invaded the muscle layer of the bladder. Approximately 70-80% of newly diagnosed bladder cancers are classified as NMIBC, which correspond to stage Ta, T1 and Tis tumors according to the 2017 TNM classification. NMIBCs are characterized by a high risk of recurrences which occur in 50 to 75% of cases, after transurethral resection, and the progression to MIBC, which occurs in about 25- 45% of patients.

The EORTC Genito-urinary group developed a scoring system and risk tables in order to predict, separately, the short-term and long-term probabilities of disease recurrence and progression based on the six most relevant clinical and pathological factors (Stage, grade, numbers of tumours, tumour size, prior recurrence rate and CIS. The main limitation of the EORTC approach was the low number of patients treated with BCG. To overcome this limitation, the CUETO scoring system was developed to predict disease recurrence and progression in BCG-treated patients. This model is based on the analysis of seven prognostic factors (Stage, Grade, Age, Gender, prior recurrence rate, number of tumors, CIS).

The purpose of this preliminary study was to assess the utility of the EORTC risk tables and the CUETO scoring model in predicting recurrence and progression rates in NMIBC patients and to compare our results with those reported by Sylvester et al. (2006) and Fernandez-Gomez et al. (2009) In addition, Kaplan-Meier and univariate analysis using the Cox regression test were performed to validate the stratification between four groups and to evaluate the association between prognostic factors with recurrence and progression disease.

Overall, 56 NMIBC patients were analysed, and the recurrence and progression rates were higher than in EORTC and CUETO risk tables at one year. The observed rate of recurrence and progression proved to be lower than it had been predicted in both tables in 5 years. Kaplan-Meier analysis reported that only the CUETO scoring model successfully stratified our cohort into four risk groups of recurrence (*p-value* = 0.005) suggesting that the CUETO model has more power than EORTC to discriminate each recurrence risk group in the Moroccan population.

In our view, both scoring models showed the feasibility and usefulness for predicting recurrence and progression in our population. However, it is necessary to carry out a prospective study on a large and multicenter cohort with a long follow-up period in order to re-establish an optimal prognostic model for Moroccan NMIBC patients.

EJMO

DOI: 10.14744/ejmo.2022.99203
EJMO 2022;6(2):121–129

Research Article

Utility of EORTC and CUETO Scoring Models in the Estimation of Recurrence and Progression of Non-Muscle-Invasive Bladder Cancer

Chaimae Hafidi Alaoui,[1,2] Mohammed Tetou,[3,5] Ilias Hassan,[3,5] Meryem El Azzouzi,[1,5] Hajar El Ahanidi,[1,5] Abdelkarim Filali-Maltouf,[2] Imane Chaoui,[1] Mounia Bensaid,[4] Laila Benbacer,[1] Mohamed Oukabli,[4,5] Abderrahmane Al Bouzidi,[5] Mohammed Attaleb,[1] Mohammed El Mzibri,[1] Ahmed Ameur[3,5]

[1]Biology and Medical Research Unit, CNESTEN, Rabat, Morocco
[2]Microbiology and Molecular Biology Laboratory, Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco
[3]Department of Urology, Mohammed V Military Teaching Hospital of Rabat, Rabat, Morocco
[4]Department of Pathology, Mohammed V Military Teaching Hospital of Rabat, Rabat, Morocco
[5]Faculty of Medicine and Pharmacy of Rabat, Mohammed V University in Rabat, Rabat, Morocco

Abstract

Objectives: Worldwide, Non-Muscle-Invasive Bladder Cancer (NMIBC) patients are characterized by a high rate of recurrence and progression highlighting the need for a valuable prognostic estimation for better management of this disease. Thus, the present preliminary study was planned to evaluate the validation of the European Organization for Research and Treatment of Cancer (EORTC) and the Spanish Urological Club for Oncological Treatment (CUETO) risk tables to predict recurrence and progression in Moroccan patients with NMIBC.

Methods: A total of 56 NMIBC patients that have undergone transurethral resection of bladder tumor (TURBT), between January 2017 and May 2021, were recruited. The recurrence and progression rates at 1 and 5 years were calculated for each patient using EORTC and CUETO scoring models and compared to EORTC and CUETO risk tables. Kaplan-Meier was performed to validate stratification and difference between the four groups obtained. A univariate analysis using the Cox regression test was realized to evaluate the association between prognostic factors with recurrence and progression of the disease.

Results: For the 56 NMIBC patients, the median follow-up duration was 49.68 months. In this cohort, 43 patients had recurrent tumors, and 27 showed progression to an advanced stage and/or grade. At 1-year progression and recurrence rates were higher than the values predicted by the EORTC and CUETO risk tables, while both tables overestimate the long-term risk probabilities of recurrence and progression. Only the CUETO model successfully stratified our patients with statistically significant differences between the four groups of recurrence (p=0.005). Of particular interest, univariate Cox analysis indicated that only prior recurrence rate had a significant effect on both recurrence-free survival (p=0.04) and progression-free survival (p=0.037).

Conclusion: CUETO scoring model is better than EORTC for recurrence stratification in Moroccan patients with NMIBC. Both models overestimate risk in 1-year and underestimate risk in 5-years. A prospective study should be realized in large cohorts to establish an ideal prognosis model for Moroccan NMIBC patients.

Keywords: EORTC, CUETO, recurrence, progression, NMIBC, Morocco

Cite This Article: *Hafidi Alaoui C, Tetou M, Hassan I, El Azzouzi M, El Ahanidi H, Filali-Maltouf A, et al. Utility of EORTC and CUETO Scoring Models in the Estimation of Recurrence and Progression of Non-Muscle-Invasive Bladder Cancer. EJMO 2022;6(2):121–129.*

Address for correspondence: Mohammed El Mzibri, MD. Centre National de l'Energie, des Sciences et techniques Nucléaires (CNESTEN). BP 1382 RP. 10001 Rabat, Maroc
Phone: +212 537 71 27 51 **E-mail:** mzibri@yahoo.com

Submitted Date: March 21, 2022 **Accepted Date:** June 03, 2022 **Available Online Date:** June 06, 2022

©Copyright 2022 by Eurasian Journal of Medicine and Oncology - Available online at www.ejmo.org
OPEN ACCESS This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License.

Bladder cancer (BC) is the eleventh most common cancer worldwide, with more than 573 278 new cases diagnosed in 2020.[1] Approximately 75 % of bladder cancer cases are non-muscle-invasive cancers (NMIBC), and 20% of them progress to muscle-invasive types during patients' lifetimes.[2]

Superficial bladder tumors are a group of tumors characterized by histological and prognostic heterogeneity, including papillary tumors that respect the basement membrane (pTa), Lamina propria (pT1) containing connective tissues between urothelium and detrusor muscle, and carcinoma in situ (CIS), characterized by low coherence and adherence of epithelial cells.[3,4] In this field, the main problems faced by clinicians are the difficulty to compare the efficacy of treatment modalities and proposing unified treatment recommendations.

An updated version of the non-invasive muscle bladder cancer (NIMBC) guidelines was released in 2021 by The European Association of Urology (EAU) to provide practical recommendations on clinical management and to harmonize treatment approaches. Accordingly, patients are stratified into four groups: low-, intermediate-, high- and very high-risk groups, and patients' risk groups can be determined using the European Organization for Research and Treatment of Cancer (EORTC) risk tables and EAU risk group calculator to predict the risk of tumor recurrence and progression after Transurethral resection of Bladder Tumor (TURBT).[5]

In 2006, the EORTC organization proposed a table model to assess the score for Ta-T1 BC and to predict the risk of recurrence and progression in each patient in both the short and long terms. This approach is the most frequently applied and validated model and is based on 6 clinical and pathologic factors: stage, grade, tumor size, number of tumors, previous disease recurrence, and concomitant in situ carcinoma (CIS).[6] The main limitation of the EORTC approach is still the difficulty of predicting the small number of patients treated with BCG.[7,8] Moreover, it was reported that EORTC taboverestimatemate both the recurrence and the progression of BCG-treated patients.[9]

To overcome this limitation, the Spanish Urological Club for Oncological Treatment (CUETO) group has created and validated a scoring model focusing on BCG-treated patients.[10] This approach was developed and validated to predict the short- and long-term risks of recurrence and progression in patients treated with BCG. The CUETO scoring system is based on the evaluation of seven prognostic factors: Age, gender, prior recurrence, number of tumors, tumor stage and grade, and presence of concomitant CIS.[11]

The aim of the present study was to externally validate the risk stratification schemes of EORTC and CUETO scoring models on a cohort of Moroccan patients with NMIBC. The two models were applied to calculate each patient their estimated recurrence and progression rates compared to the real risk of patients to highlight the opportunity to introduce these risks' assessment in the global management of bladder cancer in Morocco.

Methods

Patients

A retrospective study, based on a prospective cohort, was designed to analyze data of 73 patients treated with transurethral resection of a bladder tumor (TUR-BT) at the Urology Department of the Military Hospital of Instruction Mohamed V (MHIMV) in Rabat - Morocco, between the January 2017 and May 2021, with up to 19 years follow-up. The following inclusion criteria were taken into account during the revision process: (a) Patients had to be diagnosed, for the first time, with primary Ta/T1 urothelial bladder tumor or in situ carcinoma, and (b) the grades and stages of tumors were determined and confirmed by histo-pathological reports, (c) follow-up duration of recruited patients was more than 12 months. Patients diagnosed with a muscle-invasive type of bladder cancer (MIBC) were excluded from this study.

The study protocol was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat – Morocco (Ref 82/19), and written informed consents were obtained from all recruited patients.

Follow-up

In this retrospective study, information collected was used to generate a subsequent database that included the clinico-pathological factors used in the elaboration of the EORTC and CUETO models, including sex, age, tumor size (<3 cm or ≥3 cm), number of tumors (single or multiple), the prior recurrence rate, T category, presence of concomitant in situ carcinoma, grade, and intravesical therapy. Cases with pathologically confirmed recurrence were noted in the follow-up database at the time of the first recurrence. Tumors were defined as progressive profiless if they progress into muscle-invasive tumors, metastatic diseases, or from low to high grade.

Recurrence-free survival (RFS) was calculated from the first treatment assignment to the date of the first recurrence or the last follow-up visit. Progression-free survival (PFS) was calculated from the first treatment assignment to the date of the first progression detected, the date of death, or the last follow-up visit.

A recurrence event was defined as the time between the first TURBT treatment and the occurrence of the recurrence or progression. Progression event was accepted athe s initiation of progresion, or death of the patient due to disease progression. Patients, noted without recurrence or progression were confirmed at the time of the last cystoscopy and urinary cytology for recurrence and progression analysis. The time of recurrence and progression disease was performed by two urologists and confirmed by another urologist.

Statistical Analysis

The scores of progression and recurrence risks at 1 and 5 years were estimated for all 56 patients using the EORTC (https://www.eortc.be/tools/bladdercalculator) and CUETO (https://www.aeu.es/Cueto.html) models.[6,7] Accordingly, all patients were classified into the four risk groups: low-risk, Intermediate-risk, high-risk and very high-risk. Progression and recurrence risks were evaluated for all patients and the subgroup of BCG-treated patients after TURBT.

The EORTC scoring model was performed with respect to the following criteria: the number of tumors (single, 2–7 or ≥8), tumor size (<3 cm or ≥3 cm), prior recurrence rate (primary, ≤1 recurrence/year, >1 recurrence/year), T stage (Ta or T1), concomitant CIS (yes/no), and grade (1, 2 or 3).[6] The CUETO scoring model incorporated gender, age (<60, 60–70, >70 years), recurrent tumor (yes/no), number of tumors (≤3 or >3), T stage (Ta or T1), concomitant CIS (yes/no), and grade (1, 2, or 3).[7]

Patient scores were then stratified into 4 risk groups, and a probability of recurrence and progression risk in 1 and 5 years were estimated in order to compare them with the real rates.

Survival analysis was conducted using Kaplan-Meier methods to evaluate progression and recurrence curves obtained with EORTC and CUETO risk groups; the log-rank test was used to compare the difference between groups. A univariate analysis using the cox-regression model was performed to evaluate the association between prognostic factors with recurrence and progression of disease.

For statistical validation, IBM SPSS version 23 was used to calculate the p values, and $p<0.05$ was interpreted as statistically significant.

Results

Pathological analysis of the 73 recruited bladder cancer patients showed that 17 patients had muscle-invasive bladder cancer (MIBC) and were consequently excluded from the study. The retained 56 non-muscle-invasive bladder cancer (NMIBC) cases were mostly men (54/56) with a median age of 65.96 years.

Clinico-pathological characteristics of tumors are summarized in Table 1 and show that most patients had more than

Table 1. The clinic-pathological characteristics of tumor's patients

Socio-clinical information		Anatomo-pathological characterization	
Characteristic	**N (%)**	**Characteristic**	**N (%)**
Age		Concomitant CIS	
< 70 years	37 (66.07%)	Yes	4 (07.15%)
> 70 years	19 (33.93%)	No	52 (92.85%)
Gender		Tumor Grade	
Male	54 (96.42%)	Low (G1/G2)	27 (48.22%)
Female	2 (3.58%)	High (G3)	29 (51.78%)
T Stage		Recurrence	
pTa	20 (35.71%)	No	13 (23.22%)
pT1	36 (64.29%)	yes	43 (76.78%)
No. of Tumors		prior recurrence rate	
1	17 (30.35%)	Recurrent < = 1 per year	27 (48.22%)
2-5	16 (28.57%)	Recurrent > 1 per year	12 (21.42%)
≥ 5	23 (41.08%)	primary	17 (30.36%)
Tumor Size		Progression	
< 3 cm	25 (44.65%)	No	29 (51.78%)
≥ 3 cm	31 (55.35%)	yes	27 (48.22%)
Intravesical Therapy		Survival	
No	33 (58.92%)	Alive	48 (85.71%)
BCG	23 (41.08%)	Death	8 (14.29%)

5 multifocal tumors (41.08%), 55.35% of patients had tumor size ≥ 3 cm, and most cases were diagnosed with pT1 stage (64.29%). Anatomo-pathological analysis showed that there are as many high-grade cases (51.75%) as there are low-grade cases (48.22%,) and only 7.14% of cases exhibited concomitant CIS (4/56).

In this study, the average duration of follow-up was 49.68 months, and all recruited patients had complete data with a minimum 1-year follow-up. After an initial TUR-BT, recurrence was observed in 43 patients (73.21%), while progression was observed in 27 patients (48.21%).

Using the EORTC recurrence score of each patient, 1 (1.78%), 8 (14.28%), 23 (41.08%) and 24 (42.86%) patients were classified into the low-, intermediate, high- and very high-risk groups, respectively. Using the progression score, corresponding values were 1 (1.8%), 12 (21.4%), 25 (44.7%) and 18 (32.1%) patients using the progression score. Comparison of probabilities of progression and recurrence rates by risk group at 1 year and 5 years are reported in Tables 2 and 3, respectively, and compared to reference probabilities reported by Sylvester et al.[6] Overall, the probabilities of recurrence and progression at 1 year ranged from 0 % to 79.2% and from 0% to 41.7%, respectively. Exception was made for the low-risk group; all scores were overestimated compared to scores reported in the EORTC risk tables.

At 5 years, the probability of recurrence ranged from 0%

Table 2. Prediction of disease recurrence and progression in patients with non-muscle invasive bladder cancer using EORTC risk table at 1 year

Group of patients according to the risk score	Recurrence status		
	No. of Patients	N° of patients and rate of recurrence	Predicted recurrence rates according to EORTC risk tables % (95% CI)
Low risk	1	0 (0%)	15% (10-19%)
Intermediate risk	8	3 (37.5%)	24% (21-26%)
High risk	23	17 (73.9%)	38% (35-41%)
Very high risk	24	19 (79.2%)	61% (55-67%)
	Progression status		
	No. of patients	N° of patients and rate of progression	Predicted progression rates according to EORTC risk tables % (95% CI)
Low risk	1	0 (0%)	0.2% (0-0.7%)
Intermediate risk	12	5 (41.7%)	1% (0.4-1.6%)
High risk	25	10 (40%)	5 % (4-7%)
Very high risk	18	3 (16.7%)	17% (10-24%)

Table 3. Prediction of disease recurrence and progression in patients with non-muscle invasive bladder cancer using EORTC risk table at 5 years

Group of patients according to the risk score	Recurrence status		
	No. of Patients	N° of patients and rate of recurrence	Predicted recurrence rates according to EORTC risk tables % (95% CI)
Low risk	1	0 (0%)	31% (24-37%)
Intermediate risk	8	1 (12.5%)	46% (42-49%)
High risk	23	4 (17.4%)	62% (58-65%)
Very high risk	24	6 (25%)	78% (73-84%)
	Progression status		
	No. of patients	N° of patients and rate of progression	Predicted progression rates according to EORTC risk tables % (95% CI)
Low risk	1	0 (0%)	0.8% (0-1.7%)
Intermediate risk	12	2 (16.7%)	6% (5-8%)
High risk	25	3 (12.0%)	17% (14-20%)
Very high risk	18	3 (16.7%)	45% (35-55%)

to 25% and all obtained scores were underestimated to that reported by the EORTC table. Regarding the progression rate, the probability of progression ranged from 0 to 16.7%. This probability was underestimated for low- high- and very high-risk groups and overestimated for intermediate-risk groups.

Using the CUETO recurrence score of each patient, 10 (17.85%), 11 (19.64%), 26 (46.43%) and 9 (16.08%) patients were classified into the low-, intermediate-, high-, very high-risk groups, respectively. Using the progression score, corresponding values were 13 (23.21%), 8 (14.29%), 14 (25.00%) and 21 (37.5%) patients. Comparison of probabilities of progression and recurrence rates by risk groups at 1 year and 5 years are reported in Tables 4 and 5, respectively, and compared to reference probabilities reported by Fernandez-Gomez et al.[7] Overall, the probabilities of recurrence and progression at 1 year ranged from 10% to 88.9% and from 23.1% to 50%, respectively. The CUETO risk tables underestimated the risk of recurrence and progression in 1 year. After 5 years of the initial TRU-BT, the probability of recurrence and progression ranged from 0% to 26.9% and from 7.1% to 25%, respectively. The risk scoring of predicting recurrence in 5 years was lower in all risk groups than those described by Fernandez-Gomez et al.[7] The probabilities of progression for low- and intermediate-risk groups were higher than the reference values reported in the CUETO risk tables, and conversely, they were for high- and very high-risk groups.

Table 4. Prediction of recurrence and progression rates in patients with non-muscle invasive bladder cancer using CUETO scoring model at 1 year

Group of patients according to the risk score	Recurrence status		
	No. of patients	N° of patients and rate of recurrence	Predicted recurrence rates according to CUETO risk tables % (95% CI)
Low risk	10	1 (10%)	8.2% (5.9-10.5%)
Intermediate risk	11	9 (81.8%)	12% (8-16%)
High risk	26	21 (80.8%)	25% (20-31%)
Very high risk	9	8 (88.9%)	42% (28-56%)
	Progression status		
	No. of patients	N° of patients and rate of progression	Predicted progression rates according to CUETO risk tables % (95% CI)
Low risk	13	3 (23.1%)	1.17% (0.15-2.19%)
Intermediate risk	8	4 (50%)	3.00% (0.82- 5.18%)
High risk	14	7 (50%)	5.55% (2.73-8.37%)
Very high risk	21	7 (33.3%)	13.97 (6.64-21.30%)

Table 5. Prediction of recurrence and progression rates in patients with non-muscle invasive bladder cancer using CUETO scoring model at 5 year

Group of patients according to the risk score	Recurrence status		
	No. of expected patients	N° of patients and rate of recurrence	Predicted recurrence rates according to CUETO risk tables % (95% CI)
Low risk	10	0 (0%)	21.0 % (17.33-24.63%)
Intermediate risk	11	2 (18.2%)	35.6% (29.18-41.96%)
High risk	26	7 (26.9%)	47.6% (40.55-54.75%)
Very high risk	9	2 (22.22%)	67.61% (53.67-81.55%)
	Progression status		
	No. of expected patients	N° of patients and rate of progression	Predicted progression rates according to CUETO risk tables % (95% CI)
Low risk	13	3 (23.1%)	3.76% (1.90-5.62%)
Intermediate risk	8	2 (25%)	11.69% (7.57-15.81%)
High risk	14	1 (7.1%)	21.26% (15.85-26.67%)
Very high risk	21	2 (9.52%)	33.57% (23.06-44.08%)

Univariate Cox analysis indicated that only Prior recurrence rate had a significant effect on both recurrence-free survival (p=0.04) and progression-free survival (p=0.037); the other parameters, including gender, age, BCG treatment, grade, stage, tumor size, number of tumors and the presence of concomitant CIS, had no significant effect in this cohort (p>0.05) (Table 6).

In this study, the difference between risk groups of recurrence and progression according to EORTC and CUETO models was calculated using the Kaplan-Meier method and was evaluated by the log-rank test (Fig. 1). EORTC scoring model showed no significant difference between the recurrence (p=0.40) and progression (p=0.64) scores, respectively. Of particular interest, the CUETO risk stratification showed a worse discriminating ability for recurrence scores between risk groups (p=0.005). However, for the progression analysis, there was no significant difference between groups (p=0.43).

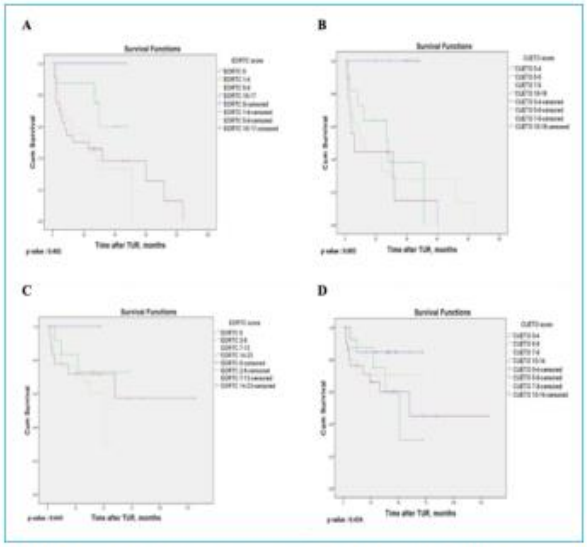

Figure 1. Kaplan-Meier survival curves of risk of recurrence, using EORTC recurrence score **(a)** and CUETO recurrence score **(b)**, and of progression using EORTC progression score **(c)** and CUETO progression score **(d)**.

Table 6. Univariate cox regression analysis for predicting recurrence and progression

	Recurrence-free survival		Progression-free survival	
	HR (95% CI)	p	HR (95% CI)	p
Gender				
Female	1.00	0.712	1.00	0.624
Male	1.457 (0.197 - 10.762)		21.247 (0.000 – 4307808.777)	
Age				
≥70	1.00	0.724	1.00	0.087
<70	1.006 (0.975-1.037)		1.042 (0.994 – 1.092)	
BCG				
Not treated	1.00	0.244	1.00	0.860
Treated	1.535 (0.746 – 3.160)		0.921 (0.367 – 2.308)	
Stages				
pTa	1.00	0.763	1.00	0.587
pT1	1.113 (0.555 - 2.231)		1.309 (0.496 – 3.457)	
Grades				
LG	1.00	0.542	1.00	0.231
HG	0.809 (0.409 - 1.599)		1.770 (0.695 – 4.503)	
Tumor size				
< 3 cm	1.00	0.093	1.00	0.572
≥ 3 cm	1.835 (0.903 – 3.731)		0.771 (0.313 – 1.900)	
N° of tumors				
1	1.00		1.00	0.618
2-5	0.631 (0.276 – 1.446)	0.521	1.148 (0.668 – 1.973)	
≥ 5	0.743 (0.327 – 1.684)		-	
Concomitant CIS				
No	1.00	0.934	1.00	0.623
Yes	0.948 (0.270 – 3.333)		0.600 (0.078 – 4.602)	
Prior recurrence rate				
Primary	1.00		1.00	0.037
Recurrent < 1 per year	0.035 (0.004 – 0.269)	0.004	2.034 (1.044 – 3.964)	
Recurrent > 1 per year	0.578 (0.282 – 1.183)		-	

Discussion

In Morocco, growing interest is given to bladder cancer as there's an increasing number of diagnosed cases and related deaths. In 2020, there were 2268 new cases diagnosed with bladder cancer, with 1268 deaths.[1] Most new cases are diagnosed as NMIBC, being a group with a high risk of recurrence and progressive rates.[12] Thus, predicting recurrence and progression risks is of great interest in the context of target therapy for better management of bladder cancer disease.

Currently, EORTC and CUETO models are widely used for risk stratification of patients with NMIBC providing specific quantitative estimates of the short- and long-term risks of recurrence and progression, making them highly promising tools for a personalized therapeutic approach.[6,7]

The main advantages of these risk stratification models are that they use socio-clinical variables that are available in patients' records, and the estimation of recurrence and progression rates is very easy and practical for clinical purposes.

To the best of our knowledge, this is the first external validation in Morocco and was conducted on a cohort of 56 patients with NMIBC with up to 19 years of follow-up.

In this study, progression and recurrence risk at one year were higher than risks probabilities presented in the EORTC and CUETO risk rates, except for progression/recurrence rates of the low-risk group.

Our results clearly showed that the EORTC risk table overestimated the risk of recurrence and progression for only the low-risk group, and the CUETO risk table underestimated the risk for recurrence and progression. This funding is in disagreement with results reported by Dovey et al. showing that both risk tables tended to overestimate both recurrence and progression rates at 1 year.[13]

For both models, our long-term finding, at 5 years, was underestimated as compared to the predicted values in the EORTC and CUETO risk tables, except in the intermediate-risk group of patients. Similar results were obtained on 123 Belgian patients[14] and 205 Brazilian patients,[15] highlighting that EORTC and CUETO tables overestimated the recurrence and progression risks at 5 years. Conversely, the study conducted by Almeida et al. on 205 Brazilian patients with bladder cancer showed that using the EORTC scoring model, they had an overestimation of the risk of recurrence in 1 year and an underestimation in 5 years.[16]

The Kaplan-Meier method was used to assess the probabilities of recurrence and progression based on both models, and the difference between the risk groups was compared with the log-rank test. Accordingly, using the EORTC risk table, no significant difference was reported in recurrence-free survival and progression-free survival rates between the risk groups. Nevertheless, previous studies have reported that the EORTC tool has high discrimination for predicting recurrence and progression.[17–19] This difference could be mainly due to the low number of cases in our study that would be considered as a sampling bias.

Of particular interest, the CUETO model showed a significant difference between groups for RFS rate ($p=0.005$), unlike PFS ($p=0.436$). These findings are in accordance with the results of Chung and Coll. Showing a great association with recurrence ($p<0.001$) but not with progression ($p=0.423$).[20] Our results are in contrast with a previous study showing that CUETO estimates progression better than recurrence.[21]

Our results have indicated that the EORTC risk scores did not enable stratification, neither the risk of recurrence nor the risk of progression. In contrast, the CUETO model successfully stratified our patients into 4 groups for recurrence, unlike for the progression risks in our Moroccan population. The ability of EORTC and CUETO to stratify risk groups is controversial, depending on the studied populations. Dalkilic and Coll.[19] have reported that the EORTC and CUETO models successfully stratified the recurrence and progression into 4 risk groups in their cohorts, whereas Xu and Coll.[9] showed that only the EORTC model was able to stratify patients with statistically different probability for recurrence and progression.

The univariate Cox regression analysis showed that most studied prognostic factors, including age, gender, BCG immunotherapy, tumor size, number of tumors, stage, and grade, are not related to recurrence and progression. Interestingly, the prior recurrence rate showed a significant association with both recurrence and progression and could be a promising predictor factor for bladder cancer follow-up.

In this study, BCG treatment was not applied to some patients who were at high risk due to adverse effects or had contraindications to BCG medication. Among the 56 recruited NMIBC cases, 23 have received BCG treatment during the study period (41.1%). This sample is too low to assess the recurrence and progression rates of BCG-treated patients according to EORTC and CUETO scoring systems.

This preliminary study is very informative and clearly showed the feasibility, usefulness and ease of use of both EORTC and CUETO scoring models for progression and recurrence prediction in Morocco. However, the study pres-

ents some limitations, mainly due to the low number of recruited patients, that has largely affected the power of the study. A large study with a consistent and multicenter cohort with a long-term follow-up is needed to really appreciate the high value of these scoring tables and to construct a valid prognostic model for Moroccan patients with NMIBC. Additionally, to better predict the cancer progression, it will be interesting to consider the cis type in future explorations.

Disclosures

Ethics Committee Approval: Hospital name: Mohammed V Military Hospital in Rabat-Morocco, Date: December 2017, number of patients: 56 (Ref 82/19).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – M.A., A.A.B.; Design – M.E.M., A.A.; Supervision – M.E.M., M.A.; Materials – C.H.A., M.E.A., H.E.A.; Data collection and/or processing – C.H.A., M.T., I.H., M.B., M.O.; Analysis and/or interpretation – C.H.A., L.B., A.F.M., M.A.; Literature search – C.H.A., C.I.; Writing – C.H.A., M.A., M.O.; Critical review – M.E.M., A.A.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA A Cancer J Clin. 2021;71:209–49. [CrossRef]
2. Cumberbatch MGK, Jubber I, Black PC, Esperto F, Figueroa JD, Kamat AM, et al. Epidemiology of bladder cancer: a systematic review and contemporary update of risk factors in 2018. Eur Urol 2018;74:784–95. [CrossRef]
3. Greene FL, Balch CM, Fleming ID, Fritz A, Haller DG, Morrow M, et al. AJCC cancer staging handbook: TNM classification of malignant tumors. New York: Springer Science & Business Media; 2002. [CrossRef]
4. Anastasiadis A, de Reijke TM. Best practice in the treatment of nonmuscle invasive bladder cancer. Ther Adv Urol 2012;4:13–32. [CrossRef]
5. Sylvester RJ, Rodríguez O, Hernández V, Turturica D, Bauerová L, Bruins HM, et al. European Association of Urology (EAU) prognostic factor risk groups for non-muscle-invasive bladder cancer (NMIBC) incorporating the WHO 2004/2016 and WHO 1973 classification systems for grade: an update from the EAU NMIBC guidelines panel. Eur Urol 2021;79:480–8. [CrossRef]
6. Sylvester RJ, van der Meijden APM, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, et al. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol 2006;49:466–5; discussion 475–7.
7. Fernandez-Gomez J, Madero R, Solsona E, Unda M, Martinez-Piñeiro L, Gonzalez M, et al. Predicting nonmuscle invasive bladder cancer recurrence and progression in patients treated with bacillus Calmette-Guerin: the CUETO scoring model. J Urol 2009;182:2195–203. [CrossRef]
8. Fahmy N, Lazo-Langner A, Iansavichene AE, Pautler SE. Effect of anticoagulants and antiplatelet agents on the efficacy of intravesical BCG treatment of bladder cancer: A systematic review. Can Urol Assoc J 2013;7:E740–9. [CrossRef]
9. Xu T, Zhu Z, Zhang X, Wang X, Zhong S, Zhang M, et al. Predicting recurrence and progression in Chinese patients with nonmuscle-invasive bladder cancer using EORTC and CUETO scoring models. Urology 2013;82:387–93.
10. Vedder MM, Márquez M, de Bekker-Grob EW, Calle ML, Dyrskjøt L, Kogevinas M, et al. Risk prediction scores for recurrence and progression of non-muscle invasive bladder cancer: an international validation in primary tumours. PLoS One 2014;9:e96849. [CrossRef]
11. Gontero P, Sylvester R, Pisano F, Joniau S, Vander Eeckt K, Serretta V, et al. Prognostic factors and risk groups in T1G3 non-muscle-invasive bladder cancer patients initially treated with Bacillus Calmette-Guérin: results of a retrospective multicenter study of 2451 patients. Eur Urol 2015;67:74–82.
12. Garg M. Urothelial cancer stem cells and epithelial plasticity: current concepts and therapeutic implications in bladder cancer. Cancer Metastasis Rev 2015;34:691–701. [CrossRef]
13. Dovey Z, Pfail J, Martini A, Steineck G, Dey L, Renström L, et al. Bladder Cancer (NMIBC) in a population-based cohort from Stockholm County with long-term follow-up; A comparative analysis of prediction models for recurrence and progression, including external validation of the updated 2021 E.A.U. model. Urol Oncol 2022;40:106.e1–106.e10. [CrossRef]
14. Holz S, Albisinni S, Gilsoul J, Pirson M, Duthie V, Quackels T, et al. Risk factor assessment in high-risk, bacillus Calmette-Guérin-treated, non-muscle-invasive bladder cancer. Res Rep Urol 2017;9:195–202.
15. Busato Júnior WFS, Almeida GL, Ribas CAPM, Ribas Filho JM, De Cobelli O. EORTC risk model to predict progression in patients with non–muscle-invasive bladder cancer: is it safe to use in clinical practice? Clinical Genitourinary Cancer 2016;14:176–82. [CrossRef]
16. Almeida GL, Busato WF Jr, Ribas CM, Ribas JM Filho, De Cobelli O. External validation of EORTC risk scores to predict recurrence after transurethral resection of brazilian patients with non -muscle invasive bladder cancer stages Ta and T1. Int Braz J Urol 2016;42:932–41. [CrossRef]
17. van Rhijn BWG, Zuiverloon TCM, Vis AN, Radvanyi F, van Leenders GJLH, Ooms BCM, et al. Molecular grade (FGFR3/MIB-1) and EORTC risk scores are predictive in primary non-muscle-invasive bladder cancer. Eur Urol 2010;58:433–41.

18. Altieri VM, Castellucci R, Palumbo P, Verratti V, Sut M, Olivieri R, et al. Recurrence and progression in non-muscle-invasive bladder cancer using EORTC risk tables. Urol Int 2012;89:61–6.
19. Dalkilic A, Bayar G, Kilinc MF. A comparison of EORTC and CUETO risk tables in terms of the prediction of recurrence and progression in all non-muscle-ınvasive bladder cancer patients. Urol J 2019;16:37–43.
20. Chung JW, Kim JW, Lee EH, Chun SY, Park DJ, Byeon KH, et al. Prognostic significance of the neutrophil-to-lymphocyte ratio in patients with non-muscle ınvasive bladder cancer treated with ıntravesical bacillus calmette-guérin and the relationship with the CUETO scoring model. Urol J. 2021 Oct 16. Doi: 10.22037/uj.v18i.6765. [Epub ahead of print].
21. Zhang G, Steinbach D, Grimm MO, Horstmann M. Utility of the EORTC risk tables and CUETO scoring model for predicting recurrence and progression in non-muscle-invasive bladder cancer patients treated with routine second transurethral resection. World J Urol 2019;37:2699–705. [CrossRef]

Second part:

Evaluation of the mutational status of four genes: hTERT, PIK3CA, AKT1 and Tp53 in biopsies and urine sediments

Introduction

Bladder tumors are the eleventh most mutated cancer among 166 other cancers. Several genetic alterations play important roles In BC tumorigenesis and progression. Cells with such mutations are associated with tumor heterogeneity with different molecular phenotypes that represent a major challenge in the implementation of precision cancer therapies, which promises to transform cancer care with molecular biomarkers for diagnosis, prognosis and guide treatment (Lavallee et al., 2021).

Expression of telomerase is absent in normal cells, while reactivation of *hTERT* expression is required for replicative senescence and proliferation in immortal malignant cells (Masutomi & Hahn, 2003). *hTERT* reactivation is up-regulated by somatic *hTERT* promoter mutations (C250T and C228T) and/or epigenetic modulations through *hTERT* promoter methylation or changes in the chromatin. Mutations of *hTERT* promoter are found in 60-80% cases of all stages and grades of UC (Griewank et al., 2013; Akincilar et al., 2016).

Somatic mutations in genes involved in PIK3CA/AKT1/mTOR signaling pathway are identified in BC (Kachrilas et al., 2019). *PIK3CA* Mutations are mainly of the missense type and grouped in exons 9 and 20, while mutations in *AKT1* gene are clustered across exons 1 and 14. These mutations have oncogenic properties by activating a multi-step protein cascade, promoting protein synthesis and regulating cancer cell proliferation (Hemmings & Restuccia, 2012).

Tp53 missense mutations are the hallmark of cancer cells (Liu et al., 2014). These alterations are grouped in the DNA-binding domain, disrupting its principal function as a transcriptional factor and providing the P53 protein with new oncogenic functions (Muller & Vousden et al., 2013). *Tp53* alterations are more frequently found in MIBC compared to NMIBC. Thus, *Tp53* mutations appear to be the key driver of metastasis in advanced BC patients (Fujimoto et al., 1992).

These genes play crucial roles in BC carcinogenesis and progression. They may serve as potential biomarkers for future diagnosis to complement cystoscopy and urine cytology tools usually used in clinical practice, effective monitoring of patient health and can improve therapeutic response. The goal of part II of this study is to evaluate the mutational status of four key genes in biopsies and urine sediments and to assess the usefulness of urine samples as a body fluid matrix for a non-invasive approach.

Evaluation of TERT promoter mutations in tumor biopsies and urine sediment of Moroccan bladder cancer patients

VI.2 Evaluation of *TERT* promoter mutations in tumor biopsies and urine sediments of Moroccan bladder cancer patients

Telomeres are nucleoprotein structures that maintain genomic stability in normal cells, and the progressive shortening of telomeric sequences through successive cell divisions induces chromosomal instability and apoptosis. In humans, germ cells, stem cells and some immune cell types have high levels of telomerase activity. In contrast, most somatic cells do not have detectable telomerase activity. Most types of cancer achieve proliferative immortality by reactivating telomerase. In tumor cells, mutations in the core promoter region of the *hTERT*, result in hyperactivation of *hTERT*. Two recurrent mutations of the *TERT* promoter region (C228T and C250T), which are the most reported mutations with high frequency, occur in multiple cancer types, including bladder cancer.

The aim of this study was to identify the genetic alterations in the *hTERT* gene promoter in bladder cancer biopsies and matched urines, and assess the association between the findings and the clinico pathological data of patients. *hTERT* promoter mutations were identified in 60% of cancer biopsies (42/70). Four hotspot mutations, type C228T (34/42), C250T (7/42), A161C and G149T (1/42), were reported in our cohort. No correlation was found between *TERT* promoter mutations and clinicopathological parameters. On the other hand, no association was reported between the mutational status of this promoter and overall survival and recurrence-free survival among NMIBC patients.

During the last decades, a great interest was given to the research for alternative non-invasive tests for the detection, diagnosis and surveillance of BC. Therefore, detecting mutations genes in urine, as the most suitable biological fluid for examination, has proven to be useful in various types of cancer. In this way, we have used parried urine sediments for each patient to test the sensibility and specificity of this gene. Mutations of *TERT* promoter were detected in 30% of matched urine sediments and demonstrated high specificity of 100% and a moderate sensitivity of 50%.

In summary, *TERT* promoter mutations are the most common and highly prevalent genetic event in bladder cancer, making it a promising biomarker of BC and potentially a new therapeutic target. Its moderate detection in exfoliated cells renders it practically a less effective biomarker. However, the ability to identify alterations of this gene in urine makes it a promising non-invasive biomarker for the diagnosis and follow-up of bladder cancer. In this regard, to validate its potential utility as a non-invasive biomarker for screening and prognosis, further investigations should be conducted with a larger sample and multicentric studies.

Ann. Cancer Res. Ther. Vol. 30, No. 1, pp. 1-7, 2022

Original papers

Evaluation of TERT promoter mutations in tumor biopsies and urine sediment of Moroccan bladder cancer patients

Chaimae Hafidi Alaoui[1,2], Hajar El Ahanidi[1,4], Meryem El Azzouzi[1,4], Abdelkarim Filali-Maltouf[2], Imane Chaoui[1], Mounia Bensaid[3], Laila Benbacer[1], Mohammed Tetou[3,4], Ilias Hassan[3,4], Mohamed Oukabli[3,4], Ahmed Ameur[3,4], Abderrahmane Al Bouzidi[4], Mohammed Attaleb[1], Mohammed El Mzibri[1]

[1] *Biology and Medical Research Unit, CNESTEN, Rabat, Morocco*
[2] *Microbiology and Molecular Biology Laboratory, Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco*
[3] *Mohammed V Military Hospital, Rabat, Morocco*
[4] *Faculty of Medicine and Pharmacy of Rabat. Mohammed V University in Rabat, Rabat, Morocco*

Abstract

Background: Human telomerase reverse transcriptase (hTERT) promoter mutations are common genetic events in bladder cancer (BC) and have been recognized as potential biomarkers for BC diagnosis and prognosis. Detection of TERT promoter mutations as urine-based tests has previously been reported to detect primary and recurrent BC. This study was planned to evaluate hTERT promoter mutations in both biopsies and urines from BC patients to assess the interest and the usefulness of introducing these biomarkers for better management of BC in Morocco.

Methods: In a cohort study involving a series of BC 70 patients with different stages and grades, hTERT promoter mutations were identified in both fresh biopsies and urine sediments by PCR amplification and DNA sequencing.

Results: Overall, hTERT promoter mutations were reported in 60% of cancer biopsies (42/70), the hotspot mutations C228T and C250T were respectively identified in 80.95% (34/42) and 16.67% (7/42) of positive cases. Other mutations: A161C and G149T were also reported and were obtained in 2.38% (1/42) and 2.38% (1/42), respectively. hTERT promoter mutations were identified in 30% of matched urine sediments (21/70) and showed an overall sensitivity of 50% and specificity of 100%. In patients with non-muscle invasive bladder cancer, no statistically significant association was detected between TERT promoter mutations and recurrence-free survival (HR: 1.224, 95% CI: 0.373–4.011, $p = 0.739$), and overall survival (HR: 0.363, 95% CI: 0.041–3.244, $p = 0.364$).

Conclusion: hTERT promoter mutations are early events occurring with high frequencies and can be identified in the exfoliated cells, making them an interesting non-invasive biomarker for diagnosis and follow-up of bladder cancer.

Keywords: *TERT* promoter region, Bladder cancer, Mutations, Non-invasive detection, Urine sediment

(Received November 1, 2021; Accepted January 5, 2022)

Introduction

Bladder urothelial carcinoma (BUC) is the most common malignancy of the urinary system and is a fatal invasive malignancy. In the world, bladder cancer ranks 12th, with about 573,278 cases and 212,536 deaths in 2020[1]. In Morocco, as reported by the regional cancer register of Casablanca, bladder cancer is a major public health problem with an estimation of 1540 new cases yearly and is affecting more men than women[2]. Overall, 75–80% of bladder cancer cases are classified as non-muscle-invasive bladder cancer (NMIBC), compromising stages Tis, Ta, and T1, and most of them have a high recurrence rate within five years and up to 20% progress to muscle-invasive bladder cancer (MIBC)[3].

Worldwide, NMIBC detection and monitoring are mainly based on cystoscopy and urine cytology, showing many limitations. Cystoscopy is an invasive and expensive method and cannot accurately identify small or flat in situ carcinoma (CIS) lesions. Moreover, it is more difficult to detect and distinguish between benign reactive lesions and malignancy, particularly for patients undergoing transurethral Resection (TURBT) or intravesical BCG treatment[4,5]. Urine cytology is a high specific microscopic evaluation widely used to detect bladder lesions but is poorly sensitive and depends on the pathologist's interpretation[6].

The authors position of Mohammed Attaleb and Mohammed El Mzibri as equal last-position.

Corresponding author: Dr. Mohammed El Mzibri, Centre National de l'Energie, des Sciences et techniques Nucléaires (CNESTEN). BP 1382 RP. 10001 Rabat, Morocco. TEL: +212.537.71.27.51, E-mail: mzibri@yahoo.com

Recent advances in molecular biology have highlighted the presence of various genetic and epigenetic biomarkers that could be used in molecular diagnosis and prognosis of bladder cancer and therapeutic targets. Interestingly, these molecular biomarkers are usually assessed in clinical specimens and urine samples as a simple, non-invasive and effective method for early detection, diagnosis, and follow-up of bladder cancer patients.

In cancer cells, telomerase activation plays a crucial role in cellular immortalization and cancer development[7, 8]. In tumor cells, telomerase activation is promoted by genetic mutations in the gene promoter region. Currently, two recurrent mutations of TERT promoter gene (C228T and C250T) are the most detected mutations associated with telomerase dysregulation and are reported in several human cancers[9]. In bladder cancer, TERT is mutated in approximately 60–85% of cases and are detected in all clinical stages and tumor grades, suggesting that these alterations are early events in bladder carcinogenesis and are considered as promising non-invasive urinary biomarkers for the detection and surveillance of bladder cancer[9-11]. Recent publications highlight the interest in detecting hTERT gene mutations as a urinary-based biomarker and report a higher sensitivity and specificity in primary and recurrent bladder cancer patients, varying between 46.4% and 80.5% and from 73% to 96%, respectively[12-15].

In this context, the present study was planned to evaluate the presence of genetic mutations of *hTERT* gene promoter in bladder cancer cases and their association with cancer clinicopathological parameters to assess the interest and the usefulness of these biomarkers in the global management of bladder cancer in Morocco.

Materials and Methods

Study design and specimens collection

A total of 70 patients' specimens from the Urology Department of the Mohammed V Military Hospital in Rabat-Morocco were collected between 2017 and 2019 with a retrospective and prospective follow-up between January 2015 and May 2021. For these patients, urine samples were obtained at the transurethral resection of bladder tumor (TUR-BT). The bladder tumor staging and grading were performed according to the TNM (tumor node metastasis) classification and World Health Organization (WHO) /International Society of Urologic Pathology criteria, respectively[16]. hTERT mutational status was assessed in both bladder cancer biopsies and voided urine samples for patients with NMIBC (≤ PT1) and MIBC (> PT1).

The study protocol was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat – Morocco (Ref 82/19), and written informed consents were obtained from all recruited patients.

Genomic DNA extraction

Genomic DNA was extracted from fresh-frozen samples and urine cell sediments using the phenol/chloroform method[17]. All extracted DNA was quantified with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). DNA samples were used immediately or stored at -20°C until use.

Mutation Detection

The mutational status was assessed by PCR – sequencing. A 193 bp fragment of TERT promoter was amplified using TERT-F (5'-CACCCGTCCTGCCCCTTCACCTT-3') and TERT-R (5'-GGCTTCCCACGTGCGCAGCAGGA-3') primers[18]. The amplification reaction was performed in a total volume of 25 μL, containing 10 μM of each consensus primer, 10 μM of each dNTP (dATP, dCTP, dGTP, and dUTP), 1.5 mM $MgCl_2$, 5 units *Taq* DNA polymerase per μL, and 2 μL of DNA sample in 1x *Taq* polymerase buffer. The mixture was first denatured at 95°C for 5 min. Then, thirty-five cycles of PCR were performed with denaturation at 94°C for 30 sec, primer annealing for 40 sec at 60°C, and primer extension for 40 sec at 72°C. At the end of the last cycle, the mixture was incubated at 72°C for 7 min. Negative control in which DNA template was omitted from the amplification mixture is included for every reaction.

Purification of PCR products was done using the ExS-Pure™ enzymatic PCR cleanup kit (Nimagen, The Netherlands) and was sequenced on an ABI 3130XL DNA analyzer using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequencing reactions were performed in a final volume of 10 μL, containing 1 μL of 2.5X Big Dye ready reaction mix v.3.1, 10 pmol of forward primer, and 100 ng of purified PCR product. The mixtures were incubated at 96°C for 1min, and 25 cycles were performed: denaturation at 96°C for 10 sec, primer annealing at 50°C for 5 sec and extension at 60°C for 4 min. Sequencing reaction products were finally purified using Sephadex G-50 gel-exclusion chromatography (GE Healthcare Life Sciences). Obtained sequences were then matched with the gene reference sequence collected from the GenBank database (NCBI Reference Sequence: NG_009265.1). The sequence alignments were performed using the Clustal W program in BioEdit Software.

Statistical analysis

All statistical analyses were carried out using SPSS for Windows, version 23 (SPSS Inc, Chicago). The correlation between mutation status of *hTERT* gene promoter and clinical-histopathological parameters (tumor's stage, grade, age of patients, smoking status, and recurrence/

Table 1 Clinicopathological characteristics

Parameter	Total	Percentage	Parameter	Total	Percentage
Sex			**Tumour stage**		
Male	68	97.14	≤ PT1	52	74.29
Female	2	2.86	>PT1	18	25.71
Age			**Tumour grade**		
			Low grade	27	38.57
< 50	1	1.43	High grade	43	61.43
50–70	44	62.86	**Tumour recurrence**		
> 70	25	35.71	Yes	12	23.08
			No	40	76.92
Smoker			**Tumour progression**		
Yes	28	40	Yes	5	9.62
No	42	60	No	47	90.38

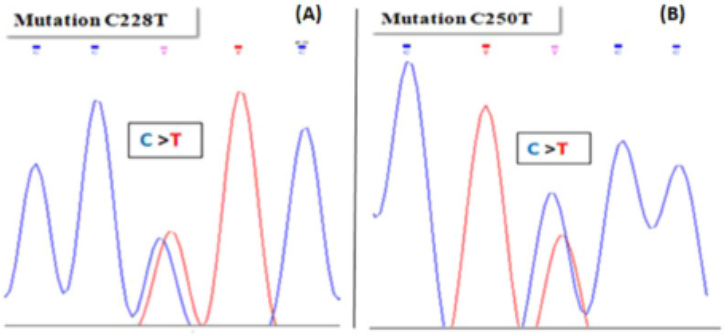

Fig. 1 Representative sequencing electropherograms of the human TERT promoter with mutations C228T (A) and C250T (B).

progression) was evaluated by $\chi 2$, and *p values* < 0.05 were interpreted as statistically significant.

Univariate and multivariate Cox regression models were performed to compare variables and outcomes. Evaluation of the association between TERT promoter mutations / clinicopathological factors, Overall Survival (OS), and Recurrence-Free Survival (RFS) was performed by univariate cox regression to estimate the hazard ratio and p-values of each covariate. Recurrence-free survival was calculated from the first treatment assignment to the date of first recurrence or the last follow-up visit. Progression-free survival was calculated from the first treatment assignment to the date of the first progression detected, the date of death, or the last follow-up visit. Overall survival was defined as the interval from the date of diagnostic to the date of death from any cause or last follow-up.

A multivariate analysis was performed if the p-value of one or more variables was less than <0.05. Survival curves were performed by the Kaplan-Meier method, and the follow-up period was defined from the date of first TUR-BT treatment to the moment of death for deceased cases or the date of the last follow-up for survivors.

Results

Characteristics of the study population

Clinico-pathological characteristics of the 70 recruited patients are reported in Table 1. Overall, 68 patients were male with a sex ratio of 34. The mean age of the patients was 67, with extreme ages at 47 and 85 years. Moreover, 40% of patients were smokers. Clinico-pathological characteristics of patients showed that 74.29% of cases were staged ≤ PT1 (52/70), and 61.43% had high tumor grades (43/70). Among NMIBC cases, 23.08% have recurred (12/52), and 9.62% have progressed upon relapse (5/52).

Evaluation of TERT promoter mutational status

All bladder cancer specimens have shown successful amplification and direct sequencing of the hTERT promoter fragment. An example of a nucleotide sequence of hTERT promoter, showing C228T and C250T mutations, is reported in Fig. 1. Overall, mutations in the hTERT promoter were reported in 60% of the bladder cancer specimens (42/70), C228T mutation prevailed and was obtained in 80.95% of positive cases (34/42). Other mutations: C250T, A161C, and G149T were also reported and were obtained in 16.67% (7/42), 2.38% (1/42), and 2.38% (1/42), respectively. Of particular interest, 1 case haro-

Table 2 Distribution of TERT gene promoter mutations according to clinicopathological parameters

Parameter	N	Overall Mutations in hTERT				*p*
		+	%	-	%	
Gender						
Male	68	41	60.29	27	39.71	0.751
Female	2	1	50.00	1	50.00	
Age						
< 50	1	1	100.00	0	0.00	
50–70	44	28	63.64	16	36.36	0.154
> 70	25	13	52.00	12	48.00	
Smoking						
Yes	28	18	64.29	10	35.71	0.941
No	42	24	57.14	18	42.86	
Stages						
≤ PT1 **(NMIBC)**	52	31	59.62	21	40.38	0.860
> PT1 **(MIBC)**	18	11	61.11	7	38.89	
Grades						
LG	27	15	55.56	12	44.44	0.538
HG	43	27	62.79	16	37.21	
Tumour recurrence						
Yes	12	7	58.33	5	41.67	0.647
No	40	24	60.00	16	40.00	
Tumour progression						
Yes	5	1	20.00	4	80.00	0.083
No	47	30	63.83	17	36.17	

Table 3 Univariable Cox regression results for the association of clinical-pathological parameter with overall survival (OS) and recurrence-free survival (RFS) among NMIBC patients

	Overall Survival		Recurrence-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
***TERT* Mutation**				
Wild type	1.00	0.364	1.00	0.739
Mutant	0.363 (0.041–3.244)		1.224 (0.373–4.011)	
Gender				
Female	1.00	0.052	1.00	0.687
Male	8.919 (0.978–81.369)		0.047 (0.000–135528.454)	
Age				
≥ 70	1.00	0.119	1.00	0.285
< 70	0.175 (0.20–1.565)		2.040 (0.552–7.543)	
Stages				
≤ PT1 **(NMIBC)**	1.00	0.305	1.00	0.301
> PT1 **(MIBC)**	0.318 (0.35–2.842)		1.835 (0.581–5.798)	
Grades				
LG	1.00	0.18	1.00	0.474
HG	0.223 (0.025–1.998)		1.566 (0.458–5.350)	

boured both C228T and C250T mutations.

The correlation between TERT promoter mutations and patients' characteristics is summarized in Table 2. No significant association was obtained between the mutational profile and patients' age, gender, and smoking status ($p > 0.05$). The mutations frequencies were similar in early and advanced stages and high and low grades. The distribution of TERT mutations according to the cancer evolution was assessed in the 52 NMIBC cases and showed that TERT mutations were found in both cases with and without recurrence ($p = 0.647$). Among the 5 patients showing progressive tumors, TERT C228T mutation was reported in 1 case (20%, whereas TERT mutations were reported in 63.83% of cases with no progressive tumors. However, the difference was not statistically significant due to the very low number of cases with progressive tumors ($p = 0.083$).

Patients outcomes

The results of univariate and multivariate Cox regression analysis are reported in Table 3. In the univariate analysis, TERT mutations and clinicopathological features do not significantly influence the Recurrence-Free Survivals ($p > 0.05$).

The univariate analysis also indicated that TERT mutations, age, tumors' grade, and stage do not significantly influence overall survival ($p > 0.05$).

These results are confirmed by the Kaplan-Meier sur-

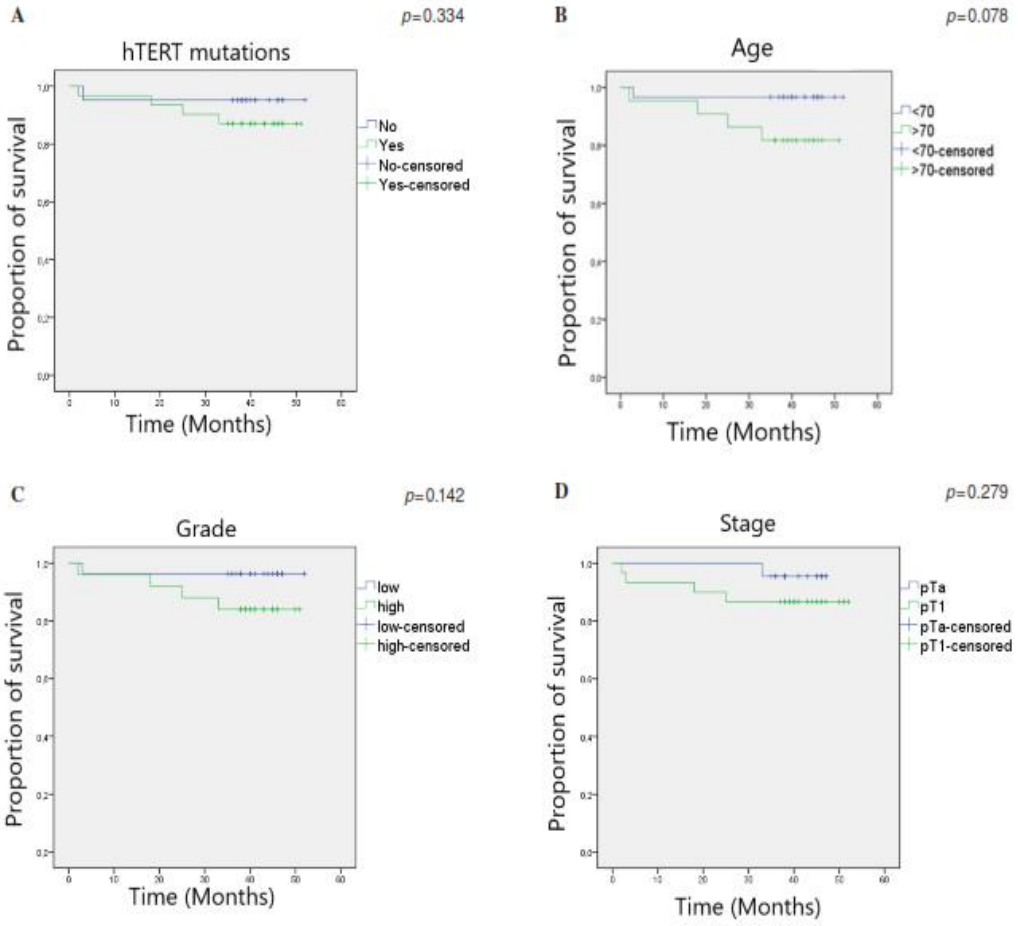

Fig. 2 Overall Survival correlated to TERT promoter mutational status (A), Age (B), tumours grade (C) and tumours stage (D)

Table 4 Frequency of mutations detected in biopsies and urine for different stages and grades types

Parameter	N	Overall Mutations in hTERT			Overall Mutations in hTERT		
		+	%	p	+	%	p
Stages							
≤ PT1 **(NMIBC)**	52	31	59.62	0.860	16	30.77	
> PT1 **(MIBC)**	18	11	61.11		15	27.78	
Grades							
LG	27	15	55.56	0.538	7	25.93	
HG	43	27	62.79		14	32.56	
Sensitivity of Urinary test [95% CI]				50% [34.2%–65.8%]			
Specificity of Urinary test [95% CI]				100%			

vival curves estimating survival rates for all cases with TERT promoter wild-type (WT) and mutant sequences and with respect to clinicopathological features (Fig. 2). Indeed, a borderline significance was obtained with age ($p = 0.078$), and no significant association was reported between TERT promoter mutations and the remaining clinicopathological features, including tumors stage, tumors grade, and Overall Survival ($p > 0.05$).

Evaluation of TERT promoter mutations in matched urine sediments

In this study, TERT Promoter mutational status was also assessed in matched urine sediments from all the 70 recruited patients, and the results are summarised in Table 4. TERT mutations were obtained in 30% of specimens (21/70). TERT mutations were detected in urines from both high and low grades cases and both cases with early and advanced stages, with no statistically signifi-

cant difference ($p > 0.05$).

Comparison between TERT promoter mutations in bladder biopsies and paired urine sediments was also assessed and is reported in Table 4. All cases showing TERT promoter mutations in urine sediments have mutations of TERT promoter in the corresponding DNA from bladder biopsies. Meanwhile, among the 42 TERT mutations harboring cases, only 21 were detected in urine samples. Accordingly, the specificity and sensitivity of detecting TERT promoter mutations in urine sediments was 100% and 50%, respectively.

Discussion

The main problem clinicians face in bladder cancer diagnosis is the low sensitivity of cytology and high invasiveness of cystoscopy, the two most widely used tools for cancer diagnosis and follow-up[19]. Recent advances in molecular oncology highlighted the presence of biomarkers of particular interest in diagnosis, prognosis, and follow-up. In this field, genetic events, and DNA mutations, in particular, are cancer hallmarks that show promise as sensitive and specific molecular markers for urological cancers. In bladder cancer, the TERT gene, particularly the promoter region, is widely reported to harbor many point mutations associated with cancer development[9, 10, 20]. The main goal of this study was to assess the prevalence of mutations in the promoter region of the *TERT* gene in Moroccan cases with bladder cancer in tumor biopsies and voided urine samples, enabling their potential use as biomarkers as an alternative strategy to cystoscopy and urine cytology for early detection, patient monitoring, and disease prognosis.

Overall, 60% of patients with bladder cancer had a mutation in the TERT promoter region. These results are in agreement with widely reported data showing that TERT is the most frequently mutated gene in bladder cancer[12, 21, 22].

The two widely reported mutations considered to be associated with bladder cancer development, C228T and C250T[12, 23], were reported as hotspot mutations in our study. C228T was found in 80.95% and C250T in 16.67% of bladder cancer cases. Other 2 point mutations, A161C and G149T, considered rare events, were obtained in 1 case each. Similar results were reported by Huang et al. that have identified C228T and C250T mutations in 39.7% (116/292) and 14.4% (42/292) of bladder cancer cases, respectively. Interestingly, they have also identified 2 patients with A161C and G149T mutations[24].

No significant difference was found between TERT promoter mutations and clinicopathological features, grade, and stage in our study. This result is shared by many authors suggesting that point mutations in TERT promoter region are early events in the cancer development and suggesting their interesting use as biomarkers for the detection and monitoring of bladder cancer[23].

In this study, the presence of mutations was not related to overall and recurrence-free survivals of bladder cancer. These results are supported by the finding of Allory et al.[12], showing that overall survival was not associated with TERT promoter mutations. However, contrary results were reported elsewhere, showing that TERT promoter mutations significantly decreased overall survival[25] and recurrence-free survival[26]. Our results were also confirmed by the Kaplan Meier curve, and cox regression model performed on the 52 cases with NMIBC.

The Cox regression model revealed an independent association of recurrence-free survival with TERT promoter mutations, age at diagnosis, grade, and stage.

In this study, TERT promoter mutational status was also evaluated in voided urine samples. Mutations in exfoliated cells were detected with a specificity of 100% but a sensitivity of 50%. Higher sensitivity ranging from 80.5% to 81.3% was already reported[13, 27]. The difference could be due to the cell sampling, preparation, DNA/RNA extraction, and protocol used for point mutations identification. Indeed, several analytical approaches are used for detecting mutations in TERT promoters. The difference could also be due to the rate of cancer exfoliated cells that could be recovered from the urine. The small urine volume may affect the number of exfoliated cells, and consequently, the quantity of DNA extracted from the malignant cells, causing the lack of detecting mutations in the urine sample. In line with this, Zuiverloon and colleagues have reported that the sensitivity of this test based on detection of mutations in the FGFR3 gene in urine increases with the volume of the urine sample[28]. Of particular interest, the number of exfoliated cells depends on the tumor grade and stage. In this study, only 62.79% of recruited cases were diagnosed with high grades. Kinde et al. have reported a sensitivity of 79% on exfoliated cells recovered from 86.7% of HG cases[11].

It is currently widely accepted that TERT promoter mutations are interesting biomarkers for bladder cancer diagnosis and follow-up, but recent investigations showed that telomerase inhibition is a promising therapeutic target. The discovery of some inhibitors, antisense oligonucleotides, and some immunotherapeutics are under clinical trials for many cancer, including bladder cancer, to increase the therapeutic arsenal for better cancer management[29].

This study is very informative and clearly showed that mutations in TERT promoter region are an interesting biomarker that could be used for early diagnosis for better management of bladder cancer. However, the main limitations of the study are: (1) the small sampling size, especially the low number of NMIBC patients not allowing to accurately estimate the association between TERT promoter mutations and cancer recurrence; (2) The very

low number of women patients; (3) The follow-up period is short, causing a missing recurrence and progression data for newly collected cases.

Conclusion

hTERT promoter mutations are early events occurring with high frequencies and can be identified in the exfoliated cells, making them an interesting non-invasive biomarker for diagnosis and follow-up of bladder cancer. A large study on a high number of cases will be of great interest to accurately assess the usefulness of this biomarker in bladder cancer management.

References

1) Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA A Cancer J Clin. 2021;71:209-49.
2) Benider A, Bendahhou k. Registre des cancers de la région du grand Casablanca 2008-2012. Ed 2016. 2016.
3) Siddiqui MR, Grant C, Sanford T, et al. Current clinical trials in non-muscle-invasive bladder cancer. Urol Oncol. 2017;35:516-27.
4) Jichlinski P, Leisinger H-J. Fluorescence cystoscopy in the management of bladder cancer: a help for the urologist! Urol Int. 2005;74: 97-101.
5) Yafi FA, Brimo F, Steinberg J, et al. Prospective analysis of sensitivity and specificity of urinary cytology and other urinary biomarkers for bladder cancer. Urol Oncol. 2015;33:66.e25-31.
6) Alameddine M, Nassir A. The influence of urine cytology on our practice. Urol Ann. 2012;4:80.
7) Holysz H, Lipinska N, Paszel-Jaworska A, et al. Telomerase as a useful target in cancer fighting—the breast cancer case. Tumor Biol. 2013;34:1371-80.
8) Jafri MA, Ansari SA, Alqahtani MH, et al. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. Genome Med. 2016;8:69.
9) Liu T, Yuan X, Xu D. Cancer-Specific Telomerase Reverse Transcriptase (TERT) Promoter Mutations: Biological and Clinical Implications. Genes. 2016;7:38.
10) Günes C, Wezel F, Southgate J, et al. Implications of TERT promoter mutations and telomerase activity in urothelial carcinogenesis. Nat Rev Urol. 2018;15:386-93.
11) Kinde I, Munari E, Faraj SF, et al. TERT promoter mutations occur early in urothelial neoplasia and are biomarkers of early disease and disease recurrence in urine. Cancer Res. 2013;73:7162-7.
12) Allory Y, Beukers W, Sagrera A, et al. Telomerase reverse transcriptase promoter mutations in bladder cancer: high frequency across stages, detection in urine, and lack of association with outcome. Eur Urol. 2014;65:360-6.
13) Descotes F, Kara N, Decaussin-Petrucci M, et al. Non-invasive prediction of recurrence in bladder cancer by detecting somatic TERT promoter mutations in urine. Br J Cancer. 2017;117:583-7.
14) Hayashi Y, Fujita K, Matsuzaki K, et al. Diagnostic potential of TERT promoter and FGFR 3 mutations in urinary cell-free DNA in upper tract urothelial carcinoma. Cancer Sci. 2019;110:1771-9.
15) Ou Z, Li K, Yang T, et al. Detection of bladder cancer using urinary cell-free DNA and cellular DNA. Clin Trans Med. 2020;9:4.
16) Kotake T. [TNM classification for urological cancer]. Gan To Kagaku Ryoho 1998;25:2293-2301.
17) Sambrook J, Fritsch E, Maniatis T. Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 1989.
18) Xie H, Liu T, Wang N, et al. TERT promoter mutations and gene amplification: Promoting TERT expression in Merkel cell carcinoma. Oncotarget 2014; 5: 10048-57.
19) Raitanen MP, Leppilahti M, Tuhkanen K et al. Routine follow-up cystoscopy in detection of recurrence in patients being monitored for bladder cancer. Ann Chir Gynaecol 2001; 90: 261-5.
20) Leiblich A. Recent Developments in the Search for Urinary Biomarkers in Bladder Cancer. Curr Urol Rep. 2017;18:100.
21) Critelli R, Fasanelli F, Oderda M, et al. Detection of multiple mutations in urinary exfoliated cells from male bladder cancer patients at diagnosis and during follow-up. Oncotarget. 2016;7:67435-48.
22) Yang A, Cross CN, Townsend JP. Non-Coding Mutations in Urothelial Bladder Cancer: Biological and Clinical Relevance and Potential Utility as Biomarkers. BLC. 2020;6:211-3.
23) Zvereva M, Pisarev E, Hosen I, et al. Activating Telomerase TERT Promoter Mutations and Their Application for the Detection of Bladder Cancer. Int J Mol Sci. 2020;21:E6034.
24) Huang FW, Bielski CM, Rinne ML, et al. TERT promoter mutations and monoallelic activation of TERT in cancer. Oncogenesis. 2015;4(12):e176.
25) Russo IJ, Ju Y, Gordon NS, et al. Toward Personalised Liquid Biopsies for Urothelial Carcinoma: Characterisation of ddPCR and Urinary cfDNA for the Detection of the TERT 228 G>A/T Mutation. BLC. 2018;4:41-8.
26) Leão R, Lee D, Figueiredo A, et al. Combined genetic and epigenetic alterations of the TERT promoter affect clinical and biological behavior of bladder cancer. Int J Cancer. 2019;144:1676-84.
27) Yahyazadeh R, Bashash D, Ghaffari P, et al. Evaluation of hTERT, KRT7, and survivin in urine for noninvasive detection of bladder cancer using real-time PCR. BMC Urol. 2021;21:64.
28) Zuiverloon TCM, Tjin SS, Busstra M, et al. Optimization of non-muscle invasive bladder cancer recurrence detection using a urine based FGFR3 mutation assay. J Urol. 2011;186:707-12.
29) Wan S, Liu X, Hua W, et al. The role of telomerase reverse transcriptase (TERT) promoter mutations in prognosis in bladder cancer. Bioengineered. 2021;12:1495-1504.

AKT1 and *PIK3CA* activating mutations in Moroccan bladder cancer patients´ biopsies and matched urines

VI.3 *AKT1* and *PIK3CA* activating mutations in Moroccan bladder cancer patients´ biopsies and matched urines

The phosphatidylinositol 3-kinase (*PI3K*)/ protein Kinase B (*AKT*) signalling pathway has become a major focus of a number of recent studies. This intra-cellular pathway was reported to be frequently over-activated in all human cancers and also appears to play a central role in bladder carcinogenesis.

The *PI3K-AKT* pathway is an important signaling pathway involved in the maintenance of cell growth, proliferation, migration and survival. It can be activated via extracellular signaling upstream of receptor tyrosine kinases, hormones and growth factors or by intracellular signalling (transformation or over-expression of proteins involved in the signal transduction), which is essential for its function in bladder cancer and other related diseases. Activating mutations in *PIK3CA* and *AKT1* genes are recurrent in BC and are promising targets to improve cancer management.

This prospective study was planned to analyse the mutational status of *PIK3AC* and *AKT1* for the possible existence of mutations in bladder cancer biopsies and urine sediments and to reliably assess their use as a urinary-based biomarkers for patients' diagnosis and monitoring. In a serie of 70 tumors and matched urines, we detected one E17K pathogenic mutation in exon 1 on the *AKT* gene (1.14%) and 11 mutations in exon 9 and 20 of the *PIK3CA* gene (15.71%), mostly seen in early stages and high-grade tumors. Sequencing of urine sediment-derived DNA for both genes showed high specificity and moderate sensitivity ranged from 66.7% for *PIK3CA* to 100% for *AKT1* genes.

Although alteration of the *PI3K/AKT* pathway is a prevalent event in bladder cancer, our findings show a low prevalence of *AKT1* and *PIK3AC* mutations. Consequently, it can be classified as a potential biomarker for the management of BC in Morocco. Furthermore, larger and multicentric studies are needed to overcome the lack of significant statistical association between mutational status and clinical outcomes.

Considering the ability to detect mutations of these genes in urine-exfoliated cells and the high specificity of this non-invasive method, we strongly suggest that these biomarkers should be taken into account in future genetic investigations in bladder cancer patients.

Article

PanAfrican Medical Journal

Research

AKT1 and PIK3CA activating mutations in Moroccan bladder cancer patients´ biopsies and matched urine

Hajar El Ahanidi, Meryem El Azzouzi, Housna Arrouchi, Chaimae Hafidi Alaoui, Mohammed Tetou, Mounia Bensaid, Mohamed Oukabli, Ahmed Ameur, Abderrahmane Al Bouzidi, Mohammed El Mzibri, Mohammed Attaleb

Corresponding author: Mohammed Attaleb, Biology and Medical Research Unit, The National Center for Energy and Nuclear Science and Technology, Rabat, Morocco. attaleb_mohammed@yahoo.fr

Received: 24 Aug 2021 - **Accepted:** 14 Dec 2021 - **Published:** 20 Jan 2022

Keywords: Bladder cancer, PIK3CA, AKT1, activating mutations, Moroccan patients

Copyright: Hajar El Ahanidi et al. Pan African Medical Journal (ISSN: 1937-8688). This is an Open Access article distributed under the terms of the Creative Commons Attribution International 4.0 License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Cite this article: Hajar El Ahanidi et al. AKT1 and PIK3CA activating mutations in Moroccan bladder cancer patients´ biopsies and matched urine. Pan African Medical Journal. 2022;41(59). 10.11604/pamj.2022.41.59.31383

Available online at: https://www.panafrican-med-journal.com//content/article/41/59/full

AKT1 and PIK3CA activating mutations in Moroccan bladder cancer patients´ biopsies and matched urine

Hajar El Ahanidi[1,2], Meryem El Azzouzi[1,2], Housna Arrouchi[2], Chaimae Hafidi Alaoui[1], Mohammed Tetou[3], Mounia Bensaid[3], Mohamed Oukabli[2,3], Ahmed Ameur[2,3], Abderrahmane Al Bouzidi[2], Mohammed El Mzibri[1], Mohammed Attaleb[1,&]

[1]Biology and Medical Research Unit, The National Center for Energy and Nuclear Science and Technology, Rabat, Morocco, [2]Rabat Medical and Pharmacy School, Mohammed V University in Rabat, Rabat, Morocco, [3]Military Hospital Mohammed V, Rabat, Morocco

[&]**Corresponding author**
Mohammed Attaleb, Biology and Medical Research Unit, The National Center for Energy and Nuclear Science and Technology, Rabat, Morocco

Abstract

Introduction: *in cancer cells, activating mutations in PIK3CA and AKT1 genes, major players of PI3K-AKT-mTOR signalling pathway, are widely reported in many cancers and present attractive targets for the identification of new therapeutics and better cancer management. The present study was planned to evaluate the mutational status of PIK3CA and AKT1 genes in bladder cancer patients and to assess the association between these mutations and patients´ clinico-pathological features.* ***Methods:*** *in this prospective study, bladder cancer biopsies and matched urine sediments samples were collected form 70 patients. Mutations were assessed by deoxyribonucleic acid (DNA) sequencing and correlation with clinico-pathological data was performed using SPSS software.* ***Results:*** *AKT1 alterations were poorly detected. Only one patient with pT1 stage and high-grade tumour carried the E17K mutation. In PIK3CA exon 9, 2 point mutations, E545K and Q546E, and a SNP (E547E) were reported, whereas in exon 20, 2 point mutations (L989V and H1047R) and 2 SNPs (I1022I and T1025T) were detected. PIK3CA mutations were mainly observed in early stages and high-grade tumours. Statistical analysis showed no significant association between the studied AKT1 and PIK3CA mutations and patients´ clinico-pathological parameters ($p > 0.05$). Detection of these mutations in voided urine samples showed a high specificity (100%) for both genes and a moderate sensitivity: 100% for AKT1 and 66.7% for PIK3CA genes.* ***Conclusion:*** *this study shows clearly that mutations in AKT1 and PIK3CA are rare events and could not be considered as valuable biomarkers for bladder cancer management.*

Introduction

Bladder cancer (BC) is the ninth most commonly diagnosed cancer in the world, accounting for approximately 420,000 new cases each year [1]. In Morocco, bladder cancer is a very significant public health problem, in terms of prevalence, mortality, impact on quality of life and economic cost [2]. According to the regional cancer registers of Casablanca and Rabat, the incidence of BC reached 5.8 and 11.3 per 100,000 persons, respectively [3,4]. Of particular interest, BC is most frequently diagnosed in males and the most frequent histological type is by far transitional cell carcinoma, usually diagnosed at stages I and II [5]. The most common presentation of bladder cancer is visible, or gross, hematuria, but patients can also present with isolated microscopic hematuria. The risk of bladder cancer is approximately 4% in patients with microscopic hematuria and 16.5% in those with gross hematuria [6]. In Morocco, the treatment of BC is based on the clinic-pathologic features [7] and the current standard method for urothelial BC detection in both the incident and surveillance settings are cystoscopy and conventional urine cytology [8]. Cystoscopy is an invasive approach that provides essential prognostic information, but shows insufficient power to precisely predict the patient outcome. Cytology is a largely used non-invasive method that presents some limits, likely related to its low sensitivity for low grade tumors and the user-dependent cytological interpretation [9]. These disadvantages are underlining the urgent need to implement more reliable and objective tools to diagnose and monitor BC patients. Currently, the development of molecular biology tools has given access to explore molecular alterations in bladder cancer and identifying potential diagnostic, prognostic and therapeutic targets; and numerous promising biomarkers have been identified, including genomic alterations and transcriptional subtypes [10]. In this field, frequent activating mutations have been identified, and multiple studies have focused on hotspot mutations markers for disease surveillance using urine sediments as promising diagnostic tool for non-invasive BC diagnosis [11, 12].

In cancer cells, PI3K-AKT-mTOR signalling pathway plays a major role in growth, proliferation and cellular survival. This pathway is either activated by extracellular signals (receptors with tyrosine kinase activity) or by intracellular ones through the transformation or the over expression of proteins

involved in the signal transduction [13, 14]. In BC, 50-70% of tumours contain mutations that are predicted to activate this pathway, including activating mutations in PIK3CA, a member of PI3K family, and in AKT1 genes [15]. PI3Ks (Phosphoinositide 3-kinases) are a class of enzymes that phosphorylate a series of membrane phospholipids playing a crucial role in tumour occurrence as part of the PI3K-AKT-mTOR signalling pathway [16]. In many cancers, a great interest has been focused on the identification of new inhibitors targeting class-IA PI3Ks, including PI3Ka and PI3K ß and their downstream pathways [17]. PIK3CA mutations can occur in any of the four domains of the gene: the adaptor-binding domain, C2 domain, helical domain and catalytic (kinase) domain. However, hot spot mutations occur mainly in exon 9 of the helical domain (E542 and E545) and in exon 20 of the kinase domain (H1047) [18]. AKT is an evolutionarily conserved kinase, also known as protein kinase B. Obviously, there are three members of the AKT family (AKT1-3), encoded by separate genes, but with over 80% amino-acid sequence identity which has both distinct and overlapping functions. AKT occupies a key regulatory node in the PI3K pathway, influencing significantly a wide range of cellular processes [19]. The AKT1 E17K mutation can activate the PI3K/AKT/mTOR pathway in a similar manner as PIK3CA alterations and promotes carcinogenesis through increasing cell proliferation or survival by phosphorylating several direct substrates that have crucial roles in cellcycle regulation. These substrates include cell cycle inhibitors and transcription factors [20, 21]. The present prospective study was planned to explore the mutational status of PIK3CA and AKT1 genes in BC biopsies and matched urines and to assess their use as anon-invasive molecular biomarker for patients´ diagnosis and monitoring.

Methods

Patients and study design

A total of 70 patients with bladder cancer were recruited at the Urology Department of the Military Hospital Mohammed V in Rabat - Morocco. Each biopsy was cut into two parts; one part was used for anatomy-pathological diagnosis and the other one was kept in -80°C for molecular biology explorations. Paired voided urine samples were obtained before surgery from all patients. Staging and grading were done according to the TNM classification (NMIBC includes Ta, T1, and Tis; and MIBC includes T2, T3, and T4) and World Health Organization criteria. Recurrence was defined as reappearance of primary NMIBC, with a lower or the same pathological stage, and progression was defined as a disease with a higher TNM stage upon relapse [9]. The study protocol was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat - Morocco (Ref 82/19). All recruited patients have agreed to participate voluntarily in the study, been informed on the study and its objectives, and signed written consents. With respect to ethical rules, rights of confidentiality and anonymity of participants have been respected.

Genomic DNA extraction

Genomic DNA was extracted from fresh frozen tumours and urine cell sediments using phenol/chloroform method [22]. All extracted DNA was quantified with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were used immediately or stored at -20°C until use.

Evaluation of PIK3CA and AKT1 mutational status

Specific DNA primers were used to amplify exons 9 (F: AATCATCTGTGAATCCAGAGG; R: ATGCTGAGATCAGCCAAAAT) and 20 (F: CATTTGAGCAAAGACCTGAAGG; R: TGAGCTTTCATTTTCTCAGTTATC) of the PIK3CA gene and exon 1 of the AKT1 gene (F: TAGAGTGTGCGTGGCTCTCA; R: CTGAATCCCGAGAGGCCAA). DNA Amplification was performed on ProFlex PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 5 min at 95°C for initial denaturation, 35 cycles of 30s at 94°C for

denaturation, 40s at specific annealing temperature of each primer set (54°C for PIK3CA exon 9, 58°C for PIK3CA exon 20 and 60°C for AKT1 exon 1 amplifications) and 40s at 72°C for DNA synthesis. At the end of the last cycle, a final elongation step of 7 min at 72°C was performed. PCR products were purified by ExS-Pure™ enzymatic PCR clean-up kit (NimaGen BV, Nijmegen, The Netherlands) and the sequencing was performed with BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer´s protocol, on an ABI 3500 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were matched with the genes references sequences (Genes ID: 5290 and 207 for PIKCA and AKT1 respectively) collected from the GeneBank database. The sequence alignment was performed using Clustal W program in Bio-Edit Software.

Statistical analysis

Statistical analyses were performed using IBM SPSS software version 23. The mutation status of studied genes in both tumours and urine samples was statistically evaluated using percentages and confidence intervals (CI) of 95%. The correlation between mutational status and clinico-histo-pathological parameters (tumours stage, grade, recurrence and progression) was evaluated using chi-squared test (χ^2 test). Differences were considered significant at p values less than 0.05.

Results

Characteristics of the study population

Among the 70 patients with bladder cancer, 68 were male and 2 were female, with a sex ratio of 34. The mean age of patients was 67 years, ranging from 47 to 85 years. The clinico-pathological characteristics of tumour specimens are summarized in Table 1. Most cases were staged = PT1 (74.29%) and have high grade tumours (61.43%). In NMIBC, 23.08% of cases recurred and 9.62% progressed upon relapse (Table 1).

Evaluation of the mutational status of PIKCA and AKT1 genes in bladder biopsies

AKT1 alterations were poorly detected in our cohort and were obtained in only in 1.4% of samples (1/70).AKT1 E17K mutation was found in a man with pT1 stage and high-grade tumour (Table 2). Mutations in PIK3CA gene were found in 12.6% of patients (9/70). In PIK3CA exon 9, 2 point mutations, E545K and Q546E, were reported in 3 and 1 cases, respectively, and a SNP (E547E) was reported in 1 case. In PIK3CA exon 20, 2 point mutations, L989V and H1047R, were found in 1 and 2 cases, respectively. Moreover, I1022I SNP was found in 2 cases and T1025T SNP in 1 case. Of note, 2 cases have 2 point mutations each; the first one have 1 SNP and 1 point mutation (E547E/Q546E) in exon 9 and the second harboured 2 SNPs (I1022I/T1025T) in the exon 20. The relationship between mutations and patient clinico-phatological features is summarized in Table 2, and clearly showed that mutations of PIK3CA were mainly observed in low tumour stages (= pT1), reported in 15.4 % of cases (8/52), and in high grade tumours, reported in 14% of cases (6/43). Of particular interest, PIK3CA and AKT1 point mutations weren´t detected in any recurrent or progressive case (Table 2). Statistical analysis showed no significant association between the studied AKT1 and PIK3CA point mutations and patients´ clinico-pathological parameters ($p > 0.05$) (Table 2).

Evaluation of the mutational status of PIK3CA and AKT1 genes in matched urine sediments

In urine sediments, PIK3CA and AKT1 gene mutations were reported in 8.6% (6/70) and 1.4% (1/70) of samples, respectively. Comparison between mutational status of the studied genes in bladder biopsies and paired urine sediments DNA was assessed and is reported in Table 3. Interestingly, all cases showing mutations in urine sediments have been diagnosed with BC after haematuria episode and have aberrant mutations in the corresponding DNA from bladder biopsies, and reciprocally, no mutation was detected in urine

sediments of patients showing the absence of point mutations in bladder biopsies, suggesting a specificity of mutation detection in urine sediments of 100% for both AKT and PIK3CA genes. Point mutation detection in urine sediments showed high sensitivity for AKT1 gene (100%); the only point mutation obtained in AKT1 gene was reported in both patient´s biopsy and urine samples. Moderate mutation detection sensitivity in urine samples was obtained for PIK3CA gene (66.7%); point mutations were detected in only 6 urine sediments samples among the 9 patients who harboured point mutations in the corresponding biopsies. Additionally, all mutated cases have been diagnoses with bladder cancer after presenting hematuria episode (Table 3).

Discussion

During the last decades, a great interest was given to the molecular events in bladder tumorigenesis as determinant of cancer progression, and worldwide published data have identified molecular events that could be of interest in patients´ diagnosis, prognosis and target therapy [23, 24]. In the present study, mutation of AKT1 gene is a rare event in bladder cancer in Moroccan patients. Among 70 patients, only 1.4% (1/70) harboured the E17K point mutation. This results is in agreement with reported finding in breast cancer (4.3%) [25], ovarian 2% and colorectal 6% cancers [26]. Therapies targeting AKT1 mutated cancers showed that treatment with AZD5363, an ATP-competitive pan-AKT kinase inhibitor, yielded durable responses and tumour regressions across a variety of tumour types harbouring the E17K mutation including breast, endometrial, cervical, and lung cancers. Mutations as D323H in AKT1 and W80C in AKT2 are relatively resistant to the allosteric AKT inhibitor MK-2206 suggesting that the vast majority of rare AKT variants are passenger mutations with no effect on drug sensitivity [27, 28].

PIK3CA mutations of the helical domain has been reported in up to 26% of breast cancers, E542K and E545K being the most prevalent point mutations and were reported in 20% and 11% of cases, respectively [29]. In our study, PIK3CA mutations were found in 12.6% (9/70) cases distributed in exon 9 (3 E545K, 1 Q546E and 1 E547E) and 20 (1 L989V; 2 I1022I;1 T1025T and 2H1047R). Interestingly, among the 9 positive cases, 2 harboured 2 point mutations; 1 case has Q546E point mutation and E547E SNP in exon 9 and the second carried the I1022I and T1025T SNPs. In a cohort study of 61 patients, with two proliferative skin lesions lacking malignant potential, the typical cancer-associated PIK3CA mutations E542K, E545K and H1047R were revealed in 16% of the cases [30]. In this study, the H1047R mutation affecting the catalytical domain of PIK3CA gene was found only in two patients supporting the widely reported data showing that PIK3CA alterations affecting the catalytical domain mutations, includingH1047R, were relatively rare (16.7%), whereas mutations affecting the helical domain, including E545K/D and E542K, prevail and are reported in relatively high number of cases [31]. In urothelial tumours somatic mutations in PIK3CA gene may be of use for early detection of primary and recurrent tumours in urine-based assays, not only for prognosis prediction, but also as molecular biomarkers for targeted therapies [32]. Specific knockdown of PIK3CA inhibited proliferation, migration, anchorage-independent growth and in vivo tumour growth of cells with PIK3CA mutations [15]. Of particular interest, E542K and E545K were respectively identified in two cell lines: BLCAb001 and BLCAb002. BLCAb001 is less responsive to cisplatin than BLCAb002, suggesting that the presence of activating PIK3CA mutations may not necessarily predict in vivo treatment response to PI3K targeted therapies, while specific gene alterations may be predictive for cisplatin response in BC models and, potentially, in patients as well [33].

In BC, numerous studies have highlighted the possibility to detect bladder oncogene mutations in DNA isolated from urine sediments, giving the opportunity to use a non-invasive approach for genetic studies and molecular diagnosis [34]. Of particular interest, it was shown that assessment of some genetic alterations in urine sediments adds

diagnostic value to urine cytology and confers high sensitivity and specificity for the detection of urothelial carcinoma [35]. In this context, we have evaluated the mutational status of PIK3CA and AKT1genes in bladder cancer biopsies and matched urines, and assess their use as anon-invasive molecular biomarker for patients´ diagnosis and monitoring. The use of DNA obtained from urine sediment; as template to detect mutations, provides sensitivities of 66.7% and 100% for PIK3CA and AKT1 genes, respectively. No false-positive result was detected and the mutations in exfoliated cells were observed only in cases showing mutations in corresponding tumours, giving a specificity of 100% for both PIK3CA and AKT1 genes. Sensitivity of detecting mutations of PIK3CA gene in urine sediments is equal to 66.7% and the only 1 mutation in AKT was reported in both the patient´s biopsy and voided urine sediment. These results must be considered with a lot of precaution, as the number of positive cases is low. In Morocco, as in most developing countries, the treatment of BC is based on the clinico-pathologic features, and the main challenge faced by clinicians is to overcome the treatment inefficiency and prevent the development of disease´ recurrence and/or resistance to chemo and/or radiotherapy [36, 37]. The main limitation of the study is the low number of cancer cases that will not reflect the real mutational status of these genes in BC and consequently will not be useful for better assessment of the association between the mutational status and clinico-pathological features.

Conclusion

In conclusion, this study is very informative and clearly showed that mutations in AKT1 and PIK3CAare rare events and could not be considered as valuable biomarkers for bladder cancer management. Other studies are needed to explore the impact of these mutations in the overall PI3K-AKT-mTOR signalling pathway and bladder cancerogenesis.

What is known about this topic

- *PI3K-AKT-mTOR signalling pathways play a major role in tumour growth, proliferation and cellular survival;*
- *Urine cytology has a low detection sensitivity, especially for low-grade tumours.*

What this study adds

- *AKT1 mutation is a rare event in bladder cancer;*
- *Detecting mutations in urine sediment is a promising and reliable method for non-invasive diagnosis and monitoring.*

Competing interests

The authors declare no competing interests.

Authors' contributions

HEA performed the experiments, analysed the data and drafted the paper; MEA participated in data analysis and contributed to the paper drafting; HA participated in the study experiments; CHA participated in bio-statistical analysis; MT and AA were in charge of patients recruitment, sampling and clinical explorations; MB, MO and AAB were in charge of the anatomo-pathological explorations; MEM contributed to the study design and revised the paper draft; MA participated in the design and coordination, supervised all the experiments of the project and review of the final manuscript.

Tables

Table 1: tumour characteristics of people with bladder cancer
Table 2: distribution of PIK3CA and AKT1 genes mutations according to clinicopathological
Table 3: comparison between mutational status in bladder biopsies and voided urine sediments

References

1. Wong MCS, Fung FDH, Leung C, Cheung WWL, Goggins WB, Ng CF. The global epidemiology of bladder cancer: a joinpoint regression analysis of its incidence and mortality trends and projection. Sci Rep. 2018 Jan 18;8(1): 1129. **PubMed**| **Google Scholar**
2. Brausi M, Olaru V. Management of high-risk non-muscle invasive bladder cancer. Minerva Urol Nefrol. 2012 Dec;64(4): 255-60. **PubMed**
3. Benider A, Bendahhou K. Registre des cancers de la région du grand Casablanca 2008-2012. Ed 2016. 2016.
4. Tazi MA, Er-Raki A, Benjaafar N. Cancer incidence in Rabat, Morocco: 2006-2008. Ecancermedicalscience. 2013 Aug 8;7: 338. **PubMed**| **Google Scholar**
5. Payandeh M, Sadeghi M, Sadeghi E. Characteristics of patients with transitional cell carcinoma of the urinary Bladder in Kermanshah Province, Iran. Iran J Cancer Prev [Internet]. 2015 Dec 23;8(6). Cited 2021 Dec 2.
6. Edwards TJ, Dickinson AJ, Natale S, Gosling J, Mcgrath JS. A prospective analysis of the diagnostic yield resulting from the attendance of 4020 patients at a protocol-driven haematuria clinic. BJU Int. 2006 Feb;97(2): 301-5; discussion 305. **PubMed**| **Google Scholar**
7. Bowa K, Mulele C, Kachimba J, Manda E, Mapulanga V, Mukosai S. A review of bladder cancer in Sub-Saharan Africa: A different disease, with a distinct presentation, assessment, and treatment. Ann Afr Med. Jul-Sep 2018;17(3): 99-105. **PubMed**| **Google Scholar**
8. Noon AP, Zlotta AR. Urothelial Bladder Cancer Urinary Biomarkers. EJIFCC. 2014 Apr 28;25(1): 99-114 eCollection 2014 Apr. **PubMed**| **Google Scholar**
9. Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BWG, Compérat E *et al*. EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2013. European Urology. 2013 Oct;64(4): 639-53. **PubMed**| **Google Scholar**
10. Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer. 2015 Jan;15(1): 25-41. **PubMed**| **Google Scholar**
11. Hayashi T, Fujita K, Hayashi Y, Hatano K, Kawashima A, McConkey DJ *et al*. Mutational Landscape and Environmental Effects in Bladder Cancer. IJMS. 2020 Aug 23;21(17): 6072. **PubMed**| **Google Scholar**
12. Hong M, He G, Goh S, Low AWX, Tay KJ, Lim TKH, et al. Biomarkers for Precision Urothelial Carcinoma Diagnosis: Current Approaches and the Application of Single-Cell Technologies. Cancers. 2021 Jan 12;13(2): 260. **PubMed**| **Google Scholar**
13. Hassan B, Akcakanat A, Holder AM, Meric-Bernstam F. Targeting the PI3-Kinase/Akt/mTOR Signaling Pathway. Surgical Oncology Clinics of North America. 2013 Oct;22(4): 641-64. **PubMed**| **Google Scholar**
14. Brotelle T, Bay J-O. La voie de signalisation PI3K-AKT-mTOR?: description, développement thérapeutique, résistances, marqueurs prédictifs/pronostiques et applications thérapeutiques en cancérologie. Bulletin du Cancer. 2016 Jan;103(1): 18-29. **Google Scholar**
15. Ross RL, McPherson HR, Kettlewell L, Shnyder SD, Hurst CD, Alder O *et al*. PIK3CA dependence and sensitivity to therapeutic targeting in urothelial carcinoma. BMC Cancer. 2016 Jul 28;16: 553. **PubMed**| **Google Scholar**
16. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. Oncogene. 2008 Sep 18;27(41): 5497-510. **PubMed**| **Google Scholar**
17. Riquelme I, Tapia O, Espinoza JA, Leal P, Buchegger K, Sandoval A *et al*. The Gene Expression Status of the PI3K/AKT/mTOR Pathway in Gastric Cancer Tissues and Cell Lines. Pathol Oncol Res. 2016 Oct;22(4): 797-805. **PubMed**| **Google Scholar**

18. Ligresti G, Militello L, Steelman LS, Cavallaro A, Basile F, Nicoletti F *et al*. PIK3CA mutations in human solid tumors: Role in sensitivity to various therapeutic approaches. Cell Cycle. 2009 May 1;8(9): 1352-8. **PubMed**| **Google Scholar**
19. Askham JM, Platt F, Chambers PA, Snowden H, Taylor CF, Knowles MA. AKT1 mutations in bladder cancer: identification of a novel oncogenic mutation that can co-operate with E17K. Oncogene. 2010 Jan 7;29(1): 150-5. **PubMed**| **Google Scholar**
20. Shorning BY, Dass MS, Smalley MJ, Pearson HB. The PI3K-AKT-mTOR Pathway and Prostate Cancer: At the Crossroads of AR, MAPK, and WNT Signaling. IJMS. 2020 Jun 25;21(12): 4507. **PubMed**| **Google Scholar**
21. Liang J, Slingerland JM. Multiple Roles of the PI3K/PKB (Akt) Pathway in Cell Cycle Progression. Cell Cycle. Jul-Aug 2003;2(4): 339-45. **PubMed**| **Google Scholar**
22. Eschbach S, Hofmann C, Maerz M, Maier U-G, Sitte P. Molecular Cloning. A Laboratory Manual. 2. Auflage. Hrsg. von J. Sambrook, E. F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press, Cold Spring Harbour 1989, $ 115. ISBN 0-87969-309-6. Biologie in unserer Zeit. 1990 Dec;20(6): 285-285.
23. McConkey DJ, Choi W. Molecular Subtypes of Bladder Cancer. Curr Oncol Rep. 2018 Aug 20;20(10): 77. **PubMed**| **Google Scholar**
24. Tan TZ, Rouanne M, Tan KT, Huang RY-J, Thiery J-P. Molecular Subtypes of Urothelial Bladder Cancer: Results from a Meta-cohort Analysis of 2411 Tumors. European Urology. 2019 Mar;75(3): 423-432 Epub 2018 Sep 10. **PubMed**| **Google Scholar**
25. Kim MS, Jeong EG, Yoo NJ, Lee SH. Mutational analysis of oncogenic AKT E17K mutation in common solid cancers and acute leukaemias. Br J Cancer. 2008 May 6;98(9): 1533-5. **PubMed**| **Google Scholar**
26. Carpten JD, Faber AL, Horn C, Donoho GP, Briggs SL, Robbins CM *et al*. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature. 2007 Jul 26;448(7152): 439-44. **PubMed**| **Google Scholar**
27. Hyman DM, Smyth LM, Donoghue MTA, Westin SN, Bedard PL, Dean EJ *et al*. AKT Inhibition in Solid Tumors With AKT1 Mutations. JCO. 2017 Jul 10;35(20): 2251-2259. **PubMed**| **Google Scholar**
28. Yi KH, Lauring J. Recurrent AKT mutations in human cancers: functional consequences and effects on drug sensitivity. Oncotarget. 2016 Jan 26;7(4): 4241-51. **PubMed**| **Google Scholar**
29. Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, et al. PIK3CA Mutations Correlate with Hormone Receptors, Node Metastasis, and ERBB2, and Are Mutually Exclusive with PTEN Loss in Human Breast Carcinoma. Cancer Res. 2005 Apr 1;65(7): 2554-9. **PubMed**| **Google Scholar**
30. Hafner C, Lopez-Knowles E, Luis NM, Toll A, Baselga E, Fernandez-Casado A *et al*. Oncogenic PIK3CA mutations occur in epidermal nevi and seborrheic keratoses with a characteristic mutation pattern. Proceedings of the National Academy of Sciences. 2007 Aug 14;104(33): 13450-4. **PubMed**| **Google Scholar**
31. Dueñas M, Martínez-Fernández M, García-Escudero R, Villacampa F, Marqués M, Saiz-Ladera C et al. PIK3CA gene alterations in bladder cancer are frequent and associate with reduced recurrence in non-muscle invasive tumors: PIK3CA Gene Alterations In Non-Muscle Invasive Bladder Cancer. Mol Carcinog. 2015 Jul;54(7): 566-76 Epub 2013 Dec 18. **PubMed**| **Google Scholar**
32. Kompier LC, Lurkin I, van der Aa MNM, van Rhijn BWG, van der Kwast TH, Zwarthoff EC. FGFR3, HRAS, KRAS, NRAS and PIK3CA Mutations in Bladder Cancer and Their Potential as Biomarkers for Surveillance and Therapy. Cheriyath V, editor. PLoS ONE. 2010 Nov 3;5(11): e13821. **PubMed**| **Google Scholar**
33. Wei L, Chintala S, Ciamporcero E, Ramakrishnan S, Elbanna M, Wang J *et al*. Genomic profiling is predictive of response to cisplatin treatment but not to PI3K inhibition in bladder cancer patient-derived xenografts. Oncotarget. 2016 Nov 22;7(47): 76374-76389. **PubMed**| **Google Scholar**

34. Ferro M, La Civita E, Liotti A, Cennamo M, Tortora F, Buonerba C *et al*. Liquid Biopsy Biomarkers in Urine: A Route towards Molecular Diagnosis and Personalized Medicine of Bladder Cancer. JPM. 2021 Mar 23;11(3): 237. **PubMed**| **Google Scholar**
35. Fantony JJ, Longo TA, Gopalakrishna A, Owusu R, Lance RS, Foo W-C *et al*. Urinary NID2 and TWIST1 methylation to augment conventional urine cytology for the detection of bladder cancer. Cancer Biomark. 2017;18(4): 381-387. **PubMed**| **Google Scholar**
36. Mari A, D´Andrea D, Abufaraj M, Foerster B, Kimura S, Shariat SF. Genetic determinants for chemo- and radiotherapy resistance in bladder cancer. Transl Androl Urol. 2017 Dec;6(6): 1081-1089. **PubMed**| **Google Scholar**
37. Roh Y-G, Mun M-H, Jeong M-S, Kim W-T, Lee S-R, Chung J-W *et al*. Drug resistance of bladder cancer cells through activation of ABCG2 by FOXM1. BMB Rep. 2018 Feb;51(2): 98-103. **PubMed**| **Google Scholar**

Table 1: tumour characteristics of people with bladder cancer

Parameter	Total cases	Percentage (%)
Gender		
Male	68	97.1
Female	2	2.9
Age		
<50	1	1.4
50-70	44	62.9
>70	25	35.7
Smoking		
Yes	28	40
No	42	60
Tumour stage		
≤PT1	52	74.3
>PT1	18	25.7
Tumour grade		
Low grade	27	38.6
High grade	43	61.4
Tumour recurrence		
Yes	12	23.1
No	40	76.9
Tumour progression		
Yes	5	9.6
No	47	90.4

Table 2: distribution of PIK3CA and AKT1 genes mutations according to clinicopathological parameters				
Parameter	**PIK3CA**		**AKT1**	
	Overall n=9		**Overall n=1**	
	+	**-**	**+**	**-**
Gender				
Male	9	59	1	67
Female	0	2	0	2
P value	0.999		1	
Age				
< 50	0	1	0	1
50 -70	7	36	1	43
> 70	2	23	0	25
P value	0.703		0.410	
Smoking				
Yes	3	25	1	27
No	6	36	0	42
P value	0.525		0.997	
Tumour stage				
≤ PT1	8	44	1	51
>PT1	1	17	0	18
P value	0.998		0.998	
Grade				
LG	3	24	0	27
HG	6	37	1	42
P value	0.512		0.998	
Recurrence				
Yes	0	12	0	12
No	9	31	1	39
P value	0.999		0.999	
Progression				
Yes	0	5	0	5
No	9	38	1	46
P value	0.999		0.999	

Table 3: comparison between mutational status in bladder biopsies and voided urine sediments							
Genes	**Mutations in fresh biopsies**		**Mutations in urine sediments**		**Sensitivity % (95% CI)**	**Specificity % (95% CI)**	**Hematuria % (95% CI)**
	N	**% (95% CI)**	**N**	**% (95% CI)**			
PIK3CA	9	12.6 (6.1-23)	6	8.6 (3.2-17.7)	66.7 (29.9-92.5)	100 (94.9-100)	100 (66.4-100)
AKT1	1	1.4 (0-7.7)	1	1.4 (0-7.7)	100 (2.5-100)	100 (94.9-100)	100 (2.5-100)
CI = confidence interval. The sensitivity reflects the fraction of patients in which the urine DNA was mutated among cases with mutation in matched tumour DNA of the same genes.							

Mutational analysis of *Tp53* gene exons 4-9 in tumours biopsies from Moroccan bladder cancer patients

VI.4 Mutational analysis of *p53* gene exons 4-9 in tumours biopsies from Moroccan bladder cancer patients

P53 is a DNA-binding transcription factor that plays a prominent role in halting the cell cycle in response to many molecular stresses; its loss of function often results in genomic or chromosomal instability that leads to dysregulated proliferation in cancer.

Mutations in the *p53* gene occur in more than half of all human malignancies, and some *P53* mutation hotspots have been revealed to play a major role in carcinogenesis. In bladder cancer, the *Tp53* gene is mutated in 50 to 60% of muscle-invasive tumors with a high risk for BC (tumours classified as T2, Ta disease and high-grade tumour associated with CIS). In contrast, mutations in the *p53* are thought to be a strong predictor of tumor recurrence and are related to invasive tumors in bladder cancer.

To get a better understanding of the genetic basis of bladder cancer in the Moroccan population, we investigated the mutational profile analysis of the *p53* gene (exons 4-9) in 70 bladder cancer tumors of all stages and grades at initial diagnosis by performing a PCR-sequencing analysis on the whole cohort. Additionally, we analyzed the prognostic significance of the identified mutations regarding clinical and pathological parameters of bladder cancer.
The present study revealed very high frequencies of mutations with a prevalence of 100%, and at least one mutation was detected for each case in our cohort. Also, alterations in this gene were detected in all stages and grades of BC. The most frequent mutation was found in exon 4, the R72P polymorphism, and was detected in 91.43% of bladder cancer specimens. Most of the mutation types found are located in exon 8 of the *p53* gene in comparison with the other exons, and only the correlation between mutations in exon 8 and grade has been observed (p-value = 0.034).

Our results suggest that mutations of the *p53* gene may play a major role in bladder cancer development. As *p53* mutations are detected in early and advanced stages, they can be used as a biomarker for early diagnosis or as a prognostic marker. The findings also suggest that the relationship between bladder cancer risk and mutations in *p53* deserves further investigation. Moreover, in different types of human cancers, a number of specific *p53* mutation hotspots have been identified and can be used as a "probe" for clinical practice to be better-informed on the complete remission of the tumor in response to treatment and the possibility of its recurrence.

Mutational analysis of p53 gene exons 4-9 in tumours biopsies from Moroccan bladder cancer patients.

Chaimae Hafidi Alaoui[a,b], Meryem El Azzouzi[a,d], Hajar El Ahanidi[a,d], El Founini Younes [a,e], Bouchra Belkadi[b], Imane Chaoui[a], Laila Benbacer[a], Mohammed Tetou[c], Ilias Hassan[c], Mohamed Oukabli[d,c], Ahmed Ameur[d,c], Ab derrahmane Al Bouzidi[d], Mohammed Attaleb[a]*, Mohammed El Mzibri[a]*
* Senior contribution

a. Biology and Medical Research Unit, CNESTEN, Rabat, Morocco.
b. Faculty of Sciences, Mohammed V University. Rabat, Morocco.
c. Mohammed V Military Hospital, Rabat, Morocco
d. Faculty of Medicine and Pharmacy of Rabat. Mohammed V University. Rabat, Morocco.
e.Cellular and Molecular Pathology Laboratory, Faculty of Medicine and Pharmacy, Hassan II University, Casablanca, Morocco

Submitted to Cancer Genetic Journal

*** Corresponding author**:
Dr. Mohammed Attaleb
Head of Biology and Medical Research Unit
Centre National de l'Energie, des Sciences et techniques Nucléaires (CNESTEN).
BP 1382 RP. 10001 Rabat, Maroc

Tel	+212.537.71.27.51
Office:	+212.537.71.18.46
Fax:	attaleb_mohammed@yahoo.fr,
E-mail:	attaleb@cnesten.org.ma

ABSTRACT

Bladder cancer (BC) is one of the most common malignant tumors of the urinary system and in which several molecular and pathological pathways are altered. The *Tp53* tumor suppressor gene is commonly mutated in various cancers, including bladder cancer, and some T*p53* mutations have been revealed to play a major role in carcinogenesis.

To find relationships between the mutation patterns in *Tp53* gene and bladder cancer, we studied mutational profiling on Exons 4-9 of the T*p53* gene in 70 bladder cancer tumors of all stages and grades at initial diagnosis by performing a PCR-sequencing analysis on the whole cohort. Additionally, we analyzed the prognostic significance of the identified mutations regarding bladder cancer's clinical and pathological parameters.

From the sequence analysis, at least one mutation was observed for each case in our cohort. Thirty-four (34) mutations were identified with more prevalent mutations in exon 4 (91.43%). Most of the mutation types were located in the DNA-binding domain (Exon 5- 8). No alteration was detected in exon 7, whereas a change at intron 7 was found. The $\chi 2$ test results showed a correlation between changes in exon 8 and grade (*p-value* = 0.034).

Findings from this preliminary study revealed that *Tp53* gene mutations may be useful as a biomarker for early diagnosis and follow-up of bladder cancer. A study of *Tp53* expression, in the same population, is needed to understand the involvement of this mutation in *Tp53* inactivation in bladder cancer patients.

Keywords: Mutations, P53, the R72P polymorphism, Bladder cancer, Morocco.

INTRODUCTION

Bladder cancer is one of the most common malignancies in the world (Dadhania et al., 2015). In Morocco, BC is still a major public health problem, it's the second most common cancer of the urinary system, after prostate cancer in men, and it's a cause of cancer death in the elderly. BC ranks as the eighth most commonly diagnosed cancer in the Moroccan population, with an estimated 2268 new cases in both sexes, accounting for 3.8% of total new cases (Bouchbika et al., 2013; GLOBOCAN, 2022). About 75% of BC patients are diagnosed with non-muscle invasive bladder cancers (NMIBC), while the other types are muscle-invasive bladder cancers type (MIBC) or develop into metastatic disease (Kassouf et al., 2015).

MIBCs are molecularly heterogeneous, characterized by high genetic instability, manifesting by genetic alterations at the molecular level, including mutations, chromosomal hybridization, and loss of heterozygosity (Matulewicz & Steinberg, 2020). While many genes can be mutated, inactivating *TP53*, *RB1*, and *PTEN* tumor suppressor genes is extremely frequent in MIBC (Cordon-Cardo, 2008), hence the importance of determining a molecular profile that can play a crucial role in predicting prognosis and evaluating therapeutic choice (Dall'Era et al., 2012).

TP53 protein is a nuclear phosphoprotein of 375 amino acids, which is commonly referred to as the "guardian of the genome" (Chang et al., 1993). In a normal cell, the primary function of Tp53 is to regulate the expression of several target genes as a transcription factor. It is considered an essential mediator of cellular response mechanisms to various stresses. The activation of Tp53, in the context of these advanced stresses, conditions a transient arrest of cell proliferation and damage repair (Ozaki & Nakagawara, 2011). The domain structures of these 53KDa proteins can be divided into three principal regions: the transactivation domain encoded by exons 2-4, the DNA binding domain encoded by exons 5 to 8 and the oligomerization domain encoded by exons 9 and 10. Each domain has a significant role in TP53 activities (Végran et al., 2013).

The *Tp53* tumor suppressor gene is commonly mutated in various cancers, including bladder cancer; and mutated *Tp53* gene plays a significant role in carcinogenesis (Kandoth et al., 2013; Ozaki & Nakagawara, 2011). Recent studies have demonstrated the essential role of mutations in the *Tp53* gene in the progression and prognosis of MIBC (Li et al., 2021). Indeed, the loss of *Tp53* function, with the gain of function mut-p53, promotes the process of tumorigenesis, increased tumor progression, and decreased survival rate. Mutations in this gene have been reported in over 66% of cases with BC and were found to be correlated with a high grade (Chen

et al., 2019; Li et al., 2021). Other effects attributed to mutant *Tp53* overexpression are the promotion of progression and metastatic potential through its effect on numerous signalling pathways leading to receptor recycling, thereby regulating metastatic gene expression (Muller & Vousden, 2014).

To better understand the genetic basis of bladder cancer in the Moroccan population, we investigated the analysis of the mutational profile of *Tp53* in 70 bladder cancer tumors of all stages and grades at initial diagnosis by performing a PCR-sequencing analysis of the whole cohort. Additionally, we analyzed the prognostic significance of the identified mutations regarding bladder cancer's clinical and pathological parameters.

MATERIALS AND METHODS

Study design and specimens' collection

Transurethral resection of the bladder (TURB) treatment was performed in the Urology Department of Military Hospital of Instruction Mohamed V (MHIMV) in Rabat, Morocco, between January 2017 and January 2019 for a panel of 70 patients studied. Fresh tissue samples were used as biological material for mutational analysis and stored at - 80°C until use.

Histological analysis of the collected tissues was conducted in the anatomy pathology department of MHIMV, and the tumor origin, staging and grading were done according to the TNM classification and World Health Organization (WHO) / International Society of Urologic Pathology criteria.

All selected patients were given clear and appropriate information on the present study and signed a written authorization in accordance with the recommendations of the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat- Morocco (Ref 82/19).

Genomic DNA extraction

Genomic DNA was extracted from fresh frozen tumors using a standard phenol/chloroform method. All extracted DNA was quantified with a NanoDrop™ 200 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) (Sambrook et al, 1989). DNA samples were stored at -20°C until analysis.

TP53 mutation detection

Exons 4-9 of the *TP53* gene were assessed by PCR-sequencing using the following primers **(Table I)**. The PCR reaction was realized in a total volume of 25 µl contained 0.5 units of *Taq*

DNA polymerase, 10 mM of each consensus primer, 1.5 mM $MgCl_2$, 10 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), and 2 µl of DNA sample in 1x *Taq* polymerase buffer.

DNA Amplification was performed on ProFlex-Applied Biosystems, starting with a denaturation step at 95°C for 5min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing was carried out for 30 seconds at a specific annealing temperature of each primer set **(Table I)**, then an extension at 72°C for 30 seconds. At the end, the mixture was incubated for a final elongation step at 72°C for 7 min. For every reaction, negative control in which the DNA template was omitted from the amplification mixture was included.

PCR products were subjected to electrophoresis on a 1-1.5%% agarose gel before proceeding to the purification of PCR products step using the ExS-Pure™ enzymatic PCR clean-up kit (NimaGen BV, Nijmegen, The Netherlands). Sequencing reactions were performed with BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in a volume of 10 µl, including 1 µl of 2.5X BigDye ready reaction mix v.3.1, 10 pmol of forward primer, and 100ng of purified PCR product. The mixtures were incubated according to the manufacturer's protocol. Sequencing reaction products were finally purified using Sephadex G-50 gel-exclusion chromatography (GE Healthcare, Life Sciences). Obtained sequences were aligned using the Clustal W program of the BioEdit software. They were then compared to the reference sequence of the gene collected in the GenBank database (NCBI Reference Sequence. NG_017013.2).

Table V. Primers and annealing temperature for PCR reaction

Gene	Forward Primer 5'–3'	Reverse Primer 5'–3'	Product size (pb)	Tm (°C)
P53/exon4	**TCT GAC TCT TTT CAC CC**	**CCA GGC ATT GAA GTC ATG GAA G**	**353**	**63**
P53/exon5	**TGT TTG TTT CTT TGC TGC CGT GT**	**CAA CCA GCC CTG TCG TCT CT**	**310**	**65**
P53/exon6	**TCC GCG CCA TGG CCA TCT AC**	**AAC CAC CCT TAA CCC CTC CT**	**331**	**62**
P53/exon7	**GGC TCT GAC TGT ACC ACC AT**	**GGA AGA AAT CGG TAA GAG G**	**201**	**55**
P53/exon8	**CTG CCT CTT GCT TCT CTT TT**	**GAG GCA AGG AAA GGT GAT AA**	**255**	**61**
P53/exon9	**GGA GGA GAC CAA GGG TGC AGT T**	**ATG CCC CAA TTG CAG GTA AAA CA**	**235**	**59**

Statistical analysis

The correlation between mutation status of the *Tp53* gene and clinico-pathological parameters was evaluated by $\chi 2$ test, and *p values* <0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS software version 23.

RESULTS

Clinical data and patient characteristics

Clinico-pathological data of 70 patients enrolled in this study are shown in **Table II.** Most of the patients included in this cohort were male (68/70), with a mean age of 67 years old (age ranging from 47 to 85 years), and 60 % of patients were ever-smokers. Using histological examination, 52 (74.28%) cases were NMIBC and 18 (25.71%) cases were MIBC.

According to the WHO classification, the proportions of each clinical stage were 74.28 % for stage I, 17.14 % for stage II, 7.15 % for stage III, and 1.43 % for stage IV. A total of 27 tumors were low-grade (38.57%), whereas 43 were high-grade (61.43%). The mean duration of follow-up was 44 months; During the follow-up period and after an initial TUR-BT, the recurrence was seen in 12 patients (23.08 %), and progression was noted in 5 patients (9.62%).

Table II. Tumour characteristics of people with bladder cancer according to clinical staging

Clinical characteristics	Stage				Total
	pTa/pT1	pT2	pT3	pT4	
Number	52	12	5	1	70
Gender					
Male	50	12	5	1	68 (97.14%)
femelle	2	0	0	0	2 (2.86%)
Age					
≤70	30	7	3	1	41 (58.57%)
>70	22	5	2	0	29 (41.43%)
Grade					
High-grade	25	12	5	1	43 (61.43%)
Low-grade	27	0	0	0	27 (38.57%)
Smoker					
Yes	34	8	5	0	47 (67.15%)
No	18	4	0	1	23 (32.85%)
BCG treatment					
Yes	22	9	3	0	34 (48.57%)
No	30	3	2	1	36 (51.43%)
Recurrence					
Yes	12	0	0	0	12 (17.15%)
No	40	12	5	1	58 (82.85%)
Progression					
Yes	5	4	5	0	14 (20%)
No	47	8	0	1	56 (80%)

Mutational status of the *Tp53* gene in BC patients

Mutation status analysis of exons 4 to 9 in the Tp53 gene showed that for each patient, at least one type of mutation was detected for all analyzed exons **(Table III).** Two insertions mutations were identified in exon 6, five deletions were detected in exon 8, and three deletions were also found in exon 9. In addition, the C→A transition was located in intron 7, and an insertion mutation was shown in intron 9. The majority of detected mutations were found in exon 8 (19

mutations,) followed by exon 6 (4 mutations) and exon 9 (6 mutations). Two types of mutations were detected in exons 4 and 5. No mutation was detected in exon 7.

Table III. P53 mutations and polymorphism detected in our sample's cohort (n=70)

***Tp53* gene mutation**					
Exon / intron	**Codon**	**Amino acid change**	**Nucleotide change**	**Type of mutation**	**Frequency %**
4	43	L43M	c.127T>A	Transversion	57.14%
4	72	R72P	c.216C>G	Transversion	91.43%
5	181	R181S	c.541C>A	Transversion	02.85%
5	176	C176R	c.526T>C	Transition	01.43%
6	218	V218G	c.653_654insC	frameshift	01.43%
6	192	Q192*	c.574G>A	Transition	01.43%
6	214	H214*	c.640 A>G	Transition	02.85%
6	221	E221*	c.661_663insT	frameshift	02.85%
Intron 7	g.7674096		c.782 C>T	Transition	80%
8	275	C275S	c.823T>A	Transversion	01.43%
8	279	G279E	c.836C>T	Transition	01.43%
8	280	R280Q	c.838A>T	Transversion	01.43%
8	281	D281G	c.842T>C	Transition	24.29%
8	282	R282R	c.846C>T	Transition	05.71%
8	284	T284*	c.850delC	frameshift	07.14%
8	285	E285K	c.853C>T	Transition	01.43%
8	286	E286D	c.858A>C	Transversion	04.29%
8	289	L289I	c.865G>T	Transversion	01.43%
8	287	E287k	c.859C>T	Transition	01.43%
8	292	K292Q	c.874T>G	Transversion	01.43%
8	294	E294A	c.881T>G	Transversion	02.85%
8	296	H296L	c.887T>A	Transversion	08.57%
8	297	H297L	c.890T>A	Transversion	07.14%
8	299	L299L	c.897C>G	Transversion	04.29%
8	302	G302G	c.906C>G	Transversion	02.85%
8	304	T304P	c.910T>G	Transversion	17.14%
8	305	K305N	c.915G>T	Transversion	07.14%
8	306	R306*	c.916C>T	Transition	07.14%
9	307	A307*	c.919delA	frameshift	04.29%
9	311	N311H	c.931A>C	Transversion	07.14%
9	322	P322H	c.964C>A	Transversion	01.43%
9	316	P316S	c.946C>T	Transition	01.43%

9	312	T312S	c.935C>G	Transversion	05.71%
Intron 9		g.7673523	c.993+12insC		04.29%

Polymorphism R72P

The R72P polymorphism is the most frequent in exon 4, found in 91.42% cases. Three possible genotype combinations were detected [pro] Pro/Pro (8.57%), [Arg] Arg/Pro (48.57%) and [Arg] Arg/Arg (42.86%). Allelic frequencies showed the presence of two alleles, C, and G, in 32.85% and 67.15% of patients respectively **(Table VI).**

Table VI: Genotypes distribution and allelic frequencies within BC patients

Amino acids / Genotype	**No. of patients**	**%**	**Allele**	**%**
P72R				
[pro] CC	6	8.57	C	32.85
[Arg] GC	34	48.57	G	67.15
[Arg] GG	30	42.86		

Association between *Tp53* gene mutation status and clinico-pathological parameters

Exon 4 was the most mutated in 64/70 of the cases, while exons 5, 6, 8, and 9 were mutated in 4.29% (3/70), 8.57% (6/70), 65.71% (46/70) and 15.71% (11/70) of cases, respectively.

A single transition C→A was detected in intron 7 in 80% (56/70) of patients. Generally, the mutation rate in all exons was higher in NMIBC cases, and high-grade tumors (**Table V** and **Table IV)**.

The correlation between mutations detected in exons encoding for transactivation, DNA binding, and oligomerization domains and the clinico-pathological outcomes is described in **Table IV.** Mutations found in transactivation and the oligomerization domain were unrelated to clinical staging and grading (*p-value* > 0.05). One Association was found between exon 8 mutations and grade with a *p-value* = 0.034.

Table V. Repartition of *Tp53* mutations status according to demographic and environmental characteristics.

Parameters	**N=70**	**Transactivation domain**	**DNA binding domain**				**Oligomerization Domain**
		Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9
Gender							
Male	68	62 (91.18%)	3 (4.41%)	6 (8.82%)	54 (79.41%)	45 (66.17%)	10 (14.70%)
Female	2	2 (100%)	0	0	2 (100%)	1 (50%)	1 (50%)

Age							
< 50	1	1 (100%)	0	0	1 (100%)	1 (100%)	1 (100%)
50 -70	44	40 (90.91%)	2 (4.54%)	2 (4.54%)	34 (77.27%)	28 (63.63%)	9 (20.45%)
> 70	25	11 (88%)	1 (4%)	4 (16%)	21 (84%)	17 (68%)	1 (4%)
Smoking							
Yes	47	42 (96.43%)	3 (6.38%)	5 (10.63%)	37 (78.72%)	30 (63.82%)	5 (10.63%)
No	23	22 (85.71%)	0	1 (4.34%)	19 (82.60%)	16 (69.56%)	6 (26.08%)

Table IV. Association of *Tp53* mutations status with clinicopathological parameters.

Parameters	**N=70**	**Transactivation domain**	**DNA binding domain**				**Oligomerization Domain**
		Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9
Stage							
≤ PT1	52	47 (90.38%)	2 (3.84%)	6 (11.54%)	41 (78.84%)	34 (65.38%	9 (17.30%)
>PT1	18	17 (88.88%)	1 (5.55%)	0	15 (83.33%)	12 (66.66%)	2 (11.11%)
p-value		0.602	0.762	0.136	0.687	0.923	0.540
Grade							
Bas grade	27	26 (96.30%)	2 (7.40%)	3 (11.11%)	22 (81.48%)	14 (51.85%)	5 (18.51%)
Haut grade	43	38 (88.37%)	1 (2.32%)	3 (6.97%)	34 (79.06%)	32 (74.41%)	6 (13.95%)
p-value		0.284	0.178	0.503	0.903	**0.034**	0.541

DISCUSSION

In Morocco, bladder cancer ranks ninth in terms of incidence (GLOBOCAN, 2022b). However, its management affects public health, mainly due to the difficulty of diagnosis and treatment. BC is usually diagnosed using cystoscopy and cytology. Both methods are limited by poor detection for low-stage and low-grade tumors (Yafi et al., 2015). Hence the need to develop a more powerful alternative and to identify new tumor biomarkers will allow the development of non-invasive tests for the diagnosis, prognosis, and therapeutic management of BC (Kompier et al., 2010b).

In bladder cancer, the *Tp53* mutations is associated with MIBC and high-grade tumors, indicating a poor prognosis that results in a low survival rate. A study conducted on the Belarusse population found that alterations affecting p53 were associated only with advanced tumors, and no alterations were detected in early-stage (pTa) (Smal et al., 2014). These findings are in discordance with our results; in our cohort, *Tp53* mutations were detected in primary tumors ≤PT1 and also in advanced tumors (>PT1) (Smal et al., 2014).

In our cohort, the R72P polymorphism was detected in 91.43% of cases; this polymorphism is the most frequent mutation shown in exon 4 of the *Tp53* gene. In this sense, two other studies have shown that 70% (Schlichtholz, 2004) and 58% (Bodoor et al., 2017) of the studied populations with BC harbor this polymorphism. These percentages are in agreement with the theory indicating that the mutated allele is altered during carcinogenesis by inhibiting the

homolog of *Tp53*, resulting in the inhibition of apoptosis. Another study performed on the Indian population confirms the association of the R72 polymorphism and BC; they also showed that the R72P G>C polymorphism with a heterozygous genotype (GC) is associated with a reduced risk of recurrence compared to the wild type (GG) in patients with NMIBC receiving BCG treatment (Srivastava et al., 2011). Regarding the L43M mutation detected in exon 4, no study reports its implication in BC, which is not in line with our research. Some studies showed that this alteration is involved in multiple cancers, including colorectal cancer and, more specifically, in pT2 stage tumors of the Taiwanese population (Chang et al., 2019), as well as in pancreatic adenocarcinoma and non-small cell lung cancer (Lowder et al., 2020; Zhang et al., 2021).

Alterations in exons 5 and 6 were noted as rare mutations in our cohort. One among these mutations has been reported to block or destabilize zinc binding; others are known to be implicated in structural destabilization, especially in the DNA binding domain, and consequently lead to loss of TP53 function (Martin et al., 2002). Another colorectal cancer study identified the g.7674096 (c.782) mutation detected in our cohort in intron 7. Its effect can be explained by the results of splicing mutations of *Tp53* on the expression of transcription variants. Intron binding, which is manifested by a frameshift, can be the origin of the generation of a truncated protein (Smeby et al., 2019).

Analysis of the mutational status of exon 8 revealed the highest number of mutations in this cohort. Nine of these alterations are rarely detected in our population, but all the mutations detected were present in the IARC database (IARC *Tp53* Database http://p53.iarc.fr/TP53GeneVariations.aspx); In addition, another study confirms that the majority of *Tp53* mutations occur in exon 8 in colorectal cancer (Li. X et al., 2015). All mutations reported in this exon may affect the core domain of the TP53 protein. Among the mutations frequently found, we have the alteration of residue G281D detected in 17 BC patients (24.28%); this site is commonly mutated in human cancer, and it has been reported that it increases the risk of mortality and invasion of the lung cancer cells (Olszewski et al., 2019; Wright, 2002). In accordance with the data previously reported in this work, it is found that the T304* and K305 mutations have been previously described in multiple types of cancers, namely in the case of transitional cell carcinoma of the bladder (Caruana et al., 1997; Zerdoumi et al., 2017). Furthermore, Maeng et al reported that the R306* alteration is among the *Tp53* hotspots, and this mutation is more predominant in BC (Maeng et al., 2012).

Analysis of exon 9 revealed 3 mutations, A307* silent mutation, N311 and T312. A307 variant was reported in another study concerning Indian patients with squamous cell carcinoma of the oral cavity (Kannan et al., 1999). N311 mutation is involved in another cancer, such as cortical adenocarcinoma (Wasserman et al., 2015). At the same time, the T312 alteration was associated with bladder tumors with T1G3 in the Spanish population (López-Knowles et al., 2006).

There was no significant association between *Tp53* mutations in each protein domain and clinicopathological outcome, except for the correlation between mutations in exon 8 and grade (p-value = 0.034). Also, p53 alterations in our analysis were seen in 47/52 of patients with NMIBC and 17/18 of MIBC cases. According to our findings, some previous studies have demonstrated the association between *Tp53* mutations and high-grade invasive tumors (Rentsch et al., 2017; The Cancer Genome Atlas Research Network, 2014). Other studies have reported that the p53 pathway is inactivated in most T1G3 bladder tumors and does not predict the outcome (López-Knowles et al., 2006).

CONCLUSION

In summary, we have evaluated the mutational status of the tumor suppressor *Tp53* in the Moroccan population with BC. Analyse of exons 4 to 9 demonstrated that at least one mutation was detected for each case in our cohort, with a prevalence of 100%. The P72R polymorphism in exon 4 was seen in 91.43% of samples. Most of the mutation types found are located in exon 8 of the *Tp53* gene. In addition, *Tp53* alterations were observed in our specimens' histological types 47/52 NMIBC and 17/18 MIBC. Only a correlation between mutations in exon 8 and grade was observed. A larger population study is needed to assess the precise correlation between *Tp53* mutation status and bladder cancer disease. This may affect the findings obtained in this study and confirm the usefulness of this gene as a biomarker for clinical use.

REFERENCES

Bodoor, K., Al-Ghabkari, A., Matalka, I., et al. Assessment of p53 mutations, expression and prognosis in bladder cancer patients from Jordan: Identification of novel deletion mutations in the DNA-binding domain. *Meta Gene*, 2017; *12*, 33–42. https://doi.org/10.1016/j.mgene.2017.01.002

Bouchbika, Z., Haddad, H., Benchakroun, N., et al. Cancer incidence in Morocco: Report from Casablanca registry 2005-2007. *Pan African Medical Journal, 16;* 2013. https://doi.org/10.11604/pamj.2013.16.31.2791

Caruana, S. M., Zwiebel, N., Cocker, R., McCormick, S. A., Eberle, R. C., & Lazarus, P. P53 alteration and human papilloma virus infection in paranasal sinus cancer. *Cancer*, 1997; *79*(7), 1320–1328.

Chang, F., Syrjänen, S., Kurvinen, K., & Syrjänen, K. The p53 tumor suppressor gene as a common cellular target in human carcinogenesis. *The American Journal of Gastroenterology*, 1993; *88*(2), 174–186.

Chang, Y., Lee, C., Ke, T., Chang, C., Chao, D., Huang, H., & Chang, J. Molecular characterization of colorectal cancer using whole-exome sequencing in a Taiwanese population. *Cancer Medicine*, 2019; *8*(8), 3738–3747. https://doi.org/10.1002/cam4.2282

Chen, L., Liu, Y., Zhang, Q., et al. P53/PCDH17/Beclin-1 Proteins as Prognostic Predictors for Urinary Bladder Cancer. *Journal of Cancer*, 2019; *10*(25), 6207–6216. https://doi.org/10.7150/jca.37335

Cordon-Cardo, C. Molecular alterations associated with bladder cancer initiation and progression. *Scandinavian Journal of Urology and Nephrology*, 2008; *42*(sup218), 154–165. https://doi.org/10.1080/03008880802291915

Dadhania, V., Czerniak, B., & Guo, C. C. Adenocarcinoma of the urinary bladder. *American Journal of Clinical and Experimental Urology*, 2015; *3*(2), 51–63.

Dall'Era, M. A., Cheng, L., & Pan, C.-X. Contemporary management of muscle-invasive bladder cancer. *Expert Review of Anticancer Therapy*, 2012; *12*(7), 941–950. https://doi.org/10.1586/era.12.60

GLOBOCAN, 2020. *Bladder cancer in Morocco. Cancer today; 2022.* https://gco.iarc.fr/today/data/factsheets/populations/504-morocco-fact-sheets.pdf

Kandoth, C., McLellan, M. D., Vandin, F., et al. Mutational landscape and significance across 12 major cancer types. *Nature*, 2013; *502*(7471), 333–339. https://doi.org/10.1038/nature12634

Kannan, K., Munirajan, A. K., Krishnamurthy, J., Bhuvarahamurthy, V., Mohanprasad, B. K., Panishankar, K. H., Tsuchida, N., & Shanmugam, G. Low incidence of p53 mutations in betel quid and tobacco chewing-associated oral squamous carcinoma from India. *International Journal of Oncology*, 1999. https://doi.org/10.3892/ijo.15.6.1133

Kassouf, W., Traboulsi, S. L., Kulkarni, G. S., et al. CUA guidelines on the management of non-muscle invasive bladder cancer. *Canadian Urological Association Journal*, 2015; *9*(9–10), 690. https://doi.org/10.5489/cuaj.3320

Kompier, L. C., Lurkin, I., van der Aa, M. N. M., van Rhijn, B. W. G., van der Kwast, T. H., & Zwarthoff, E. C. FGFR3, HRAS, KRAS, NRAS and PIK3CA mutations in bladder cancer and their potential as biomarkers for surveillance and therapy. *PloS One*, 2010; *5*(11), e13821. https://doi.org/10.1371/journal.pone.0013821

Li, H., Lu, H., Cui, W., Huang, Y., & Jin, X. A TP53-based immune prognostic model for muscle-invasive bladder cancer. *Aging*, 2021; *13*(2), 1929–1946. https://doi.org/10.18632/aging.202150

Li, X.-L., Zhou, J., Chen, Z.-R., & Chng, W.-J. P53 mutations in colorectal cancer—Molecular pathogenesis and pharmacological reactivation. *World Journal of Gastroenterology*, 2015; *21*(1), 84–93. https://doi.org/10.3748/wjg.v21.i1.84

López-Knowles, E., Hernández, S., Kogevinas, M., et al. The p53 Pathway and Outcome among Patients with T1G3 Bladder Tumors. *Clinical Cancer Research*, 2006; *12*(20), 6029–6036. https://doi.org/10.1158/1078-0432.CCR-06-0206

Lowder, C. Y., Dhir, T., Goetz, A. B., et al. A step towards personalizing next line therapy for resected pancreatic and related cancer patients: A single institution's experience. *Surgical Oncology*, 2020; *33*, 118–125. https://doi.org/10.1016/j.suronc.2020.02.003

Maeng, C. H., Lee, J., van Hummelen, P., et al. High-Throughput Genotyping in Metastatic Esophageal Squamous Cell Carcinoma Identifies Phosphoinositide-3-Kinase and BRAF Mutations. *PLoS ONE*, 2012; *7*(8), e41655. https://doi.org/10.1371/journal.pone.0041655

Martin, A. C. R., Facchiano, A. M., Cuff, A. L., Hernandez-Boussard, T., Olivier, M., Hainaut, P., & Thornton, J. M. Integrating mutation data and structural analysis of the TP53 tumor-suppressor protein. *Human Mutation*, 2002; *19*(2), 149–164. https://doi.org/10.1002/humu.10032

Matulewicz, R. S., & Steinberg, G. D. Non-muscle-invasive Bladder Cancer: Overview and Contemporary Treatment Landscape of Neoadjuvant Chemoablative Therapies. *Reviews in Urology*, 2020; *22*(2), 43–51.

Muller, P. A. J., & Vousden, K. H. Mutant p53 in cancer: New functions and therapeutic opportunities. *Cancer Cell*, 2014; *25*(3), 304–317. https://doi.org/10.1016/j.ccr.2014.01.021

Olszewski, M., Pruszko, M., Snaar-Jagalska, E., Zylicz, A., & Zylicz, M. Diverse and cancer type-specific roles of the p53 R248Q gain-of-function mutation in cancer migration and invasiveness. *International Journal of Oncology 2019.* https://doi.org/10.3892/ijo.2019.4723

Ozaki, T., & Nakagawara, A. Role of p53 in Cell Death and Human Cancers. *Cancers* 2011; *3*(1), 994–1013. https://doi.org/10.3390/cancers3010994

Rentsch, C. A., Müller, D. C., Ruiz, C., & Bubendorf, L. Comprehensive Molecular Characterization of Urothelial Bladder Carcinoma: A Step Closer to Clinical Translation? *European Urology* 2017; *72*(6), 960–961. https://doi.org/10.1016/j.eururo.2017.06.022

Schlichtholz, B. Clinical implications of p53 mutation analysis in bladder cancer tissue and urine sediment by functional assay in yeast. *Carcinogenesis*, 2004; *25*(12), 2319–2323. https://doi.org/10.1093/carcin/bgh256

Smal, M. P., Rolevich, A. I., Polyakov, S. L., Krasny, S. A., & Goncharova, R. I. FGFR3 and TP53 mutations in a prospective cohort of Belarusian bladder cancer patients. *Experimental Oncology* 2014; *36*(4), 246–251.

Smeby, J., Sveen, A., Eilertsen, I. A., et al. Transcriptional and functional consequences of TP53 splice mutations in colorectal cancer. *Oncogenesis* 2019; *8*(6), 35. https://doi.org/10.1038/s41389-019-0141-3

Srivastava, P., Jaiswal, P. K., Singh, V., & Mittal, R. D. Role of p53 gene polymorphism and bladder cancer predisposition in northern India. *Cancer Biomarkers* 2011; *8*(1), 21–28. https://doi.org/10.3233/DMA-2011-0816

The Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature*, 2014; *507*(7492), 315–322. https://doi.org/10.1038/nature12965

Végran, F., Rebucci, M., Chevrier, S., Cadouot, M., Boidot, R., & Lizard-Nacol, S. Only missense mutations affecting the DNA binding domain of p53 influence outcomes in patients with breast carcinoma. *PloS One* 2013; *8*(1), e55103. https://doi.org/10.1371/journal.pone.0055103

Wasserman, J. D., Novokmet, A., Eichler-Jonsson, C., Ribeiro, R. C., Rodriguez-Galindo, C., Zambetti, G. P., & Malkin, D. Prevalence and Functional Consequence of *TP53* Mutations in Pediatric Adrenocortical Carcinoma: A Children's Oncology Group Study. *Journal of Clinical Oncology*, 2015; *33*(6), 602–609. https://doi.org/10.1200/JCO.2013.52.6863

Wright, J. D. Factors governing loss and rescue of DNA binding upon single and double mutations in the p53 core domain. *Nucleic Acids Research*, 2002; *30*(7), 1563–1574. https://doi.org/10.1093/nar/30.7.1563

Yafi, F. A., Brimo, F., Steinberg, J., Aprikian, A. G., Tanguay, S., & Kassouf, W. Prospective analysis of sensitivity and specificity of urinary cytology and other urinary biomarkers for bladder cancer. *Urologic Oncology: Seminars and Original Investigations*, 2015; *33*(2), 66.e25-66.e31. https://doi.org/10.1016/j.urolonc.2014.06.008

Zerdoumi, Y., Lanos, R., Raad, S., Flaman, J.-M., Bougeard, G., Frebourg, T., & Tournier, I. Germline TP53 mutations result into a constitutive defect of p53 DNA binding and transcriptional response to DNA damage. *Human Molecular Genetics*, 2017; *26*(14), 2591–2602. https://doi.org/10.1093/hmg/ddx106

Zhang, T., Xu, Z., Liu, G., Jiang, B., de Bock, G. H., Groen, H. J. M., Vliegenthart, R., & Xie, X. Simultaneous Identification of EGFR,KRAS,ERBB2, and TP53 Mutations in Patients with Non-Small Cell Lung Cancer by Machine Learning-Derived Three-Dimensional Radiomics. *Cancers*, 2021; *13*(8), 1814. https://doi.org/10.3390/cancers13081814

Third part: Analysis of promoter methylation status of 4 genes hTERT, TWIST1, NID2 and VIM in biopsies and urine sediments

Introduction:

Epithelial-to-mesenchymal transition is one of the important cellular processes in most cancer types, including BC. Epithelial cells lose cell-cell junctions and confer motility, migration and invasive properties to become mesenchymal stem cells. EMT is characterized mainly by a high expression of various mesenchymal cell markers like the *TWIST* family, *VIM, Snail* and *ZEB-1*, this is accompanied by a loss of epithelial cell markers such as *E-cadherin, Nidogen* and *MUC1* (Zhao et al., 2017; Brabletz et al., 2021).

hTERT, TWIST1, NID2 and *VIM* promoter genes are hypermethylated in numerous cancers and involved in EMT and invasion phenotype in BC. Hypermethylation of CpG islands is most commonly associated with the downregulation of *TWIST1, NID2* and *VIM* genes. However, a positive correlation between hypermethylation and *hTERT* gene expression was also reported, suggesting a more diverse mechanism of epigenetic regulation (Shirahata et al., 2009; Sui et al., 2013; Yegin et al., 2013).

Epigenetic modifications were reported in BC tumors and parried urine specimens with high sensitivity and specificity (Li et al., 2016; Fantony et al., 2017). Thus, these genes can be promising biomarkers that may play an important role in the detection and monitoring of patients with bladder cancer. The third part of this dissertation aims to analyse the epigenetic profile of *hTERT, TWIST1, NID2* and *VIM* promoter genes in biopsies and urine-exfoliated cells in order to assess the possibility of using urine as a matrix to support BC diagnosis and follow-up.

Evaluation of DNA methylation in promoter regions of *hTERT*, *TWIST1*, *VIM* and *NID2* genes in Moroccan bladder cancer patients

VI.5 Evaluation of DNA methylation in promoter regions of *hTERT, TWIST1, VIM* and *NID2* genes in Moroccan bladder cancer patients

Human cancers develop due to the accumulation of both genetic and epigenetic changes that trigger inappropriate activation or inactivation of genes inducing oncogenic transformation. DNA methylation is an epigenetic mechanism that refers to the covalent addition of a methyl group to CpG islands. It generally plays a key role in repressing gene expression or inhibiting the binding of transcription factors to DNA.

Recent studies have demonstrated that epigenetic modifications are implicated in almost every step of neoplastic cell development and progression. Aberrant methylation of 5' CpG islands of the gene promoters plays an important role in modifying transcriptional activity in several types of cancer, including bladder cancer. The main challenge is to identify and select a panel of genes as potential biomarkers in diagnosis, prognosis and prediction of response to therapy.

The aim of this paper is to investigate the methylation patterns in the promoter regions of *hTERT, VIM, TWIST1* and *NID2* genes in Moroccan bladder cancer patients and to evaluate their potential use as epigenetic biomarkers for bladder cancer management. All these genes have been reported to promote the Epithelial-Mesenchymal Transition and may play a key role in various cellular functions, such as cell migration and invasion, adhesion and mortality and also promote resistance to various cell stressors.

MSP assay performed on 70 DNAs extracted from BC biopsies revelated that those four genes, *hTERT, TWIST1, VIM* and *NID2*, were frequently hypermethylated. Promoter hypermethylation of all these genes has occurred in all pathological grades and stages of bladder cancer indicating their use as promising prognostic and predictive biomarkers in bladder cancer. Moreover, hypermethylation in *hTERT, TWIST1, VIM* and *NID2* promoter genes may be an attractive therapeutic target, more useful than conventional molecular markers, for the treatment and prevention of bladder cancer progression and metastasis.

Cancer Genetics 260–261 (2022) 41–45

Contents lists available at ScienceDirect

Cancer Genetics

journal homepage: www.elsevier.com/locate/cancergen

Evaluation of DNA methylation in promoter regions of *hTERT, TWIST1, VIM* and *NID2* genes in Moroccan bladder cancer patients

Meryem El Azzouzi[a,b], Hajar El Ahanidi[a,b], Chaimae Hafidi Alaoui[a,d], Imane Chaoui[a], Laila Benbacer[a], Mohamed Tetou[c], Ilias Hassan[c], Mounia Bensaid[c], Mohamed Oukabli[b,c], Ahmed Ameur[b,c], Abderrahmane Al Bouzidi[b], Mohammed El Mzibri[a], Mohammed Attaleb[a,*]

[a] Biology and Medical Research Unit, CNESTEN, Rabat, Morocco
[b] Faculty of Medicine and Pharmacy of Rabat. Mohammed V University in Rabat, Rabat, Morocco
[c] Mohammed V Military Hospital, Rabat, Morocco
[d] Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco

ARTICLE INFO

Article history:
Received 16 July 2021
Revised 8 November 2021
Accepted 5 December 2021

Keywords:
Epigenetics
DNA methylation
Bladder cancer
Methylation specific PCR
Recurrence

ABSTRACT

Promoter hypermethylation have been reported to play a key role in bladder cancer development and progression. The aim of this study is to evaluate the methylation status of *hTERT, TWIST1, VIM* and *NID2* genes in bladder cancer. The methylation status was evaluated using the Methylation-Specific PCR (MSP) approach on 70 tumour biopsies from Moroccan bladder cancer patients. Overall, methylation frequencies of *hTERT, TWIST1, VIM* and *NID2* genes, were 90%, 85.71%, 67.14% and 67.14%, respectively. Hypermethylation of all studied genes was found in all pathological grades and stages of bladder cancer. Nevertheless, statistical analysis showed no significant association between promoter methylation of *hTERT, TWIST1, VIM* and *NID2* genes and tumours stage/grade (p value >0.05). Moreover, we have investigated the association between the methylation pattern of selected genes and the treatment outcome in a sub-group of non-muscle-invasive bladder cancer cases (52/70). Hypermethylation of *hTERT, TWIST1, VIM* and *NID2* was detected in 83.34%; 66.67%; 83.34% and 58.34% of recurrent cases, respectively, and in 80%; 80%; 80% and 60% of progressive cases, respectively. Statistical analysis highlighted a significant association between *TWIST1* hypermethylation and tumour recurrence ($p = 0.041 < 0.05$).

Our results indicate that hypermethylation of *hTERT, TWIST1, VIM* and *NID2* genes is a frequent epigenetic event in bladder cancer and could be a promising therapeutic target to prevent bladder cancer progression and metastasis.

© 2021 Elsevier Inc. All rights reserved.

Introduction

Bladder cancer (BC) is the ninth most common cancer in the world accounting for approximately 420,000 new cases each year, and the fourth most commonly diagnosed cancer in men [1]. In Morocco, according to the regional cancer register of Casablanca, the incidence of bladder cancer reached 3.7 per 100,000 persons [2]. Worldwide, most patients are diagnosed with non-muscle-invasive bladder cancer (NMIBC) and have a good prognosis [1]. Patients with muscle-invasive bladder cancer (MIBC) have a poor prognosis, with a 5-year survival rate ranging from 10 to 50% depending on the tumour stage. Unfortunately, 70% of NMIBC cases will recur after transurethral resection and 10–20% will eventually progress to MIBC [3].

Currently, bladder cancer diagnosis is mainly based on cystoscopy and urine cytology. Cystoscopy identifies most lesions and provides essential prognostic information, but it is an invasive procedure and shows insufficient power to predict precisely the patient outcome. Urine cytology is the standard non-invasive method for BC detection and disease monitoring. However, this method lacks sensitivity (35%), especially for low grades and stages [4].

Recently, cancer management have known a great evolution at both conceptual and methodological levels using different technological approaches and based on a wide range of molecular biomarkers that could be used in the cancer diagnosis, for prognosis and/or as therapeutic targets. These biomarkers have made an

* Corresponding author at: Biology and Medical Research Unit, Centre National de l'Energie, des Sciences et techniques Nucléaires (CNESTEN). BP 1382 RP. 10001 Rabat, Maroc.
E-mail address: attaleb@cnesten.org.ma (M. Attaleb).

https://doi.org/10.1016/j.cancergen.2021.12.001
2210-7762/© 2021 Elsevier Inc. All rights reserved.

outstanding contribution to our understanding of cancer initiation, development and progression [5].

Scientific evidence has shown that epigenetic events play a key role in the development of human cancer. Accordingly, a great interest was given to study the epigenetic modifications highlighting the critical role of epigenetic mechanisms in normal cellular processes and the abnormal events that lead to diseases, most notably to cancer. Epigenetic alterations, such as DNA methylation, are frequently observed in tumors [6].

DNA methylation is a covalent modification allowing the fixation of a methyl group to the 5th carbon of the cytosine ring of a CpG dinucleotide in the promoter region of a gene, playing a crucial role in the regulation and control of gene expression [7]. In bladder cancer, as for other types of cancers, CpG islands hypermethylation of gene promoters is considered as a major modification involved in carcinogenesis process and exhibit a strong correlation with clinical-pathological parameters such as tumours stage, grade, recurrence and progression [8].

In bladder cancer, scientific evidence has shown that some genes including *hTERT, TWIST1, VIM* and *NID2* play an important role in cancer development and are involved in many cellular function that favour cancer promotion and progression [9,10].

A defining feature of cancer cells is replicative immortality attained by telomerase reactivation [11]. Telomerase reactivation in cancer is often related to the methylation of catalytic subunit of the telomerase complex (TERT) and more specifically, a region located on gene promoter, termed THOR (TERT Hypermethylated Oncological Region). THOR hypermethylation is widely reported in many cancer types, including prostate, breast, blood and colon cancers [12].

TWIST1 is a highly conserved transcription factor belonging to the family of basic helix–loop–helix proteins. It is involved in epithelial-mesenchymal transition (EMT) regulation, allowing cancer cell migration and invasion. *TWIST1* contributes to dissemination of cells from premalignant lesions by EMT promotion and apoptosis inhibition; it confers the ability of a cell to self-renew and induces angiogenesis and chromosomal instability. *TWIST1* is known to be hypermethylated in various tumour types including colorectal [13] and breast cancer [14].

Of particular interest, *TWIST1* hypermethylation was also reported in BC cases and was well appreciated for cancer stratification and the overall cancer-specific survival evaluation [15].

VIM gene is reported as an important mesenchymal marker and plays a key role in the EMT in malignant tumours with regard to cellular adhesion, migration and signalling [16]. Down-regulation of *VIM* is well documented and many studies have reported that *VIM* is silenced through aberrant promoter hyper-methylation in oesophageal, gastric and colon cancers [17, 18]. Of particular interest, the methylation level in *VIM* gene promoter was significantly higher in BC tissues, compared to normal bladder mucosa, and was therefore suggested as a potential biomarker for early detection of bladder cancer [19].

NID2 is a member of Nidogen family proteins playing an important role in the maintenance of the structural integrity of basement membranes and setting up a barrier for cell movement, migration and invasion. Its involvement in cell differentiation, proliferation and survival makes this protein a key factor in cancer development. Several studies have suggested that *NID2* is a tumour-suppressor gene. Its loss contributes to the development and progression of cancer, stimulates the weakened of the cell-cell interaction and favours metastasis and invasion [20–22].

The present study was planned to evaluate the methylation status of *hTERT, TWIST1, VIM* and *NID2* genes' promoters in BC samples from Moroccan patients and to evaluate their potential use as epigenetic biomarkers for bladder cancer management in Morocco.

Material and methods

Patients and study design

A total of 70 patients with BC were recruited between September 2017 and June 2018 in the Urology Department of the University Military Hospital Mohammed V in Rabat – Morocco. Each biopsy was cut into two parts; one part was used for anatomy-pathological diagnosis and the other one was kept at

−80 °C for molecular biology explorations. Staging and grading were done according to the TNM classification (NMIBC includes Ta, T1, and Tis; and MIBC includes T2, T3, and T4) and World Health Organization criteria. All patients were followed up in accordance with standard recommendations. Recurrence was defined as reappearance of primary NMIBC with a lower or the same pathological stage, and progression was defined as a disease with a higher TNM stage upon relapse [3].

The study protocol was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat – Morocco (Ref 82/19), and written informed consent was obtained from each patient.

Genomic DNA extraction

Genomic DNA was extracted from fresh frozen tumours using phenol/chloroform method. All extracted DNAs were quantified with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were used immediately or stored at −20 °C until use.

Sodium bisulfite modification

Genomic DNA extracted from tumours was subjected to sodium bisulfite treatment to convert all unmethylated cytosine to uracil and keep the methylated cytosines unchanged. Sodium bisulfite treatment and DNA purification was performed using EZ DNA Methylation™ Kit (Zymo Research, CA, USA). Briefly, 500 ng of DNA was diluted in a final volume of 50 µl containing 5 µl of dilution buffer and 45 µl of sterile water, 100 µl of conversion reagent were added and mixtures were incubated during 12–16 h at 50 °C in the dark. Modified DNA was finally desulphoneted using desulphonation buffer and recovered in 10 µl of elution buffer [23].

Methylation-specific PCR (MSP)

The modified DNA was subject to methylation-specific PCR (MSP) using unmethylation-specific and methylation-specific primers (Table 1). The amplification reaction was carried out in a final volume of 25 µl containing 2 µl of modified DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 10 µM of each dNTP, 10 µM of each primer and 1 U Platinum Taq Polymerase (Invitrogen, USA). PCR reactions were performed in thermal cycler (Gene Amp, PCR system 9700, Applied Bio system). Mixtures were first denatured at

95 °C for 10 min. Then, 40 cycles of PCR were performed with denaturation at 95 °C for 30 s, primer annealing for 45 s at the corresponding temperature (Table 1) and primer extension for 45 s at

72 °C. At the end of the last cycle, the mixtures were incubated at 72 °C for additional 10 min. For every reaction a negative control, in which DNA template was omitted from the amplification mixture, was included. PCR products were analysed by electrophoresis on 2% agarose gel followed by staining with ethidium bromide (10 mg/ml).

Table 1
Primers and annealing temperatures for Methylation Specific PCR.

Gene		Forward Primer 5′ –> 3′	Reverse Primer 5′ –> 3′	Product size(bp)	Tm °C
hTERT	M	GAGGTATTTCGGGAGGTTTCGC	ACTCCGAACACCACGAATACCG	121	58
	U	GGGAGGTATTTTGGGAGGTTTTGT'	CAAACTCCAAACACCACAAATACCA	126	58
TWIST1	M	CCGTCGCCTTCCTCCGACGA	TGCGTTAGGGTTCGGGGGCGT	100	64
	U	TTGGATGGGGTTGTTATTGT	CCTAACCCAAACAACCAACC	79	63
VIM	M	TTATAAAAATAGCGTTTTCGGC	ATAACGCGAACTAACTCCCG	143	59
	U	GGGTTATAAAAATAGTGTTTTTGGT	ACAATAACACAAACTAACTCCCA	149	57
NID2	M	TAGTATTGGTAACGACGATAGTATC	AAATTCGAAACTAACGCGACACG	141	58
	U	TAGTATTGGTAATGATGATAGTATT	AAATTCAAAACTAACACAACACA	141	55

Table 2
Tumour characteristics of bladder cancer patients.

Parameter	Total cases	Percentage (%)
Tumour stage		
≤ PT1	52	74.3
> PT1	18	25.7
Tumour grade		
Low grade	27	38.6
High grade	43	61.4
Tumour recurrence		
Yes	12	23.1
No	40	76.9
Tumour progression		
Yes	5	9.6
No	47	90.4

Statistical analysis

Hypermethylation status of gene promoters in tumour biopsies was statistically evaluated using percentages and confidence intervals (CI) of 95%. The correlation between hypermethylation and clinico-histopathological parameters (tumours stage, grade, recurrence and progression) was evaluated using chi-squared test ($\chi 2$ test). p Values < 0.05 were considered statistically significant. Both statistical analyses were performed using IBM SPSS software version 23.

Results

Characteristics of the study population

Amongst the 70 recruited patients, 68 were male and only 2 were female with a sex ratio of 34. The mean age of patients was 67 years, ranging from 47 to 85 years. Clinico-pathological characteristics of tumour specimens are summarized in Table 2 and showed that most cases were staged ≤PT1 (74.3%) and have high tumour grade (61.4%). Amongst NMIBC cases, 23.1% recurred and 9.6% progressed upon relapse (Table 2).

Evaluation of the methylation status of selected genes in bladder cancer biopsies

Overall, hypermethylation was found in all patients in at least one of the studied genes, *hTERT* promoter region being the most methylated one; DNA hypermethylation was reported in 90% [80.5–95.9] of cases (Table 3). Hypermethylation of *TWIST1, VIM* and *NID2* promoters were reported in 85.7% [75.3–92.9], 67.1% [54.9–77.9] and 67.1% [54.9–77.9], respectively.

Distribution of promoter's hypermethylation and clinico-pathological features was also assessed and is reported in Table 3. For the studied genes, promoter's hypermethylation was found in both high and low grades cases. Indeed, *hTERT* methylation was reported in 90.7% of high grade cases (39/43) and in 88.9% in low grade cases (24/27). For *TWIST1, VIM* and *NID2* genes, methylation was reported in 88.4% (38/43), 69.8% (30/43) and 69.8% (30/43) of high grade cases, respectively and in 81.5% (22/27), 63.0% (17/27) and 63.0% (17/27) of low grade cases respectively.

Statistical analysis showed no significant association between hypermethylation of *hTERT, TWIST1, VIM* and *NID2* gene promoters and tumours grade ($p > 0.05$) (Table 3).

In this study, most hypermethylated cases were diagnosed with low tumour stages (≤ PT1). In fact, *hTERT* hypermethylation was reported in 90.4% (47/52) and *TWIST1* hypermethylation in 86.5% (45/52) of low stage cases. For *VIM* and *NID2*, hypermethylation of their promoters was reported in 69.2% (36/52) and 61.5% (32/52) of low stages cases, respectively. Nevertheless, statistical analysis showed no significant association between hypermethylation of *hTERT, TWIST1, VIM* and *NID2* gene promoters and tumours stage ($p > 0.05$) (Table 3).

Association between the methylation status and bladder cancer recurrence

In this study, tumour recurrence and progression were studied only for NMIBC cases (52/70). Overall, 12 cases showed tumour recurrence and 5 were relapsed with tumour progression (Table 4). Amongst the 12 recurrent cases, 10 have shown hypermethylation of *hTERT* promoter region (83.3%) and 10 have shown hypermethylation of *VIM* promoter region (83.3%). Hypermethylation of *TWIST1* and *NID2* was reported in 8 cases (66.7%) and 7 cases (58.3%), respectively. Statistical analysis showed a significant association between *TWIST1* promoter methylation and bladder cancer recurrence ($p = 0.041$) (Table 4).

Hypermethylation was also reported in progressive cases. Indeed, hypermethylation of *hTERT, TWIST1, VIM* and *NID2* promoters was reported in 4 (80%), 4 (80%), 4 (80%) and 3 cases (60%). Statistical analysis showed no significant association between hypermethylation of *hTERT, TWIST1, VIM* and *NID2* gene promoters and tumour progression ($p > 0.05$) (Table 4).

Discussion

During last decades, molecular genetics has known a great evolution at both conceptual and methodological levels, using new developed approaches and recent technical advances. In this field, targeted therapy, based on the evaluation of various biomarkers, has revolutionized the cancer therapy and is widely used in the routine management of all types of cancer. Currently, a growing interest is given to the identification and evaluation of new biomarkers that could be used in cancer diagnosis, prognosis and/or as therapeutic targets [24, 25].

In this study, the methylation status of the promoter regions of *hTERT, TWIST1, VIM* and *NID2* genes has been evaluated in tumour specimens from Moroccan patients with BC. Aberrant methylation patterns have been associated with cancer development, chronic inflammation and ageing and have been reported to be involved in both the disease development and progression [6].

Table 3
Distribution of promoter's hypermethylation according to clinicopathological results.

Parameter		*hTERT*		*TWIST1*		*VIM*		*NID2*	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
clinical stage									
≤ PT1	52	47 (90.4)	5 (9.6)	45 (86.5)	7 (13.5)	36 (69.2)	16 (30.8)	32 (61.5)	20 (38.5)
>PT1	18	16 (88.9)	2 (11.1)	15 (83.3)	3 (16.7)	11 (61.1)	7 (38.9)	15 (83.3)	3 (16.7)
p value		0.582		0.947		0.306		0.122	
Tumour grade									
Low grade	27	24 (88.9)	3 (11.1)	22 (81.5)	5 (18.5)	17 (63.0)	10 (37.0)	17 (63.0)	10 (37.0)
High grade	43	39 (90.7)	4 (9.3)	38 (88.4)	5 (11.6)	30 (69.8)	13 (30.2)	30 (69.8)	13 (30.2)
p value		0.627		0.452		0.253		0.782	
Total	70	63 (90)	7 (10)	60 (85.7)	10 (14.3)	47 (67.1)	23 (32.9)	47 (67.1)	23 (32.9)

Table 4
Distribution of promoter's hypermethylation according to the recurrence status.

Parameter		*HTERT*		*TWIST1*		*VIM*		*NID2*	
		+ (%)	– (%)	+ (%)	– (%)	+ (%)	– (%)	+ (%)	– (%)
Tumourrecurrence									
Yes	12	10 (83.3)	2 (16.7)	8 (66.7)	4 (33.3)	10 (83.3)	2 (16.7)	7 (58.3)	5 (41.7)
No	40	37 (92.5)	3 (7.5)	37 (92.5)	3 (7.5)	26 (65)	14 (35)	25 (62.5)	15 (37.5)
p value		0.135		**0.041**		0.352		0.369	
Tumourprogression									
Yes	5	4 (80)	1 (20)	4 (80)	1 (20)	4 (80)	1 (20)	3 (60)	2 (40)
No	47	43 (91.5)	4 (8.5)	41 (87.2)	6 (12.8)	32 (68.1)	15 (31.9)	29 (61.7)	18 (38.3)
p value		0.332		0.657		0.568		0.695	
Total	52	47 (90.4)	5 (9.6)	45 (86.5)	7 (13.5)	36 (69.2)	16 (30.8)	32 (61.5)	20 (38.5)

hTERT, TWIST1, VIM and *NID2* genes were selected for their implication in the cell division and in the mechanism of the epithelial-mesenchymal transition (EMT), playing a key role in tumorigenesis [10,16].

In this genes' panel, 100% of diagnostic coverage in all tumours' grades and stages was obtained. Overall, 90% of BC cases were hypermethylated in *hTERT* gene and 85.7% in *TWIST1* gene, whereas *VIM* and *NID2* genes have shown a hypermethylation status in 67.1% each.

In this study, *hTERT* was found to be the most frequent hypermethylated gene which is in agreement with a previously reported data showing a significant association between THOR hypermethylation and bladder cancer risk, providing a novel insight into telomere biology in cancer [9].

In this investigation, *TWIST1* hypermethylation was detected in 85.1% of Moroccan bladder cancer cases. Hypermethylation of *TWIST1* gene promoter is widely discussed and documented, and several studies have reported a high prevalence of *TWIST1* promoter hypermethylation in many cancers, including bladder cancer [10] and cervical neoplastic [26].

Several studies have investigated *VIM* promoter hypermethylation status in human cancers, including colorectal, gastric, bladder, pancreatic and cervical cancer, for their association with cancer development and promotion and results were controversial. High hypermethylation frequencies were reported in gastric carcinoma [18], colorectal carcinoma [17] and bladder cancer [21], highlighting the important role of *VIM* gene in the epithelial-mesenchymal transition with regard to cellular adhesion, migration and signalling, which are generally considered as characteristics of cancer cells [16]. In contrast, a lower frequency of *VIM* promoter hypermethylation was reported in pancreatic cancer [22], which may be due to another genetic and/or epigenetic alterations underlining the multifactorial causes of cancer disease.

In this study, *NID2* gene hypermethylation was obtained in 67.1% of BC cases. Higher methylation frequencies were reported in bladder cancer cases from Turkish patients [10]. Abnormal *NID2* promoter hypermethylation was also reported in breast cancer [27] and in gastric cancer [28]. Of particular interest, Wang et al. have shown that *NID2* methylation reduces the protein expression level and promotes the development of lung cancer. Moreover, experimental analysis on mice showed that *NID2* over-expressing or demethylation in lung cancer cells inhibited the tumorigenesis of lung cancer, highlighting the key role of *NID2* silencing in cancer development [29].

Hypermethylation of *hTERT, TWIST1, VIM* and *NID2* genes was found in both high and low tumour grade and in both high and low cancer stage with no significant difference. In such studies, the interest was usually given on the evaluation of the association between the methylation profiles and pathological parameters such as the tumour stage and grade. Our results are in agreement with findings of previous study that has also reported no association between *TWIST1* and *NID2* hypermethylation and pathological parameters [10].

In this study, we have also investigated the association between the methylation pattern of these genes and the treatment outcome in NMIBC cases (52/70). Hypermethylation of *hTERT, TWIST1, VIM* and *NID2* was detected in 83.3% (10/12); 66.7% (8/12); 83.3% (10/12); 58.3% (7/12) of recurrent cases, respectively, and in 80% (4/5); 80% (4/5); 80% (4/5); 60% (3/5) of progressive cases respectively, with a significant association between *TWIST1* hypermethylation and tumour recurrence ($P = 0.041$). These results are in agreement with data reported by Reinert et al. showing that the methylation level of *TWIST1* gene is a promising diagnostic biomarker for bladder cancer recurrence [30] and suggesting that *TWIST1* hypermethylation can be considered as a prognostic biomarker for bladder cancer recurrence.

Epigenetics alterations such DNA methylation is a reversible event. This interesting property is of a great interest in the identification of promising and attractive biomarkers that could be used in cancer treatment. As such, many assays using de-methylating agents are widely used to re-express the methylation gene and restore the physiological function.

Overall, hypermethylation of *hTERT, TWIST1, VIM* and *NID2* is a frequent epigenetic event in bladder cancer, suggesting an abnormality of the methylation mechanism in the cancer cells which-

could be a promising therapeutic target to prevent bladder cancer progression and metastasis.

Authors statement

The article entitled "**Evaluation of DNA methylation in promoter regions of *hTERT, TWIST1, VIM* and *NID2* genes in Moroccan bladder cancer patients**" is herewith submitted for publication in Cancer Genetics. It has not been published before, and it is not under consideration for publication in any other journal (s). It contains no matter that is scandalous, obscene, libellous, or otherwise contrary to law. I/We agree that copies made under these circumstances will continue to carry the copyright notice that appeared in the original published work.

When the article is accepted for publication, I/We, as author/authors, hereby agree to transfer(s), assign(s), or otherwise convey(s) all copyright ownership, including any and all rights incidental thereto, exclusively to the Journal, in the event that such work is published by the Journal. The Journal shall own the work, including 1) copyright; 2) the right to grant permission to republish the article in whole or in part, with or without fee; 3) the right to produce preprints or reprints and translate into languages other than English for sale or free distribution; and 4) the right to republish the work in a collection of articles in any other mechanical or electronic format.

The article will be published under the terms of the latest Creative Commons Attribution-Non Commercial- Share Alike License, unless the journal notifies the author otherwise in writing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments

We would like to thank everyone who has participated to the realisation of this work, specially the medical staffs of Urology and Anatomopathology Departments of the Military Hospital Mohamed V in Rabat – Morocco for their precious help and contribution.

References

[1] Wong MCS, Fung FDH, Leung C, Cheung WWL, Goggins WB, Ng CF. The Global Epidemiology of Bladder Cancer: A Joinpoint Regression Analysis of Its Incidence and Mortality Trends and Projection. Sci Rep 2018;8(1):1129. doi:10.1038/s41598-018-19199-z.

[2] Benider A, Bendahhou k. Registre Des Cancers de La Région Du Grand Casablanca 2008-2012. Ed 2016 2016.

[3] Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BWG, Compérat E, et al. European Association of Urology. EAU Guidelines on Non-Muscle-Invasive Urothelial Carcinoma of the Bladder: Update 2013. Eur Urol 2013;64(4):639–53. doi:10.1016/j.eururo.2013.06.003.

[4] NiceBladder Cancer: Diagnosis and Management of Bladder Cancer: © NICE (2015) Bladder Cancer: Diagnosis and Management of Bladder Cancer. BJU Int 2017;120(6):755–65. doi:10.1111/bju.14045.

[5] Giordano A, Soria F. Role and Efficacy of Current Biomarkers in Bladder Cancer. AME Med J 2020;5 6–6. doi:10.21037/amj.2020.02.06.

[6] Locke WJ, Guanzon D, Ma C, Liew YJ, Duesing KR, Fung KYC, Ross JP. DNA Methylation Cancer Biomarkers: Translation to the Clinic. Front Genet 2019;10:1150. doi:10.3389/fgene.2019.01150.

[7] Bird A. DNA Methylation Patterns and Epigenetic Memory. Genes Dev 2002;16(1):6–21. doi:10.1101/gad.947102.

[8] Fantony JJ, Longo TA, Gopalakrishna A, Owusu R, Lance RS, Foo W-C, Inman BA, Abern MR. Urinary NID2 and TWIST1 Methylation to Augment Conventional Urine Cytology for the Detection of Bladder Cancer. CBM 2017;18(4):381–7. doi:10.3233/CBM-160261.

[9] Leão R, Lee D, Figueiredo A, Hermanns T, Wild P, Komosa M, et al. Combined Genetic and Epigenetic Alterations of the TERT Promoter Affect Clinical and Biological Behavior of Bladder Cancer. Int J Cancer 2019;144(7):1676–84. doi:10.1002/ijc.31935.

[10] Yegin Z, Gunes S, Buyukalpelli R. Hypermethylation of TWIST1 and NID2 in Tumor Tissues and Voided Urine in Urinary Bladder Cancer Patients. DNA and Cell Biology 2013;32(7):386–92. doi:10.1089/dna.2013.2030.

[11] Reddel RR. Telomere Maintenance Mechanisms in Cancer: Clinical Implications. Curr Pharm Des 2014;20(41):6361–74. doi:10.2174/1381612820666140630101047.

[12] Lee DD, Leão R, Komosa M, Gallo M, Zhang CH, Lipman T, et al. DNA Hypermethylation within TERT Promoter Upregulates TERT Expression in Cancer. J Clin Invest 2019;129(1):223–9. doi:10.1172/JCI121303.

[13] Lin P-C, Lin J-K, Lin C-H, Lin H-H, Yang S-H, Jiang J-K, Chen W-S, Chou C-C, Tsai S-F, Chang S-C. Clinical Relevance of Plasma DNA Methylation in Colorectal Cancer Patients Identified by Using a Genome-Wide High-Resolution Array. Ann Surg Oncol 2015;22(S3):1419–27. doi:10.1245/s10434-014-4277-2.

[14] Yang X, Hu J, Shi C, Dai J. Activation of TGF-B1 Pathway by SCUBE3 Regulates TWIST1 Expression and Promotes Breast Cancer Progression. Cancer Biother Radiopharm 2020;35(2):120–8. doi:10.1089/cbr.2019.2990.

[15] López JI, Angulo JC, Martín A, Sánchez-Chapado M, González-Corpas A, Colás B, et al. A DNA Hypermethylation Profile Reveals New Potential Biomarkers for the Evaluation of Prognosis in Urothelial Bladder Cancer. APMIS 2017;125(9):787–96. doi:10.1111/apm.12719.

[16] Ivaska J, Pallari H-M, Nevo J, Eriksson JE. Novel Functions of Vimentin in Cell Adhesion, Migration, and Signaling. Exp Cell Res 2007;313(10):2050–62. doi:10.1016/j.yexcr.2007.03.040.

[17] Kitamura Y-H, Shirahata A, Sakata M, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y, Hibi K. Frequent Methylation of Vimentin in Well-Differentiated Gastric Carcinoma. Anticancer Res 2009;29(6):2227–9.

[18] Zou H, Harrington JJ, Shire AM, Rego RL, Wang L, Campbell ME, et al. Highly Methylated Genes in Colorectal Neoplasia: Implications for Screening. Cancer Epidemiol Biomarkers Prev 2007;16(12):2686–96. doi:10.1158/1055-9965.EPI-07-0518.

[19] Costa VL, Henrique R, Danielsen SA, Duarte-Pereira S, Eknaes M, Skotheim RI, et al. Three Epigenetic Biomarkers, GDF15, TMEFF2, and VIM, Accurately Predict Bladder Cancer from DNA-Based Analyses of Urine Samples. Clin Cancer Res 2010;16(23):5842–51. doi:10.1158/1078-0432.CCR-10-1312.

[20] Kaur J, Jacobs R, Huang Y, Salvo N, Politis C. Salivary Biomarkers for Oral Cancer and Pre-Cancer Screening: A Review. Clin Oral Investig 2018;22(2):633–40. doi:10.1007/s00784-018-2337-x.

[21] Nagata S, Hamada T, Yamada N, Yokoyama S, Kitamoto S, Kanmura Y, et al. Aberrant DNA Methylation of Tumor-Related Genes in Oral Rinse: A Non-invasive Method for Detection of Oral Squamous Cell Carcinoma. Cancer 2012;118(17):4298–308. doi:10.1002/cncr.27417.

[22] Schmitz S, Ang KK, Vermorken J, Haddad R, Suarez C, Wolf GT, Hamoir M, Machiels J-P. Targeted Therapies for Squamous Cell Carcinoma of the Head and Neck: Current Knowledge and Future Directions. Cancer Treatment Reviews 2014;40(3):390–404. doi:10.1016/j.ctrv.2013.09.007.

[23] Attaleb M, El hamadani W, Khyatti M, Benbacer L, Benchekroun N, Benider A, Amrani M, El Mzibri M. Status of P16^{INK4a}and E-Cadherin Gene Promoter Methylation in Moroccan Patients With Cervical Carcinoma. oncol res 2009;18(4):185–92. doi:10.3727/096504009790217416.

[24] Takeshima H, Ushijima T. Accumulation of Genetic and Epigenetic Alterations in Normal Cells and Cancer Risk. npj Precis. Onc. 2019;3(1):7. doi:10.1038/s41698-019-0079-0.

[25] Yamashita S, Kishino T, Takahashi T, Shimazu T, Charvat H, Kakugawa Y, Nakajima T, Lee Y-C, Iida N, Maeda M, Hattori N, Takeshima H, Nagano R, Oda I, Tsugane S, Wu M-S, Ushijima T. Genetic and Epigenetic Alterations in Normal Tissues Have Differential Impacts on Cancer Risk among Tissues. Proc Natl Acad Sci USA 2018;115(6):1328–33. doi:10.1073/pnas.1717340115.

[26] Missaoui N, Hmissa S, Trabelsi A, Traoré C, Mokni M, Dante R, Frappart L. Promoter Hypermethylation of CDH13, DAPK1 and TWIST1 Genes in Precancerous and Cancerous Lesions of the Uterine Cervix. Pathology - Research and Practice 2011;207(1):37–42. doi:10.1016/j.prp.2010.11.001.

[27] Strelnikov VV, Kuznetsova EB, Tanas AS, Rudenko VV, Kalinkin AI, Poddubskaya EV, Kekeeva TV, Chesnokova GG, Trotsenko ID, Larin SS, Kutsev SI, Zaletaev DV, Nemtsova MV, Simonova OA. Abnormal Promoter DNA Hypermethylation of the Integrin, Nidogen, and Dystroglycan Genes in Breast Cancer. Sci Rep 2021;11(1):2264. doi:10.1038/s41598-021-81851-y.

[28] Ulazzi L, Sabbioni S, Miotto E, Veronese A, Angusti A, Gafà R, Manfredini S, Farinati F, Sasaki T, Lanza G, Negrini M. Nidogen 1 and 2 Gene Promoters Are Aberrantly Methylated in Human Gastrointestinal Cancer. Mol Cancer 2007;6(1):17. doi:10.1186/1476-4598-6-17.

[29] Wang J, Zhao Y, Xu H, Ma J, Liang F, Zou Q, et al. Silencing NID2 by DNA Hypermethylation Promotes Lung Cancer. Pathol Oncol Res 2020;26(2):801–11. doi:10.1007/s12253-019-00609-0.

[30] Reinert T, Borre M, Christiansen A, Hermann GG, Ørntoft TF, Dyrskjøt L. Diagnosis of Bladder Cancer Recurrence Based on Urinary Levels of EOMES, HOXA9, POU4F2, TWIST1, VIM, and ZNF154 Hypermethylation. PLoS One 2012;7(10):e46297. doi:10.1371/journal.pone.0046297.

Exploring urine sediments as a non-invasive method for DNA methylation detection in bladder cancer

VI.6 Exploring urine sediments as a non-invasive method for DNA methylation detection in bladder cancer

Urine cytology coupled with cystoscopy examination remains the mainstay test for diagnosis and surveillance of BC. Cystoscopy is an endoscopic procedure that provides information on the number, microscopic appearance and size of the tumour, it has good sensitivity but can also give a "false negative" result due to operator error or for tumors with small diameters, such as carcinoma *in situ*. A urine cytology test is performed on fresh or fixed urine specimens to look for abnormal cells; it has inversely a high specificity but low sensitivity, especially for low-grade tumors.

It is, therefore, necessary to use a molecular approach using urine as a non-invasive test to check many different physiological and pathological processes. In fact, proteins present in the urine may reflect a disorder of cells of the urinary tract, but also other dysfunctions by the passage of the majority of proteins from the blood into the urine during glomerular filtration. Many genetic and epigenetic markers have been identified for detecting and monitoring bladder cancer, and several urine assays and tests have already been developed for detecting some of these markers.

For this work, we have focused on determining the methylation status of a panel of genes promoters that is shown to be involved in bladder cancer in tumors biopsies and their paired urine exfoliated cells in order to evaluate the usefulness of urine samples as matrix for non-invasive test for methylation status assessment.

Our study demonstrates that the detection in voided urine samples of a set of four hypermethylated genes *TWIST1*, *hTERT*, *NID2* and *VIM* is highly sensitive ($\geqslant$82.8%) with excellent specificity (100%). This suggests that these four methylated biomarkers are ideally suitable for a non-invasive urine-based alternative method for the detection of BC disease, including low-grade and low-stage tumours.

The use of urinary biomarkers has potential value and facilitates not only diagnosis but also monitoring of this disease. It also plays a crucial role in reducing or replacing the need for invasive cystoscopy and associated health care costs. Surveillance biomarkers can be used to prevent the recurrence and progression of NMIBC and to predict therapeutic response in bladder cancer after intravesical instillation, neoadjuvant chemotherapy or adjuvant chemotherapy.

El azzouzi et al. African Journal of Urology (2022) 28:31
https://doi.org/10.1186/s12301-022-00298-3

African Journal of Urology

ORIGINAL RESEARCH Open Access

Exploring urine sediments as a non-invasive method for DNA methylation detection in bladder cancer

Meryem El azzouzi[1,2], Hajar El ahanidi[1,2], Chaimae Hafidi Alaoui[1,5], Imane Chaoui[1], Laila Benbacer[1], Mohamed Tetou[2,3], Ilias Hassan[2,3], Mounia Bensaid[4], Mohamed Oukabli[2,4], Ahmed Ameur[2,3], Abderrahmane Al bouzidi[2], Mohammed El mzibri[1] and Mohammed Attaleb[1*]

Abstract

Background: The main epigenetic event occurring during the bladder carcinogenesis process is DNA methylation, affecting genes involved in various metabolic pathways and cell regulation. The use of biological fluids such as urine sediments could be used as a non-invasive approach to enhance bladder cancer management. In this study, we aim to determine the promoter methylation status of a panel of genes in bladder cancer on tumor biopsies and urine sediments to evaluate the usefulness of urine samples as a non-invasive approach for methylation status assessment.

Methods: Using the methylation-specific PCR technique, we explored the promoter methylation status of *hTERT*, *TWIST1*, *VIM* and *NID2* genes in 40 tumor biopsies and their paired urine samples from Moroccan bladder cancer patients.

Results: In this study, bladder tumors showed promoter hypermethylation frequency of individual genes as 90%, 85%, 62.5% and 72.5% in *TWIST1*, *hTERT*, *NID2* and *VIM* genes, respectively.

Interestingly, the specificity of methylation detection in urine samples was 100% and the sensitivity to detect hypermethylation of *TWIST1*, *hTERT*, *NID2* and *VIM* genes reached 91.7%; 97.1%; 84% and 82.8%, respectively.

Conclusions: Our results clearly show that the assessment of promoter hypermethylation in urine samples is highly specific and has high sensitivity. Furthermore, urine sediments would be a useful approach to detect the DNA methylation status of genes and its potential association with bladder cancer development.

Keywords: DNA methylation, Non-invasive test, Bladder cancer, Urine sediments

1 Background

Worldwide bladder cancer (BC) is the tenth most commonly diagnosed cancer, with approximately 570,000 new cases per year and more than 210,000 deaths annually [1].

Currently, BC modalities for diagnosis and follow-up are cystoscopy and urine cytology; Cystoscopy is highly sensitive for most tumors but it may fail to identify smaller and flat tumors such as in situ carcinoma. Moreover, it is an expensive and an invasive procedure that shows insufficient power to predict precisely the patient outcome. Conversely, urinary cytology is a noninvasive and highly specific modality, but it has a poor sensitivity for low-grade and well-differentiated lesions [2]. Therefore, other non-invasive, rapid and accurate alternatives with high sensitivity and specificity are needed to improve the BC management.

In the recent years, extensive efforts are made to identify a molecular signature of BC to improve patients'

*Correspondence: attaleb_mohammed@yahoo.fr; attaleb@cnesten.org.ma
[1] Biology and Medical Research Unit, CNESTEN, Rabat, Morocco
Full list of author information is available at the end of the article

Springer Open

© The Author(s) 2022. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

treatments and prognosis prediction. In this context, many epigenetic changes have been reported to correlate with cancer malignancy and progression. The main epigenetic event occurring during the bladder carcinogenesis process is DNA methylation; which affects genes involved in various metabolic pathways and cells regulation [3]. Furthermore, the use of biological fluids such as urine sediments has attracted much attention as a non-invasive method of bladder cancer detection and surveillance.

Accordingly, several studies have shown that urine fluid is a reliable matrix to detect molecular alterations in all stages and grades of bladder cancer [4], and could be used as a non-invasive approach to identify a molecular signature for enhancing bladder cancer management [5]. In this optic, the present study was planned to evaluate the promoter methylation status of a panel of genes known for their involvement in bladder carcinogenesis, in tumor biopsies and their paired urine sediments to assess the usefulness of urine samples as a non-invasive approach of promoters' methylation detection and bladder cancer management.

2 Methods

2.1 Specimens

A total of 40 fresh frozen tumor samples were obtained by transurethral resection of the bladder or cystoscopy at the Urology Department of University Military Hospital in Rabat, Morocco and paired urine samples were obtained before surgery from every patient. The protocol of the study was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat – Morocco (Ref 82/19) and written informed consent was obtained from each recruited patient.

2.2 Methylation specific PCR (MSP)

Tissue specimens were immediately stored at − 80 °C. The voided urines (50 mL fresh urines) were spun down by centrifugation at 4500 g for 15 min, and the pelleted urine sediments were washed twice with phosphate buffered saline (PBS) and stored at − 80 °C. Genomic DNA was extracted from fresh tumor biopsies and urine cell sediments using phenol/chloroform method [6]. Then, 500 ng of genomic DNA extracted from each sample was used for bisulfite modification as previously described [7]. The Sodium bisulfite modification and DNA purification were carried out by the EZ DNA Methylation Kit (Zymo Research, USA). The modified DNA was subject to methylation-specific PCR (MSP) using methylation-specific and unmethylation-specific primers of a panel of genes "*TWIST1*, *hTERT*, *VIM* and *NID2*" [8–11]. The amplification reaction was carried out in a final volume of 25 µl containing 2 µl of modified DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 10 µM of each dNTP, 10 µM of each primer and 1U Platinum Taq Polymerase (Invitrogen, USA). Mixtures were first denatured at 95 °C for 10 min. Then, 40 cycles of PCR were performed with denaturation at 95 °C for 30 s, primer annealing for 45 s at the corresponding temperature (Table 1) and primer extension for 45 s at 72 °C. At the end of the cycles, mixtures incubation was carried out at 72 °C for 10 min. A negative control, in which DNA was omitted from the amplification mix, was included in each reaction. PCR products were analyzed by electrophoresis on 2% agarose gel followed by staining with ethidium bromide (10 mg/ml). The sensitivity reflects the fraction of patients in which the urine DNA was methylated among cases with methylation in matched tumor DNA of the same gene promoter. The specificity reflects the number of methylated tumor biopsies among cases showing methylation in matched urine DNA of the same gene promoter.

2.3 Statistical analysis

The hypermethylation status of genes promoters in tumor biopsies and urine sediments was statistically evaluated using percentages and confidence intervals (CI) of 95% by IBM SPSS software version 23.

Table 1 Genes primers and corresponding annealing temperatures for MSP

Gene		Forward (5′ – > 3′)	Reverse (5′ – > 3′)	Product size (pb)	Tm (°C)
TWIST1	M	CCGTCGCCTTCCTCCGACGA	TGCGTTAGGGTTCGGGGGCGT	100	64
	U	TTGGATGGGGTTGTTATTGT	CCTAACCCAAACAACCAACC	79	63
hTERT	M	GAGGTATTTCGGGAGGTTTCGC	ACTCCGAACACCACGAATACCG	121	58
	U	GGGAGGTATTTTGGGAGGTTTTGT′	CAAACTCCAAACACCACAAATACCA	126	58
NID2	M	TAGTATTGGTAACGACGATAGTATC	AAATTCGAAACTAACGCGACACG	141	58
	U	TAGTATTGGTAATGATGATAGTATT	AAATTCAAAACTAACACAACACA	141	55
VIM	M	TTATAAAAATAGCGTTTTCGGC	ATAACGCGAACTAACTCCCG	143	59
	U	GGGTTATAAAAATAGTGTTTTTGGT	ACAATAACACAAACTAACTCCCA	149	57

Table 2 Tumor characteristics of recruited cases

Parameter	Total cases	Percentage (%)
Gender		
Male	39	97.5
Female	1	2.5
Age		
≤ 50	1	2.5
50–70	24	60
> 70	15	37.5
Smoking		
Yes	22	55
No	18	45
Tumor stage		
≤ PT1	30	75
> PT1	10	25
Tumor grade		
Low grade	14	35
High grade	26	65

Table 3 Comparison between methylation status in bladder biopsies and voided urine sediments

Genes	Hypermethylation detection in fresh tumor biopsies		Hypermethylation detection in urine sediments		Sensitivity % (95% CI)
	N	% [95% CI]	*N*	% [95% CI]	
TWIST1	36	90 [76.3–97.2]	33	82.5 [67.2–92.7]	91.7 [77.5–98.2]
hTERT	34	85 [70.2–94.3]	33	82.5 [67.2–92.7]	97.1 [84.7–99.9]
NID2	25	62.5 [45.8–77.3]	21	52.5 [36.1–68.5]	84 [63.9–95.5]
VIM	29	72.5 [56.1–85.4]	24	60 [43.3–75.1]	82.8 [64.2–94.2]

CI = confidence interval

3 Results

Clinico-pathological characteristics of tumor specimens are summarized in Table 2. The mean age of patients was 67 years, ranging from 47 to 84 years. The most recruited cases were staged ≤ PT1 and have high tumor grade (Table 2).

Promoter's methylation was assessed in tumor biopsies and matched urine sediments from all patients and results are summarized in Table 3. Globally, DNA hypermethylation was found in all bladder tumor biopsies in at least one of the studied genes. Comparison between hypermethylation of the studied genes in bladder biopsies and paired urine sediments, showed that all cases with hypermethylation in DNA from urine sediments have an aberrant methylation in the corresponding DNA from bladder biopsies; suggesting a specificity of methylation detection in urine sediments of 100%.

In this study, the sensitivity of hypermethylation detection in urine sediments was different according to the studied gene. It was higher for *hTERT* 97.1% [95% CI = 77.5–98.2] and lower for *VIM* gene 82.8% [95% CI = 64.2–94.2] (Table 3).

4 Discussion

Recent advances in oncology research report that molecular signature of tumors is a key factor for cancer stratification, personalized therapy and a reliable prognosis [12]. Molecular characterization of tumors is mainly based on the assessment of genetic alterations leading to genetic activation/inactivation and cellular pathways deregulation.

During last decade, a great interest was given to DNA methylation changes as the main epigenetic event associated with cancer development and progression [13]. In bladder cancer, DNA hypermethylation have been used as targets for detection and diagnosis of tumor cells in clinical specimens such as tissue biopsies and urine sediments [4].

In this study, we have used MSP assay to assess the promoter methylation status of a panel of genes in both tumor biopsies and urine sediments DNA from Moroccan patients with bladder cancer. The interest was focused on, *TWIST1, hTERT, NID2* and *VIM* genes for their implication in many cellular pathways and their widely reported data as highly methylated genes in bladder cancer [14–17].

In this study, bladder tumors showed promoter hypermethylation frequency of individual genes as 90%, 85%, 62.5% and 72.5% in *TWIST1, hTERT, NID2* and *VIM* genes, respectively.

These findings are in good agreement with already reported data on Moroccan patients [17]. Of interest, medium frequency of *TERT* (53%) and high frequency of *TWIST1* (98%) promoters' methylation in BC were reported by Leão et al. [14] and Yegin et al. [16], respectively.

The methylation study of each gene promoter was extended to the matched urine sediment DNA for all studied patients. Overall, the promoter hypermethylation for at least one gene was detected in 39 analyzed samples; thereby the detection coverage of methylation in urine reached 97.5% [95% CI = 86.8–99.9]. Interestingly, the sensitivity of *TWIST1* and *TERT* promoters' hypermethylation detection in urine samples was higher; reaching 91.7% and 97.1%, respectively. These results are supported by Yegin et al. (2013) and Vinci et al. (2011), who showed that *TWIST1* and *TERT* hypermethylation have high sensitivities (87.5% and 76%, respectively) and specificities (93.3% and 100%, respectively) for bladder cancer detection in urine sediments [16, 18]. Similar

results were reported by Renard et al. (2010) that have identified high sensitivity and specificity (90% and 93%, respectively) of *TWIST1* methylation detection in urine sediments [19].

In this study, sensitivity of detecting *NID2* and *VIM* hypermethylation in urine sediments was 84% and 82.8%, respectively. These results are slightly different from those reported by Yegin et al. (2013) and Costa et al. (2010), showing higher sensitivity frequencies (95.8% and 96%, respectively) in urinary bladder cancer patients [16, 20]. This difference could be due to the smaller cohort in our study. Scientific evidences have shown that the urine DNA is presumably shed from the original primary tumor or neoplastic lesions in the bladder wall, and consequently genetic and epigenetic alterations in urine sediments will reflect the whole situation in the bladder tumors' tissues. Moreover, DNA methylation assessment in urine sediments has much significant potential added value for clinicians and patients: (1) a non-invasive approach; (2) reducing the number of cystoscopies required for the diagnosis and surveillance of bladder cancer and (3) saving time and budget.

In this study, of particular interest, a specificity of 100% was obtained for the all studied genes. Indeed, the methylation was only detected in those patients whose tumor tissue also showed gene promoter methylation, and consequently no false-positive result was detected, with 100% specificity for all the studied genes. These results are of a great interest and highlighted the interest of using urine sediments for biomarkers' assessment.

This study is very informative and clearly highlights the usefulness of urine sediment as a valuable and non-invasive matrix for epigenetic studies. However, there were several limitations due to the use of the MSP technique which identifies only the qualitative level of DNA methylation and to the small scale of our study cohort. Further studies on a large population are necessary to confirm the obtained results.

5 Conclusions

Our investigation clearly showed that promoters' hypermethylation assessment in non-invasive urine samples is highly specific, shows a great sensitivity, and would be a useful approach for studying epigenetic alterations and their potential association with bladder cancer development.

Acknowledgements

We would like to thank everyone who has participated to the realization of this work, especially the medical staffs of Urology and Anatomopathology Departments of the Military Hospital Mohamed V in Rabat – Morocco for their precious help and contribution.

Author contributions

ME performed the experiments, analyzed the data and wrote the paper. HE analyzed the data and contributed to experiments and paper writing. CHA, IC and LB contributed to experiments and paper writing. MT, IH and MB provided patient samples and follow up. MO, AA and AA provided intellectual contributions and revised the manuscript. ME provided intellectual contribution critically revised the manuscript. MA supervised all the experiments, critically revised the manuscript and gave final approval to the publication. All authors read and approved the final manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee for Biomedical Research, Faculty of Medicine and Pharmacy of Rabat – Morocco (Ref 82/19) and written informed consent was obtained from each recruited patient.

Consent for publication

I declare that all participants have given their consent for publication.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Biology and Medical Research Unit, CNESTEN, Rabat, Morocco. [2]Faculty of Medicine and Pharmacy of Rabat, Mohammed V University in Rabat, Rabat, Morocco. [3]Department of Urology, Mohammed V Military Teaching Hospital of Rabat, Rabat, Morocco. [4]Department of Pathology, Mohammed V Military Teaching Hospital of Rabat, Rabat, Morocco. [5]Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco.

Received: 29 November 2021 Accepted: 7 June 2022
Published online: 16 July 2022

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA A Cancer J Clin 71(3):209–249. https://doi.org/10.3322/caac.21660
2. Bladder cancer: diagnosis and management of bladder cancer: © NICE (2015) Bladder cancer: diagnosis and management of bladder cancer. BJU Int. 2017;120(6):755–765. https://doi.org/10.1111/bju.14045.
3. Kandimalla R, van Tilborg AA, Zwarthoff EC (2013) DNA methylation-based biomarkers in bladder cancer. Nat Rev Urol 10(6):327–335. https://doi.org/10.1038/nrurol.2013.89
4. Satyal U, Srivastava A, Abbosh PH (2019) Urine biopsy—liquid gold for molecular detection and surveillance of bladder cancer. Front Oncol 9:1266. https://doi.org/10.3389/fonc.2019.01266
5. López JI, Angulo JC, Martín A, Sánchez-Chapado M, González-Corpas A, Colás B, Ropero S (2017) A DNA hypermethylation profile reveals new potential biomarkers for the evaluation of prognosis in urothelial bladder cancer. APMIS 125(9):787–796. https://doi.org/10.1111/apm.12719
6. Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
7. Attaleb M, El Hamadani W, Khyatti M, Benbacer L, Benchekroun N, Benider A, Amrani M, El Mzibri M (2009) Status of p16INK4a and E-cadherin gene promoter methylation in moroccan patients with cervical carcinoma. Oncol Res Featur Preclin Clin Cancer Ther 18(4):185–192. https://doi.org/10.3727/096504009790217416

8. Eroglu O, Cilingir O, Artan S, Aras BD (2019) Investigation of DNAmethylation of TWIST gene in breast cancer and its relationship to histopathological features. Adv Breast Cancer Res 08(01):45–59. https://doi.org/10.4236/abcr.2019.81004
9. Guo N, Cheng D, Li ZH, Zhou QB, Zhou JJ, Lin Q, Zeng B, Liao Q, Chen RF (2012) Transfection of HCVc improves hTERT expression through STAT3 pathway by epigenetic regulation in Huh7 cells. J Cell Biochem 113(11):3419–3426. https://doi.org/10.1002/jcb.24218
10. Ulazzi L, Sabbioni S, Miotto E, Veronese A, Angusti A, Gafà R, Manfredini S, Farinati F, Sasaki T, Lanza G, Negrini M (2007) Nidogen 1 and 2 gene promoters are aberrantly methylated in human gastrointestinal cancer. Mol Cancer 6(1):17. https://doi.org/10.1186/1476-4598-6-17
11. Xiong G, Liu J, Tang Q, Fan Y, Fang D, Yang K, Xie F, Zhang M, Zhang L, Liu L, Zhang C, Yao L, Yang L, Ci W, Zhao W, Gong Y, He Q, Gong K, He Z, Zhou L (2015) Prognostic and predictive value of epigenetic biomarkers and clinical factors in upper tract urothelial carcinoma. Epigenomics 7(5):733–744. https://doi.org/10.2217/epi.15.34
12. Herceg Z, Hainaut P (2007) Genetic and epigenetic alterations as biomarkers for cancer detection, diagnosis and prognosis. Mol Oncol 1(1):26–41. https://doi.org/10.1016/j.molonc.2007.01.004
13. Locke WJ, Guanzon D, Ma C, Liew YJ, Duesing KR, Fung KYC, Ross JP (2019) DNA methylation cancer biomarkers : translation to the clinic. Front Genet 10:1150. https://doi.org/10.3389/fgene.2019.01150
14. Leão R, Lee D, Figueiredo A, Hermanns T, Wild P, Komosa M, Lau I, Mistry M, Nunes NM, Price AJ, Zhang C, Lipman T, Poyet C, Valtcheva N, Oehl K, Coelho H, Sayyid R, Gomes AM, Castro PEL, Tabori U (2019) Combined genetic and epigenetic alterations of the TERT promoter affect clinical and biological behavior of bladder cancer. Int J Cancer 144(7):1676–1684. https://doi.org/10.1002/ijc.31935
15. Monteiro-Reis S, Leça L, Almeida M, Antunes L, Monteiro P, Dias PC, Morais A, Oliveira J, Henrique R, Jerónimo C (2014) Accurate detection of upper tract urothelial carcinoma in tissue and urine by means of quantitative GDF15, TMEFF2 and VIM promoter methylation. Eur J Cancer 50(1):226–233. https://doi.org/10.1016/j.ejca.2013.08.025
16. Yegin Z, Gunes S, Buyukalpelli R (2013) Hypermethylation of *TWIST1* and *NID2* in tumor tissues and voided urine in urinary bladder cancer patients. DNA Cell Biol 32(7):386–392. https://doi.org/10.1089/dna.2013.2030
17. El Azzouzi M, El Ahanidi H, Hafidi Alaoui C, Chaoui I, Benbacer L, Tetou M, Hassan I, Bensaid M, Oukabli M, Ameur A, Al Bouzidi A, El Mzibri M, Attaleb M (2022) Evaluation of DNA methylation in promoter regions of hTERT, TWIST1, VIM and NID2 genes in Moroccan bladder cancer patients. Cancer Genet 260–261:41–45. https://doi.org/10.1016/j.cancergen.2021.12.001
18. Vinci S, Giannarini G, Selli C, Kuncova J, Villari D, Valent F, Orlando C (2011) Quantitative Methylation Analysis of BCL2, HTERT, and DAPK Promoters in Urine Sediment for the Detection of Non-Muscle-Invasive Urothelial Carcinoma of the Bladder: A Prospective, Two-Center Validation Study. Urol. Oncol 29:150–156. https://doi.org/10.1016/j.urolonc.2009.01.003.
19. Renard I, Joniau S, van Cleynenbreugel B, Collette C, Naômé C, Vlassenbroeck I, Nicolas H, de Leval J, Straub J, Van Criekinge W, Hamida W, Hellel M, Thomas A, de Leval L, Bierau K, Waltregny D (2010) Identification and validation of the methylated TWIST1 and NID2 genes through real-time methylation-specific polymerase chain reaction assays for the noninvasive detection of primary bladder cancer in urine samples. Eur. Urol 58:96–104. https://doi.org/10.1016/j.eururo.2009.07.041.
20. Costa VL, Henrique R, Danielsen SA, Duarte-Pereira S, Eknaes M, Skotheim RI, Rodrigues A, Magalhães JS, Oliveira J, Lothe RA, Teixeira MR, Jerónimo C, Lind GE (2010) Three epigenetic biomarkers, GDF15, TMEFF2, and VIM, accurately predict bladder cancer from DNA-based analyses of urine samples. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research 16(23):5842–5851. https://doi.org/10.1158/1078-0432.CCR-10-1312.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen journal and benefit from:

- ► Convenient online submission
- ► Rigorous peer review
- ► Open access: articles freely available online
- ► High visibility within the field
- ► Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com

Discussion
&
Conclusion

VII. Discussion

Bladder cancer is the second most commonly urological cancer in the world after prostate cancer. In Morocco, BC is the eighth most common malignancy. Its management is an essential element in health care, and great efforts are made continuously to introduce new methods to improve diagnosis and treatment (Sung et al., 2021).

In general, about 75 % of all newly diagnosed patients of bladder cancer have NMIBC forms, which are a heterogeneous subclassification of UC; Patients with NMIBC have high recurrence and progression rates to muscle-invasive disease. The remaining 25% of cases are tumor invading the muscle layer of the bladder wall (Babjuk et al., 2022). The management of NMIBC represents a clinical challenge and can help to define prognosis factors able to determine, for each patient, the risk level of recurrence and progression in order to propose an appropriate treatment and monitoring plan for BC patients(Sylvester et al., 2021). Distinguishing low, intermediate and high-risk NMIBC patients who may recur or progress to MIBC remains the primary concern in NMIBC management, which will serve to establish a regular schedule of visits and surveillance following therapy (Babjuk et al., 2022).

Prediction of the likelihood of NMIBC recurrence and progression to MIBC remains an important topic of research. Available risk calculators and risk tables for recurrence and progression have been proposed, including EORTC and CUETO scoring models. EORTC was constructed based on 2596 patients and evaluated from 6 clinical pathological factors to provide further prognostic information (Sylvester et al., 2006). The major limitation of the EORTC calculator is its inability to predict recurrence and progression in patients treated with BCG. The CUETO scoring model was specifically created and validated to overcome this problem (Fernandez-Gomez et al., 2009).

The EORTC and CUETO scoring systems are based on clinical trial data of patients from European clinical trials. These models are not necessarily designed to precisely reflect non-European patients, including Moroccan patients. However, the uptake of these tools in Moroccan NMIBC patients may help to determine whether there is a need to enhance the existing tools to increase their applicability and usability.

In the first part of the thesis, a preliminary study was planned to evaluate the ability of the EORTC and the CUETO scoring systems to predict recurrence and progression in Moroccan

patients with NMIBC. Results clearly showed that both EORTC and CUETO risk tables underestimate the short-term probabilities of recurrence and progression. Both tables overestimate recurrence and progression rates at 5 years. Our long-term findings are in line with those of Holz et al. (Holz et al., 2017) and Busato Júnior et al. (Busato Júnior et al., 2016), who found that both tables overestimated the recurrence and progression in long-term (5 years). At one year, our results were in contrast with those of Dovey et al. reporting that the risk tables overestimated both recurrence and progression rate (Dovey et al., 2022). Also, we noted that only the CUETO model successfully stratified our patients into four risk groups of recurrence ($p = 0.005$). As shown by Chung et al. (2021), a strong association with recurrence but not with progression was noted in their research (Chung et al., 2021), which supports our findings.

The CUETO tool has more power than EORTC to discriminate each recurrence risk group, despite some limitations that arise from the smaller size than the original EORTC, CUETO and external validation studies, the short follow-up period and sometimes clinical follow-up is difficult in Morocco. This encourages us to carry out the same study in a large population to minimize the limitations (small cohort, short follow-up period…), which may improve predictive value of EORTC or/and CUETO nomograms and introduce them in clinical practice.

Moreover, the classification of patients into prognostic factor risk groups in Morocco is based on the following prognostic factors (Age, stage and grade, tumors size, number of tumors, carcinoma *in situ* and prior recurrence status), according to the French ccAFU guidelines 2020-2022 (Rouprêt et al., 2020; Babjuk et al., 2022). However, none of the EORTC and CUETO scoring models are used in clinical practice in Morocco, despite their predictive power and the fact that they are the most commonly used risk stratification models for the recurrence and progression of NMIBC. Therefore, it is worth to recommend the use of these models to provide an easy calculation of the estimated probabilities of recurrence and progression prior to the choice of proper treatment.

Several genetic, immunologic and molecular biomarkers have been recently integrated into EORTC and CURTO scores. For example, FGFR3 mutational status and MIB-1 expression, as a molecular grade, have been evaluated in a multicenter study and combined with EORTC risk scores for primary NMIBC. These molecular grade based on FGFR3 mutations status and MIB-1 expression proved highly reproductible and predictive than pathological grade (Rhijn et al., 2010). On the contrary, a further study has documented that the addition of five biomarkers (*p21, p53, p27, Kl-67* and *cyclin E1*) to both scoring systems reduces the predictive benefit, and

no association was observed with recurrence and progression (Passoni et al., 2016). Therefore, future perspectives include the combination of EORTC and CUETO scores and a panel of molecular biomarkers in a large multicentric cohort is needed to improve the discrimination of these tools in predicting the recurrence and progression of high-grade NMIBC.

During the last years, a great effort has been made to detect many predictive and diagnostic biomarkers in early and advanced BC to overcome the limitations of cystoscopy (invasive method) and urine cytology (because it fails to diagnose low-grade tumors). In particular, molecular markers used in diagnosis and follow-up, which can be incorporated into the personalized management of patients with BC (Yuanbin et al., 2011).

Considerable progress has been made in molecular biology through genomic and epigenomic profiling technologies, which allow the determination of genetic factors underlying carcinogenesis and genetic alterations caused by environmental agents. These advances led, during the past decades, to the discovery and development of molecular biomarkers as tools for cancer detection, prognosis and prediction of response to therapy (Goossens et al., 2015). Within this context, the overexpression of *HER2* has been confirmed as a predictive biomarker for targeted therapy with anti-HER2 in breast cancer (Piccart-Gebhart et al., 2005). Similarly, activating mutations in *KRAS* and *NRAS* genes predict resistance to anti-EGFR targeted therapies in metastatic colorectal cancer (Cutsem et al., 2009). These biomarkers should be detected by non-invasive methods at early-stage disease with high specificity and sensitivity. Regarding, epigenomic biomarkers, the detection of hypermethylation status of *RASSF1A*, *TWIST1, cyclin D2,* and *HIN1* genes using quantitative multiplex-methylation specific PCR (QM-MSP) is a highly sensitive method for diagnosis of breast cancer (Auwera et al., 2010). The NMP22 BladderCheck, BTA test, ImmunoCyt/uCyt+ assay and the UroVysion assay are the most common BC screening tests and are approved by FDA (Sugeeta et al., 2021).

Despite these significant discoveries, which have improved the management of patients and the effectiveness of treatments, only a few molecular biomarkers have been validated and routinely used in oncotherapeutics (Sugeeta et al., 2021). As BC is a very heterogeneous malignancy by nature with many subtypes, there is a need to identify new biomarkers in order to adapt treatments accordingly (Têtu, 2009).

In this sense, we studied the mutational status of *TERT, Tp53, PIK3CA/AKT1* genes and the methylation status of *TWIST1, hTERT, VIM* and *NID2* genes. These biomarkers could

potentially be interesting for non-invasive diagnostic tests and effective targeted therapies for BC. These studies highlight also the role of predictive tools in stratifying patients into the risk group for recurrence and progression, which is essential in the management of BC.

Accordingly, we have used a population-based series consisting of 70 patients diagnosed with bladder cancer in the urology department at Military Hospital Mohammed V, Rabat – Morocco, during 2017-2019. Among them, 68 were male and 2 females, with a sex ratio of 34. The mean age of patients was 67 years, ranging from 47 to 85 years. 28 patients (40%) were reported active or former smokers. Most cases were staged ≤ PT1 (74.29 %) and had high tumour grade (61.43%). 20/70 specimens were collected from patients with a previous history of bladder cancer, while 50/70 of collected specimens were primary tumours. Among patients with NMIBC type collected during this study, 12 experienced tumor recurrence (23.08%) and 5 (10%) were diagnosed with progression up on the 4 years of follow-up.

In this work, among 70 BC patients, only two women were diagnosed with BC. Recent epidemiologic research has elucidated the importance of demographic heterogeneity in the management of patients with BC (Shariat et al., 2010). Indeed, men are more likely to be diagnosed with BC, the gender disparity can be explained by the excessive environmental exposure to carcinogens (tobacco and industrial chemicals) in men (Hartge et al., 1990). Age is the greatest and an independent risk factor for the development of BC, this disease can occur generally in elderly people with the overall mean age at diagnosis of ≈70 years (Siroky, 2004). 74.29% of cases were diagnosed with NMIBC, which is in line with literature indicating that NMIBC accounts for over 70% of all BC cases (Matulewicz & Steinberg, 2020). The low recurrence and progression rates in our population can be explained by the small size of the cohort and the short follow-up period.

In the second part of this thesis, we conducted an analysis of the mutational status of some genetic biomarkers that may be associated with cell survival, proliferation, invasion and resistance therapies. Sequencing of *TERT*, *Tp53*, *PIK3CA* and *AKT* was successfully performed, and we published the core findings as a scientific research paper. Sequencing analysis of the *Tp53* gene revealed very high frequencies of mutations with a high prevalence of 100%, and at least one mutation was detected in each patient, followed by *hTERT* promotor (60%) and *PIK3CA* (12.6%). Only one pathogenic mutation E17K in the exon 1 on the *AKT* gene was detected.

In order to develop an appropriate targeted therapy and diagnostic test for any cancer, the molecular targets that induce carcinogenesis must be well understood. Like other forms of cancer, BC development is a process that begins with initiation, followed by promotion and progression (Hennings et al., 1993). In bladder cancer, mutations in *Tp53* and *PIK3CA/AKT1* genes are considered an advanced event in bladder tumorigenesis (Puzio-Kuter et al., 2009), whereas mutations of the *hTERT* gene are common in the early stages of cancer (initiation and promotion). The high prevalence of mutations in these genes sets suggests their use as potential biomarkers and therapeutic targets (Hosen et al., 2020). In our study, mutations in the *p53* gene appear to be correlated with tumor grade, while no correlation between *hTERT* and *PIK3CA/AKT1* genes has been shown in our cohort; this may be due to the small sample size.

Recently, a urine-based assay (the Uromonitor-V2®, U-monitor, Porto, Portugal) has been reported that can detect mutations in *TERT, FGFR3* and *KRAS* and has been shown to be more sensitive than cytology alone and could be an alternative to current follow-up methods. Although promising, this test needs to be validated in a clinical setting before its implementation (Kessel et al., 2017). Furthermore, other multicenter and prospective studies evaluating the combination of *TERT* promoter mutations, *OTX1* and *PLEKHS1* mutation panels have shown high sensitivity values, especially in low-grade tumors (Beukers et al., 2017; Xing et al., 2021).

Concerning *TP53* and *PIK3CA/AKT1* genes, no urine test is available at present. A recent study performed by Zhu et al. (2019) recommended two models of urine assay combining multiple potential markers to improve the accuracy of prediction. These assays containing *FGFR3* and *TP53, PIK3CA, ARID1A, STAG2* and *KTM2D* mutations panel, which yielded the most success and could be applied to early diagnosis and prediction of relapse in patients with NMIBC (Zhu et al., 2019). To assess the utility of urine sediment to detect genetic alterations as a suitable and non-invasive specimen for bladder cancer diagnosis and monitoring, we explored urine liquid biopsy to look for cancer mutations in *PIK3CA*, *AKT1* and *TERT* genes. For the three genes, our findings reveal a moderate sensitivity and specificity for detecting mutations in urine exfoliated cells.

However, given the moderate accuracy of mutations in the tested genes (*TERT, PIK3CA/AKT1*) in our study; it seems that the use of urine biofluids to identify genetic alterations needs more refinement and further independent evaluation in a large cohort is required to assess if any of

these non-invasive biomarkers or other accurate biomarkers could outperform current tools for BC detection and monitoring.

In addition to genetic markers, a high number of epigenetic biomarker candidates are continuously proposed, and the technologies used to analyse these promising epigenetic biomarkers are improving and becoming easier to use in clinical practice (Larsen et al., 2019).

The Epithelial-Mesenchymal Transition (EMT) is an important and highly dynamic process and one of the most notable program involved in tumor invasion and metastasis. Epithelial cells that acquire mesenchymal phenotype undergo a series of biochemical changes and can be classified into three different biological types: a primary event occurs during embryonic development; the second type is involved in tissue regeneration, and the third occurs in neoplastic cells that have previously undergone genetic and epigenetic changes (Brabletz et al., 2021).

Recently, it has been reported that promoter hypermethylation of *TWIST, VIM, NID2* and *hTERT* genes are involved in EMT regulation as an important mesenchymal marker in various tumor types, including bladder cancer. This raises the possibility of their use as a promising therapeutic target for cancer, especially in advanced diseases (Shirahata et al., 2009; Sui et al., 2013; Yegin et al., 2013). The EMT program is largely induced by a core set of various EMT activating transcription factors, including the *TWIST* families, the zinc finger E-box binding homeobox factors ZEB1 and ZEB2 and SNAIL and SLUG. In Parallel, the EMT-TFs directly or indirectly activate genes associated with a mesenchymal phenotype, including *VIM* (Vimentin), *FN1* (Fibronectin), and *CDH2* (N-cadherin) (Zhao et al., 2017).

In the present study, our goal was to investigate DNA methylation levels of CpG sites in four candidate gene promoters in tumours biopsies and urine sediments from 70 Moroccan bladder cancer patients using the Methylation-specific PCR method. We also compared the association patterns and prognostic impact of these markers.

The analyses were restricted to DNA methylation status in tumour biopsies. Here, our results indicate that methylation frequencies of *hTERT, TWIST1, VIM* and *NID2* genes were 90%, 85.71%, 67.14% and 67.14%, respectively. Statistical analysis showed a significant association between *TWIST1* hypermethylation and BC recurrence ($p = 0.041$). In Paper V, we included paired voided urine samples from the same cohort to evaluate the sensitivity and specificity of these genes as a non-invasive method for detecting cancer cells in urine. We found a higher

sensitivity and specificity to detect hypermethylation of *TWIST1, hTERT, NID2* and *VIM* genes, reaching 91.7%; 97.1%; 84% and 82.8%, respectively.

DNA methylation markers have been studied, and their aberrations are hallmarks of many human cancers, including BC. DNA hypermethylation of *TWIST1, hTERT, NID2* and *VIM* promoter genes is a common event in BC, and it is positively correlated with tumor progression and survival and thus, may serve as a prognostic marker for BC (Hong-Tao et al., 2016).

Several commercial tests that identify the DNA promoter methylation of single or gene sets are now available for the diagnosis of BC from urine or blood (AssureMDx®, Bladder CARE®, Bladder EPICHECK®), and some of these are tested as diagnostic biomarkers in clinical trials (Urakami et al., 2006). According to some studies, assessment of the methylation status of *TWIST1* and *NID2* genes in urinary sediments, in combination with cytology, has been found to increase sensitivity in BC patients (Fantony et al., 2017). In addition, *VIM* and *TERT* are also listed among potential and attractive DNA-methylated urine-based epigenetic biomarkers and may have a clinical value in BC. Nonetheless, validation of the performance of these biomarkers in well-designed multicenter clinical studies is an urgent need to demonstrate the added value over the common clinical practice (Hong-Tao et al., 2016).

The basis for urothelial carcinoma development and progression involves several genetic, epigenetic and pathological pathways which reflect varied morphological appearances. The diagnosis and monitoring of this disease are mainly based on an invasive method, cystoscopy, combined with urine cytology. Several molecular biomarkers assay is available for use in the diagnosis and management of BC and may complement cystoscopy, but there is no formal indication of their use in clinical practice (Batista et al., 2020).

In fact, at present, the use of these markers has not been yet established in clinical practice by international guidelines. Hence, further research is still needed to define the role of these promising biomarkers for cancer detection and follow-up (Giordano & Soria, 2020).

Treatment of Moroccan BC patients is based on intravesical bacillus Calmette-Guérin (BCG) immunotherapy or/and intravesical chemotherapy instillations. In addition, Atezolizumab injection is a *PD-L1* inhibitor administered to advanced or metastatic BCs unfit for cisplatin-based chemotherapy and whose tumors highly express *PD-L1* (Babjuk et al., 2022; Witjes et al., 2021). Administration of atezolizumab as an immune checkpoint inhibitor has demonstrated

a significant survival benefit compared to chemotherapy (Witjes et al., 2021). In Morocco, immunotherapy is not routinely used to treat BC patients, and some patients have benefited from these drugs under specific authorisation. The efficacy of these drugs needs to be evaluated with respect to the genetic heterogeneity of this disease. Moreover, there are no targeted therapies for genetic mutations in *TERT, P53, PIK3CA-AKT* and epigenetic aberrations in *TERT, TWIST, NID2* and *VIM*, even though the high prevalence of alterations in these genes.

The introduction of new drugs targeting these genetic and epigenetics biomarkers into clinical use become essential for an optimal treatment strategy and to improve the healthcare of BC patients nationally and worldwide.

VIII. Conclusion

Evaluation of EORTC and CUETO is performed was a preliminary study, and results clearly showed that both the EORTC and CUETO models were useful to predict recurrence and progression rates at a shot- and long-term. However, only the CUETO scoring model successfully stratified our patients into four risk groups of recurrence.

We noticed that among the genetic biomarkers tested, the prevalence of *P53* (100%) and *hTERT* (60%) mutations in biopsies was highest, followed by *PIK3AC* and *AKT1*. Also, Epigenetic analysis revealed a high frequency of aberrant methylation in the panel of studied genes *hTERT* (90%), *TWIST1* (85.71%), *VIM* (67.14%) and *NID2* (67.14%).

Identification of mutations in the *hTERT* and *PIK3CA-AKT1* genes from urine samples showed high specificity (100%) and sensitivity ranged from 50% to 100%. Furthermore, the assessment of promoters hypermethylation of *hTERT, TWIST1, VIM*, and *NID2* genes from urine samples is highly specific (100%) and has high sensitivity ranged from 82.8% to 97.1%.

It is unrealistic that the candidate biomarkers proposed in this study will be used in clinical practice in the near future, but they will be hopefully incorporated into clinical use after careful validation by several research groups. The work done in this thesis will hopefully be useful to others who want to investigate these candidate genes for the non-invasive detection of bladder cancer or other types of disease and to improve the care of urinary bladder cancer patients.

IX. Perspectives

Although our study identified hotspot mutations and aberrant methylation in the candidate genes analysed in the Moroccan population, much remains to be done. The bladder cancer series used in this thesis is currently being expanded to include women as well. New survival and clinico-pathological data from Military Hospital Mohammed V, Rabat, have been requested.

A retrospective study in a large and multicenter Moroccan population for external validation of the EORTC and CUETO scoring model to predict recurrence and progression during an extended follow-up period is planned. Results will certainly evaluate these models with more precision and accuracy for better management of BC.

Our study clearly showed a low frequency of mutations in the *hTERT* gene. Also, mutations in *PIK3CA* and *AKT1* are rare events in BC and could not be considered as reliable biomarkers for the management of the disease. Assessment of genetic and epigenetic alterations, as well as the study of the expression of studied genes and others potential genes involved in BC susceptibility (using RT-qPCR) in a large cohort, are necessary to determine if the mutational status, methylation status and expression level of these genes significantly affect the development of bladder cancers.

In addition to studying methylation as an epigenetic event, alterations in global histone modification play a crucial role during carcinogenesis, including bladder cancer. Thereby, identifying altered global levels of histone acetylation during bladder cancer progression could be helpful in identifying patients who may benefit the most from these targeted therapies.

References

References

Abern, M. R., Owusu, R., & Inman, B. A. (2014). Clinical performance and utility of a DNA methylation urine test for bladder cancer. *Urologic Oncology: Seminars and Original Investigations*, *32*(1), 51.e21-51.e26. https://doi.org/10.1016/j.urolonc.2013.08.003

Alleemudder, A., & Ballaro, A. (2016). The physiology and pharmacology of the lower urinary tract. Surgery (Oxford), 34(7), 326–332.

AHANIDI, H., HAFIDI ALAOUI, C., BENSAID, M., EL MZIBRI, M., AMEUR, A., ABBAR, M., AL BOUZIDI, A., & Mohammed ATTALEB, M. A. (2019). ASSESSMENT OF MOLECULAR BIOMARKERS FOR BLADDER CANCER DIAGNOSIS, GRADING AND PROGNOSIS. *Frontiers in Science and Engineering*, *Vol 8*, Supplment 2 (2019). https://doi.org/10.34874/IMIST.PRSM/FSEJOURNAL-V8I2.28049

Aine, M., Eriksson, P., Liedberg, F., Sjödahl, G., & Höglund, M. (2015). Biological determinants of bladder cancer gene expression subtypes. *Scientific Reports*, *5*, 10957. https://doi.org/10.1038/srep10957

Akincilar, S. C., Unal, B., & Tergaonkar, V. (2016). Reactivation of telomerase in cancer. *Cellular and Molecular Life Sciences: CMLS*, *73*(8), 1659–1670. https://doi.org/10.1007/s00018-016-2146-9

Antoni, S., Ferlay, J., Soerjomataram, I., Znaor, A., Jemal, A., & Bray, F. (2017). Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends. *European Urology*, *71*(1), 96–108. https://doi.org/10.1016/j.eururo.2016.06.010

Arianayagam, R., Arianayagam, M., & Rashid, P. (2011). Bladder cancer—Current management. *Australian Family Physician*, *40*(4), 209–213.

Autexier, C., & Lue, N. F. (2006). The structure and function of telomerase reverse transcriptase. *Annual Review of Biochemistry*, *75*, 493–517. https://doi.org/10.1146/annurev.biochem.75.103004.142412

Babjuk, M., Burger, M., Capoun, O., Cohen, D., Compérat, E. M., Dominguez Escrig, J. L., Gontero, P., Liedberg, F., Masson-Lecomte, A., Mostafid, A. H., Palou, J., van Rhijn, B. W. G., Rouprêt, M., Shariat, S. F., Seisen, T., Soukup, V., & Sylvester, R. J. (2022a). European Association of Urology Guidelines on Non-muscle-invasive Bladder Cancer (Ta, T1, and Carcinoma in Situ). *European Urology*, *81*(1), 75–94. https://doi.org/10.1016/j.eururo.2021.08.010

Babu, S., Mockler, D. C., Roa-Peña, L., Szygalowicz, A., Kim, N. W., Jahanfard, S., Gholami, S. S., Moffitt, R., Fitzgerald, J. P., Escobar-Hoyos, L. F., & Shroyer, K. R. (2019). Keratin 17 is a sensitive and specific biomarker of urothelial neoplasia. *Modern Pathology*, *32*(5), 717–724. https://doi.org/10.1038/s41379-018-0177-5

Baj, J., Korona-Głowniak, I., Forma, A., Maani, A., Sitarz, E., Rahnama-Hezavah, M., Radzikowska, E., & Portincasa, P. (2020). Mechanisms of the Epithelial–Mesenchymal Transition and Tumor Microenvironment in Helicobacter pylori-Induced Gastric Cancer. Cells, 9(4), 1055. https://doi.org/10.3390/cells9041055

Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., & Vogelstein, B. (1989). Chromosome 17 Deletions and p53 Gene Mutations in Colorectal Carcinomas. *Science*, *244*(4901), 217–221. https://doi.org/10.1126/science.2649981

Barry, M. J., & O'Leary, M. P. (1995). Advances in benign prostatic hyperplasia. The developmental and clinical utility of symptom scores. *The Urologic Clinics of North America*, *22*(2), 299–307.

Bates, A. W., & Baithun, S. I. (2000). Secondary neoplasms of the bladder are histological mimics of nontransitional cell primary tumours: Clinicopathological and histological features of 282 cases. *Histopathology*, *36*(1), 32–40. https://doi.org/10.1046/j.1365-2559.2000.00797.x

Batinac, T., Gruber, F., Lipozencić, J., Zamolo-Koncar, G., Stasić, A., & Brajac, I. (2003). Protein p53—Structure, function, and possible therapeutic implications. *Acta Dermatovenerologica Croatica: ADC*, *11*(4), 225–230.

Batista, R., Vinagre, N., Meireles, S., Vinagre, J., Prazeres, H., Leão, R., Máximo, V., & Soares, P. (2020). Biomarkers for Bladder Cancer Diagnosis and Surveillance: A Comprehensive Review. *Diagnostics*, *10*(1), 39. https://doi.org/10.3390/diagnostics10010039

Beauval, J.-B., Grange, C., & Roumiguié, M. (2015). Cystectomie et dérivations urinaires: Techniques et accompagnement. *Progrès en Urologie*, *25*(14), 900–906. https://doi.org/10.1016/j.purol.2015.09.016

Benider, A., Harif, M., Karkouri, M., Quessar, A., Sahraoui, S., Sqalli, S., & Ahmadaye, I. K. (2016). *Registre des cancers de la région du grand Casablanca pour la période 2008-2012. Foundation Lalla Salma*. https://www.contrelecancer.ma/site_media/uploaded_files/RCRGC.pdf

Beukers, W., van der Keur, K. A., Kandimalla, R., Vergouwe, Y., Steyerberg, E. W., Boormans, J. L., Jensen, J. B., Lorente, J. A., Real, F. X., Segersten, U., Orntoft, T. F., Malats, N., Malmström, P.-U., Dyrskjot, L., & Zwarthoff, E. C. (2017). *FGFR3* , *TERT* and *OTX1* as a Urinary Biomarker Combination for Surveillance of Patients with Bladder Cancer in a Large Prospective Multicenter Study. *Journal of Urology*, *197*(6), 1410–1418. https://doi.org/10.1016/j.juro.2016.12.096

Bodoor, K., Al-Ghabkari, A., Matalka, I., Haddad, Y., Alkhateeb, A., Jaradat, S., Jaradat, Z. W., Al-Ghazo, M., Abu-sheikha, A., Jalboush, S. A., & Jarun, Y. (2017). Assessment of p53 mutations, expression and prognosis in bladder cancer patients from Jordan: Identification of novel deletion mutations in the DNA-binding domain. *Meta Gene*, *12*, 33–42. https://doi.org/10.1016/j.mgene.2017.01.002

Bolla, S., Sampselle, C., & Jetti, R. (2022). Histology, Bladder. In *Treasure Island (FL): StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK540963/

Boltze, C., Schneider-Stock, R., Roessner, A., Quednow, C., & Hoang-Vu, C. (2003). Function of HSP90 and p23 in the telomerase complex of thyroid tumors. *Pathology, Research and Practice*, *199*(9), 573–579. https://doi.org/10.1078/0344-0338-00464

Boutou, E., Vlachodimitropoulos, D., Pappa, V., Sturzbecher, H.-W., & E., C. (2013). DNA Repair and Telomeres—An Intriguing Relationship. In C. Chen (Ed.), New Research Directions in DNA Repair. InTech. https://doi.org/10.5772/56115

Bork, P., Downing, A. K., Kieffer, B., & Campbell, I. D. (1996). Structure and distribution of modules in extracellular proteins. *Quarterly Reviews of Biophysics*, *29*(2), 119–167. https://doi.org/10.1017/S0033583500005783

Bouaoun, L., Sonkin, D., Ardin, M., Hollstein, M., Byrnes, G., Zavadil, J., & Olivier, M. (2016). TP53 Variations in Human Cancers: New Lessons from the IARC TP53 Database and Genomics Data. *Human Mutation*, *37*(9), 865–876. https://doi.org/10.1002/humu.23035

Bouchbika, Z., Haddad, H., Benchakroun, N., Eddakaoui, H., Kotbi, S., Megrini, A., Bourezgui, H., Sahraoui, S., Corbex, M., Harif, M., & Benider, A. (2013). Cancer incidence in Morocco: Report from Casablanca registry 2005-2007. *Pan African Medical Journal*, *16*. https://doi.org/10.11604/pamj.2013.16.31.2791

Brabletz, S., Schuhwerk, H., Brabletz, T., & Stemmler, M. P. (2021). Dynamic EMT: A multi-tool for tumor progression. *The EMBO Journal*, *40*(18). https://doi.org/10.15252/embj.2021108647

Brasure, M., Fink, H., Risk, M., Roderick, Taytana, S., Jeannine, O., Dongjuan, X., Butter, M., Kane, R. L., & Wilt, T. J. (2014). Chronic Urinary Retention: Comparative Effectiveness and Harms of Treatments. In *Comparative Effectiveness and Harms of Treatments*.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *68*(6), 394–424. https://doi.org/10.3322/caac.21492

Brems-Eskildsen, A. S., Zieger, K., Toldbod, H., Holcomb, C., Higuchi, R., Mansilla, F., Munksgaard, P. P., Borre, M., Ørntoft, T. F., & Dyrskjøt, L. (2010). Prediction and diagnosis of bladder cancer recurrence based on urinary content of hTERT, SENP1, PPP1CA, and MCM5 transcripts. *BMC Cancer*, *10*(1), 646. https://doi.org/10.1186/1471-2407-10-646

Burger, M., Catto, J. W. F., Dalbagni, G., Grossman, H. B., Herr, H., Karakiewicz, P., Kassouf, W., Kiemeney, L. A., La Vecchia, C., Shariat, S., & Lotan, Y. (2013). Epidemiology and Risk Factors of Urothelial Bladder Cancer. *European Urology*, *63*(2), 234–241. https://doi.org/10.1016/j.eururo.2012.07.033

Busato Júnior, W. F. S., Almeida, G. L., Ribas, C. A. P. M., Ribas Filho, J. M., & De Cobelli, O. (2016). EORTC Risk Model to Predict Progression in Patients With Non–Muscle-Invasive Bladder Cancer: Is It Safe to Use in Clinical Practice? *Clinical Genitourinary Cancer*, *14*(2), 176–182. https://doi.org/10.1016/j.clgc.2015.09.005

Byun, Y., Chen, F., Chang, R., Trivedi, M., Green, K. J., & Cryns, V. L. (2001). Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. *Cell Death & Differentiation*, *8*(5), 443–450. https://doi.org/10.1038/sj.cdd.4400840

Califf, R. M. (2018). Biomarker definitions and their applications. *Experimental Biology and Medicine (Maywood, N.J.)*, *243*(3), 213–221. https://doi.org/10.1177/1535370217750088

Cancer Genome Atlas Research Network. (2014). Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature*, *507*(7492), 315–322. https://doi.org/10.1038/nature12965

Caruana, S. M., Zwiebel, N., Cocker, R., McCormick, S. A., Eberle, R. C., & Lazarus, P. (1997). P53 alteration and human papilloma virus infection in paranasal sinus cancer. *Cancer*, *79*(7), 1320–1328.

Cassell, A., Yunusa, B., Jalloh, M., Mbodji, M. M., Diallo, A., Ndoye, M., Diallo, Y., Labou, I., Niang, L., & Gueye, S. M. (2019). Non-Muscle Invasive Bladder Cancer: A Review of the Current Trend in Africa. *World Journal of Oncology*, *10*(3), 123–131. https://doi.org/10.14740/wjon1210

Centers for Disease Control and Prevention (US), National Center for Chronic Disease Prevention and Health Promotion (US), & Office on Smoking and Health (US). (2010). *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General*. Centers for Disease Control and Prevention (US). http://www.ncbi.nlm.nih.gov/books/NBK53017/

Chai, A. W. Y., Cheung, A. K. L., Dai, W., Ko, J. M. Y., Ip, J. C. Y., Chan, K. W., Kwong, D. L.-W., Ng, W. T., Lee, A. W. M., Ngan, R. K. C., Yau, C. C., Tung, S. Y., Lee, V. H. F., Lam, A. K.-Y., Pillai, S., Law, S., & Lung, M. L. (2016). Metastasis-suppressing *NID2* , an epigenetically-silenced gene, in the pathogenesis of nasopharyngeal carcinoma and esophageal squamous cell carcinoma. *Oncotarget*, *7*(48), 78859–78871. https://doi.org/10.18632/oncotarget.12889

Chakraborty, A., Dasari, S., Long, W., & Mohan, C. (2019). Urine protein biomarkers for the detection, surveillance, and treatment response prediction of bladder cancer. *American Journal of Cancer Research*, *9*(6), 1104–1117.

Chang, F., Syrjänen, S., Kurvinen, K., & Syrjänen, K. (1993). The p53 tumor suppressor gene as a common cellular target in human carcinogenesis. *The American Journal of Gastroenterology*, *88*(2), 174–186.

Chang, Y., Lee, C., Ke, T., Chang, C., Chao, D., Huang, H., & Chang, J. (2019). Molecular characterization of colorectal cancer using whole-exome sequencing in a Taiwanese population. *Cancer Medicine*, *8*(8), 3738–3747. https://doi.org/10.1002/cam4.2282

Chen, L., Liu, Y., Zhang, Q., Zhang, M., Han, X., Li, Q., Xie, T., Wu, Q., & Sui, X. (2019). P53/PCDH17/Beclin-1 Proteins as Prognostic Predictors for Urinary Bladder Cancer. *Journal of Cancer*, *10*(25), 6207–6216. https://doi.org/10.7150/jca.37335

Chi, S.-W. (2014). Structural insights into the transcription-independent apoptotic pathway of p53. *BMB Reports*, *47*(3), 167–172. https://doi.org/10.5483/BMBRep.2014.47.3.261

Cho, K. H., Jeong, K. J., Shin, S. C., Kang, J., Park, C. G., & Lee, H. Y. (2013). STAT3 mediates TGF-β1-induced TWIST1 expression and prostate cancer invasion. *Cancer Letters*, *336*(1), 167–173. https://doi.org/10.1016/j.canlet.2013.04.024

Choi, J., & Lee, S. Y. (2020). Clinical Characteristics and Treatment of Immune-Related Adverse Events of Immune Checkpoint Inhibitors. *Immune Network*, *20*(1), e9. https://doi.org/10.4110/in.2020.20.e9

Chou, R., Gore, J. L., Buckley, D., Fu, R., Gustafson, K., Griffin, J. C., Grusing, S., & Selph, S. (2015). Urinary Biomarkers for Diagnosis of Bladder Cancer: A Systematic Review and Meta-analysis. *Annals of Internal Medicine*, *163*(12), 922–931. https://doi.org/10.7326/M15-0997

Chung, J.-W., Kim, J. W., Lee, E. H., Chun, S. Y., Park, D. J., Byeon, K. H., Choi, S. H., Lee, J. N., Kim, B. S., Kim, H. T., Yoo, E. S., Kwon, T. G., Ha, Y.-S., & Kim, T.-H. (2021). Prognostic Significance of the Neutrophil-to-Lymphocyte Ratio in Patients with Non-Muscle Invasive Bladder Cancer treated with Intravesical Bacillus Calmette–Guérin and the Relationship with the CUETO Scoring Model: Significance of the NLR with CUETO model. *Urology Journal*, *Instant 2021*, 6765. https://doi.org/10.22037/uj.v18i.6765

Cochard, P., & Paulin, D. (1984). Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *4*(8), 2080–2094.

Compérat, E. M., Burger, M., Gontero, P., Mostafid, A. H., Palou, J., Rouprêt, M., van Rhijn, B. W. G., Shariat, S. F., Sylvester, R. J., Zigeuner, R., & Babjuk, M. (2019). Grading of Urothelial Carcinoma and The New "World Health Organisation Classification of Tumours of the Urinary System and Male Genital Organs 2016". *European Urology Focus*, *5*(3), 457–466. https://doi.org/10.1016/j.euf.2018.01.003

Cong, Y.-S., Wright, W. E., & Shay, J. W. (2002). Human Telomerase and Its Regulation. Microbiology and Molecular Biology Reviews, 66(3), 407–425. https://doi.org/10.1128/MMBR.66.3.407-425.2002

Cordon-Cardo, C. (2008). Molecular alterations associated with bladder cancer initiation and progression. *Scandinavian Journal of Urology and Nephrology*, *42*(sup218), 154–165. https://doi.org/10.1080/03008880802291915

Costa, R., Carneiro, B. A., Taxter, T., Tavora, F. A., Kalyan, A., Pai, S. A., Chae, Y. K., & Giles, F. J. (2016). FGFR3-TACC3 fusion in solid tumors: Mini review. *Oncotarget*, *7*(34), 55924–55938. https://doi.org/10.18632/oncotarget.10482

Dadhania, V., Czerniak, B., & Guo, C. C. (2015). Adenocarcinoma of the urinary bladder. *American Journal of Clinical and Experimental Urology*, *3*(2), 51–63.

Dall'Era, M. A., Cheng, L., & Pan, C.-X. (2012). Contemporary management of muscle-invasive bladder cancer. *Expert Review of Anticancer Therapy*, *12*(7), 941–950. https://doi.org/10.1586/era.12.60

Dash, B. C., & El-Deiry, W. S. (2005). Phosphorylation of p21 in G2/M promotes cyclin B-Cdc2 kinase activity. *Molecular and Cellular Biology*, *25*(8), 3364–3387. https://doi.org/10.1128/MCB.25.8.3364-3387.2005

de Lange, T. (2002). Protection of mammalian telomeres. *Oncogene*, *21*(4), 532–540. https://doi.org/10.1038/sj.onc.1205080

Deaton, A. M., & Bird, A. (2011). CpG islands and the regulation of transcription. *Genes & Development*, *25*(10), 1010–1022. https://doi.org/10.1101/gad.2037511

Dovey, Z., Pfail, J., Martini, A., Steineck, G., Dey, L., Renström, L., Hosseini, A., Sfakianos, J. P., & Wiklund, P. (2022). Bladder Cancer (NMIBC) in a population-based cohort from Stockholm County with long-term follow-up; A comparative analysis of prediction models for recurrence and progression, including external validation of the updated 2021 E.A.U. model. *Urologic Oncology: Seminars and Original Investigations*, *40*(3), 106.e1-106.e10. https://doi.org/10.1016/j.urolonc.2021.10.008

Dratwa, M., Wysoczańska, B., Łacina, P., Kubik, T., & Bogunia-Kubik, K. (2020). TERT—Regulation and Roles in Cancer Formation. Frontiers in Immunology, 11, 589929. https://doi.org/10.3389/fimmu.2020.589929

Drayton, R. M., & Catto, J. W. F. (2012). Molecular mechanisms of cisplatin resistance in bladder cancer. *Expert Review of Anticancer Therapy*, *12*(2), 271–281. https://doi.org/10.1586/era.11.201

Dridi, W., Krabchi, K., Gadji, M., Lavoie, J., Bronsard, M., Fetni, R., & Drouin, R. (2006). Activité dominante négative des protéines p53 mutées. *Médecine/Sciences*, *22*(3), 301–307. https://doi.org/10.1051/medsci/2006223301

Dudderidge, T., Stockley, J., Nabi, G., Mom, J., Umez-Eronini, N., Hrouda, D., Cresswell, J., & McCracken, S. R. C. (2020). A Novel, non-invasive Test Enabling Bladder Cancer Detection in Urine Sediment of Patients Presenting with Haematuria—A Prospective Multicentre Performance Evaluation of ADXBLADDER. *European Urology Oncology*, *3*(1), 42–46. https://doi.org/10.1016/j.euo.2019.06.006

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., & Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell*, *75*(4), 817–825. https://doi.org/10.1016/0092-8674(93)90500-p

El Ghouzzi, V., Legeai-Mallet, L., Benoist-Lasselin, C., Lajeunie, E., Renier, D., Munnich, A., & Bonaventure, J. (2001). Mutations in the basic domain and the loop-helix II junction of TWIST abolish DNA binding in Saethre-Chotzen syndrome. *FEBS Letters*, *492*(1–2), 112–118. https://doi.org/10.1016/S0014-5793(01)02238-4

Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B., & Herman, J. G. (1999). Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Research*, *59*(1), 67–70.

Faleiro, I., Leão, R., Binnie, A., de Mello, R. A., Maia, A.-T., & Castelo-Branco, P. (2017). Epigenetic therapy in urologic cancers: An update on clinical trials. *Oncotarget*, *8*(7), 12484–12500. https://doi.org/10.18632/oncotarget.14226

Fantony, J. J., Longo, T. A., Gopalakrishna, A., Owusu, R., Lance, R. S., Foo, W.-C., Inman, B. A., & Abern, M. R. (2017). Urinary NID2 and TWIST1 methylation to augment conventional urine cytology for the detection of bladder cancer. *Cancer Biomarkers*, *18*(4), 381–387. https://doi.org/10.3233/CBM-160261

Ferguson, A. R. (1911). Associated bilharziosis and primary malignant disease of the urinary bladder, with observations on a series of forty cases. *The Journal of Pathology and Bacteriology*, *16*(1), 76–94. https://doi.org/10.1002/path.1700160107

Fernandez-Gomez, J., Madero, R., Solsona, E., Unda, M., Martinez-Piñeiro, L., Gonzalez, M., Portillo, J., Ojea, A., Pertusa, C., Rodriguez-Molina, J., Camacho, J. E., Rabadan, M., Astobieta, A., Montesinos, M., Isorna, S., Muntañola, P., Gimeno, A., Blas, M., & Martinez-Piñeiro, J. A. (2009). Predicting nonmuscle invasive bladder cancer recurrence and progression in patients

treated with bacillus Calmette-Guerin: The CUETO scoring model. *The Journal of Urology*, *182*(5), 2195–2203. https://doi.org/10.1016/j.juro.2009.07.016

Ferrari, S., Cannizzaro, L. A., Battini, R., Huebner, K., & Baserga, R. (1987). The gene encoding human vimentin is located on the short arm of chromosome 10. *American Journal of Human Genetics*, *41*(4), 616–626.

Finlay, C. A., Hinds, P. W., & Levine, A. J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. *Cell*, *57*(7), 1083–1093. https://doi.org/10.1016/0092-8674(89)90045-7

Forbes, S. A., Bindal, N., Bamford, S., Cole, C., Kok, C. Y., Beare, D., Jia, M., Shepherd, R., Leung, K., Menzies, A., Teague, J. W., Campbell, P. J., Stratton, M. R., & Futreal, P. A. (2011). COSMIC: Mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Research*, *39*(Database), D945–D950. https://doi.org/10.1093/nar/gkq929

Foresman, W. H., & Messing, E. M. (1997). Bladder cancer: Natural history, tumor markers, and early detection strategies. *Seminars in Surgical Oncology*, *13*(5), 299–306. https://doi.org/10.1002/(SICI)1098-2388(199709/10)13:5<299::AID-SSU3>3.0.CO;2-8

Freedman, N. D. (2011). Association Between Smoking and Risk of Bladder Cancer Among Men and Women. *JAMA*, *306*(7), 737. https://doi.org/10.1001/jama.2011.1142

Friedell, G. H., Parija, G. C., Nagy, G. K., & Soto, E. A. (1980). The Pathology of Human Bladder Cancer. *Cancer*, *45 Suppl 7*, 1823–1831. https://doi.org/10.1002/cncr.1980.45.s7.1823

Fujimoto, K., Yamada, Y., Okajima, E., Kakizoe, T., Sasaki, H., Sugimura, T., & Terada, M. (1992). Frequent association of p53 gene mutation in invasive bladder cancer. *Cancer Research*, *52*(6), 1393–1398.

Gao, Y., Sun, Y., Frank, K. M., Dikkes, P., Fujiwara, Y., Seidl, K. J., Sekiguchi, J. M., Rathbun, G. A., Swat, W., Wang, J., Bronson, R. T., Malynn, B. A., Bryans, M., Zhu, C., Chaudhuri, J., Davidson, L., Ferrini, R., Stamato, T., Orkin, S. H., … Alt, F. W. (1998). A Critical Role for DNA End-Joining Proteins in Both Lymphogenesis and Neurogenesis. *Cell*, *95*(7), 891–902. https://doi.org/10.1016/S0092-8674(00)81714-6

Garavís, M., González, C., & Villasante, A. (2013). On the Origin of the Eukaryotic Chromosome: The Role of Noncanonical DNA Structures in Telomere Evolution. *Genome Biology and Evolution*, *5*(6), 1142–1150. https://doi.org/10.1093/gbe/evt079

Ghouzzi, V. E., Merrer, M. L., Perrin-Schmitt, F., Lajeunie, E., Benit, P., Renier, D., Bourgeois, P., Bolcato-Bellemin, A.-L., Munnich, A., & Bonaventure, J. (1997). Mutations of the TWIST gene in the Saethre-Chotzene syndrome. *Nature Genetics*, *15*(1), 42–46. https://doi.org/10.1038/ng0197-42

Giordano, A., & Soria, F. (2020). Role and efficacy of current biomarkers in bladder cancer. *AME Medical Journal*, *5*, 6–6. https://doi.org/10.21037/amj.2020.02.06

GLOBOCAN, 2020. (2020). *Bladder cancer in the world. Cancer today.* https://gco.iarc.fr/today/online-analhttps://gco.iarc.fr/today/online-analysismap?v=2020&mode=population&mode_population=continents0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=10&group_cancer=1&include_nmsc=1&include_nmsc_other=1&projection=naturalearth&color_palette=default&map_scale=quantile&map_nb_colors=5&continent=0&show_ranking=0&rotate=%255B10%252C0%255D

GLOBOCAN, 2020. (2022). *Bladder cancer in Africa. Cancer today.* https://gco.iarc.fr/today/data/factsheets/populations/903-africa-fact-sheets.pdf

GLOBOCAN, 2020. (2022). *Bladder cancer in Morocco. Cancer today.* https://gco.iarc.fr/today/data/factsheets/populations/504-morocco-fact-sheets.pdf

Goebel, C. (1905). Ueber die bei Bilharziakrankheit vorkommenden Blasentumoren mit besonderer Berücksichtigung des Carcinoms. *Zeitschrift für Krebsforschung*, *3*(3), 369–513. https://doi.org/10.1007/BF02215122

Goossens, N., Nakagawa, S., Sun, X., & Hoshida, Y. (2015). Cancer biomarker discovery and validation. *Translational Cancer Research*, *4*(3), 256–269. https://doi.org/10.3978/j.issn.2218-676X.2015.06.04

Gort, E. H., Suijkerbuijk, K. P. M., Roothaan, S. M., Raman, V., Vooijs, M., van der Wall, E., & van Diest, P. J. (2008). Methylation of the TWIST1 Promoter, TWIST1 mRNA Levels, and Immunohistochemical Expression of TWIST1 in Breast Cancer. *Cancer Epidemiology, Biomarkers & Prevention*, *17*(12), 3325–3330. https://doi.org/10.1158/1055-9965.EPI-08-0472

Griewank, K. G., Murali, R., Schilling, B., Scholz, S., Sucker, A., Song, M., Süsskind, D., Grabellus, F., Zimmer, L., Hillen, U., Steuhl, K.-P., Schadendorf, D., Westekemper, H., & Zeschnigk, M. (2013). TERT promoter mutations in ocular melanoma distinguish between conjunctival and uveal tumours. *British Journal of Cancer*, *109*(2), 497–501. https://doi.org/10.1038/bjc.2013.312

Gripp, K. W., Zackai, E. H., & Stolle, C. A. (2000). Mutations in the humanTWIST gene. *Human Mutation*, *15*(2), 150–155. https://doi.org/10.1002/(SICI)1098-1004(200002)15:2<150::AID-HUMU3>3.0.CO;2-D

Gu, J., & Wu, X. (2011). Genetic susceptibility to bladder cancer risk and outcome. *Personalized Medicine*, *8*(3), 365–374. https://doi.org/10.2217/pme.11.15

Guerrero-Preston, R., Soudry, E., Acero, J., Orera, M., Moreno-López, L., Macía-Colón, G., Jaffe, A., Berdasco, M., Ili-Gangas, C., Brebi-Mieville, P., Fu, Y., Engstrom, C., Irizarry, R. A., Esteller, M., Westra, W., Koch, W., Califano, J., & Sidransky, D. (2011). NID2 and HOXA9 promoter hypermethylation as biomarkers for prevention and early detection in oral cavity squamous cell carcinoma tissues and saliva. *Cancer Prevention Research (Philadelphia, Pa.)*, *4*(7), 1061–1072. https://doi.org/10.1158/1940-6207.CAPR-11-0006

Guo, A., Wang, X., Shi, J., Sun, C., & Wan, Z. (2014). Bladder tumour antigen (BTA stat) test compared to the urine cytology in the diagnosis of bladder cancer: A meta-analysis. *Canadian Urological Association Journal*, *8*(5–6), 347. https://doi.org/10.5489/cuaj.1668

Hafner, A., Bulyk, M. L., Jambhekar, A., & Lahav, G. (2019). The multiple mechanisms that regulate p53 activity and cell fate. *Nature Reviews. Molecular Cell Biology*, *20*(4), 199–210. https://doi.org/10.1038/s41580-019-0110-x

Hamamori, Y., Wu, H. Y., Sartorelli, V., & Kedes, L. (1997). The basic domain of myogenic basic helix-loop-helix (bHLH) proteins is the novel target for direct inhibition by another bHLH protein, Twist. *Molecular and Cellular Biology*, *17*(11), 6563–6573. https://doi.org/10.1128/MCB.17.11.6563

Harlé, A., Lion, M., Lozano, N., & Merlin, J.-L. (2013). [Clinical, diagnostic significance and theranostic interest of PIK3CA gene mutations in breast cancer]. *Bulletin Du Cancer*, *100*(10), 947–954. https://doi.org/10.1684/bdc.2013.1827

Harley, C. B., Futcher, A. B., & Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature*, *345*(6274), 458–460. https://doi.org/10.1038/345458a0

Harris, L., Fritsche, H., Mennel, R., Norton, L., Ravdin, P., Taube, S., ... & Bast Jr, R. C. (2007). American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. Journal of clinical oncology, 25(33), 5287-5312.

Hartge, P., Harvey, E. B., Linehan, W. M., Silverman, D. T., Sullivan, J. W., Hoover, R. N., & Fraumeni, J. F. (1990). Unexplained Excess Risk of Bladder Cancer in Men. *JNCI: Journal of the National Cancer Institute*, *82*(20), 1636–1640. https://doi.org/10.1093/jnci/82.20.1636

Hayter, C. R., Paszat, L. F., Groome, P. A., Schulze, K., Math, M., & Mackillop, W. J. (1999). A population-based study of the use and outcome of radical radiotherapy for invasive bladder cancer. *International Journal of Radiation Oncology, Biology, Physics*, *45*(5), 1239–1245. https://doi.org/10.1016/s0360-3016(99)00306-5

Hemmings, B. A., & Restuccia, D. F. (2012). PI3K-PKB/Akt pathway. *Cold Spring Harbor Perspectives in Biology*, *4*(9), a011189. https://doi.org/10.1101/cshperspect.a011189

Hennings, H., Glick, A. B., Greenhalgh, D. A., Morgan, D. L., Strickland, J. E., Tennenbaum, T., & Yuspa, S. H. (1993). Critical aspects of initiation, promotion, and progression in multistage epidermal carcinogenesis. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, *202*(1), 1–8. https://doi.org/10.3181/00379727-202-43511a

Herman, J. G., & Baylin, S. B. (2003). Gene Silencing in Cancer in Association with Promoter Hypermethylation. *New England Journal of Medicine*, *349*(21), 2042–2054. https://doi.org/10.1056/NEJMra023075

Hertfordshire and Bedfordshire Urological Cancer Centre, Department of Urology, Lister Hospital, Stevenage, UK, Omorphos, N. P., Pansaon Piedad, J. C., Hertfordshire and Bedfordshire Urological Cancer Centre, Department of Urology, Lister Hospital, Stevenage, UK, Vasdev, N., Hertfordshire and Bedfordshire Urological Cancer Centre, Department of Urology, Lister Hospital, Stevenage, UK, & School of Medicine and Life Sciences, University of Hertfordshire, Hatfield, UK. (2021). Guideline of guidelines: Muscle-invasive bladder cancer. *Türk Üroloji Dergisi/Turkish Journal of Urology*, *47*(Supp1), S71–S78. https://doi.org/10.5152/tud.2020.20337

Holz, S., Albisinni, S., Gilsoul, J., Pirson, M., Duthie, V., Quackels, T., Vanden Bossche, M., & Roumeguère, T. (2017). Risk factor assessment in high-risk, bacillus Calmette-Guérin-treated, non-muscle-invasive bladder cancer. *Research and Reports in Urology*, *9*, 195–202. https://doi.org/10.2147/RRU.S143865

Hosen, M. I., Sheikh, M., Zvereva, M., Scelo, G., Forey, N., Durand, G., Voegele, C., Poustchi, H., Khoshnia, M., Roshandel, G., Sotoudeh, M., Nikmanesh, A., Etemadi, A., Avogbe, P. H., Chopard, P., Delhomme, T. M., Foll, M., Manel, A., Vian, E., … Calvez-Kelm, F. L. (2020). Urinary TERT promoter mutations are detectable up to 10 years prior to clinical diagnosis of bladder cancer: Evidence from the Golestan Cohort Study. *EBioMedicine*, *53*, 102643. https://doi.org/10.1016/j.ebiom.2020.102643

Ivaska, J., Pallari, H.-M., Nevo, J., & Eriksson, J. E. (2007). Novel functions of vimentin in cell adhesion, migration, and signaling. *Experimental Cell Research*, *313*(10), 2050–2062. https://doi.org/10.1016/j.yexcr.2007.03.040

James, N. D., Hussain, S. A., Hall, E., Jenkins, P., Tremlett, J., Rawlings, C., Crundwell, M., Sizer, B., Sreenivasan, T., Hendron, C., Lewis, R., Waters, R., & Huddart, R. A. (2012). Radiotherapy with or without Chemotherapy in Muscle-Invasive Bladder Cancer. *New England Journal of Medicine*, *366*(16), 1477–1488. https://doi.org/10.1056/NEJMoa1106106

Jan, R., & Chaudhry, G.-E.-S. (2019). Understanding Apoptosis and Apoptotic Pathways Targeted Cancer Therapeutics. *Advanced Pharmaceutical Bulletin*, *9*(2), 205–218. https://doi.org/10.15171/apb.2019.024

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians*, *61*(2), 69–90. https://doi.org/10.3322/caac.20107

Jiang, B.-H., & Liu, L.-Z. (2009). PI3K/PTEN signaling in angiogenesis and tumorigenesis. *Advances in Cancer Research*, *102*, 19–65. https://doi.org/10.1016/S0065-230X(09)02002-8

Jie, M.-M., Chang, X., Zeng, S., Liu, C., Liao, G.-B., Wu, Y.-R., Liu, C.-H., Hu, C.-J., Yang, S.-M., & Li, X.-Z. (2019). Diverse regulatory manners of human telomerase reverse transcriptase. *Cell Communication and Signaling*, *17*(1), 63. https://doi.org/10.1186/s12964-019-0372-0

Joerger, A. C., & Fersht, A. R. (2008). Structural biology of the tumor suppressor p53. *Annual Review of Biochemistry*, *77*, 557–582. https://doi.org/10.1146/annurev.biochem.77.060806.091238

Joerger, A. C., & Fersht, A. R. (2010). The tumor suppressor p53: From structures to drug discovery. *Cold Spring Harbor Perspectives in Biology*, *2*(6), a000919. https://doi.org/10.1101/cshperspect.a000919

Jones, T. D., & Cheng, L. (2021). Histologic Grading of Bladder Tumors: Using Both the 1973 and 2004/2016 World Health Organization Systems in Combination Provides Valuable Information for Establishing Prognostic Risk Groups. *European Urology*, *79*(4), 489–491. https://doi.org/10.1016/j.eururo.2021.01.005

Jung, S., Yi, L., Kim, J., Jeong, D., Oh, T., Kim, C.-H., Kim, C.-J., Shin, J., An, S., & Lee, M.-S. (2011). The role of vimentin as a methylation biomarker for early diagnosis of cervical cancer. *Molecules and Cells*, *31*(5), 405–411. https://doi.org/10.1007/s10059-011-0229-x

Kachrilas, S., Dellis, A., Papatsoris, A., Avgeris, S., Anastasiou, D., Gavriil, A., Horti, M., Tseleni Balafouta, S., Livadas, K., Stravopodis, D., Alivizatos, G., Voutsinas, G., & Deliveliotis, C. (2019). PI3K/AKT pathway genetic alterations and dysregulation of expression in bladder cancer. *Journal of B.U.ON.: Official Journal of the Balkan Union of Oncology*, *24*(1), 329–337.

Kamaraj, B., & Bogaerts, A. (2015). Structure and Function of p53-DNA Complexes with Inactivation and Rescue Mutations: A Molecular Dynamics Simulation Study. *PloS One*, *10*(8), e0134638. https://doi.org/10.1371/journal.pone.0134638

Kamat, A. M., Dickstein, R. J., Messetti, F., Anderson, R., Pretzsch, S. M., Gonzalez, G. N., Katz, R. L., Khanna, A., Zaidi, T., Wu, X., Grossman, H. B., & Dinney, C. P. (2012). Use of Fluorescence In Situ Hybridization to Predict Response to Bacillus Calmette-Guérin Therapy for Bladder Cancer: Results of a Prospective Trial. *Journal of Urology*, *187*(3), 862–867. https://doi.org/10.1016/j.juro.2011.10.144

Kamoun, A., de Reyniès, A., Allory, Y., Sjödahl, G., Robertson, A. G., Seiler, R., ... & Weinstein, J. (2020). A consensus molecular classification of muscle-invasive bladder cancer. European urology, 77(4), 420-433.

Kandoth, C., McLellan, M. D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., McMichael, J. F., Wyczalkowski, M. A., Leiserson, M. D. M., Miller, C. A., Welch, J. S., Walter, M. J., Wendl, M. C., Ley, T. J., Wilson, R. K., Raphael, B. J., & Ding, L. (2013). Mutational landscape and significance across 12 major cancer types. *Nature*, *502*(7471), 333–339. https://doi.org/10.1038/nature12634

Kannan, K., Munirajan, A. K., Krishnamurthy, J., Bhuvarahamurthy, V., Mohanprasad, B. K., Panishankar, K. H., Tsuchida, N., & Shanmugam, G. (1999). Low incidence of p53 mutations in betel quid and tobacco chewing-associated oral squamous carcinoma from India. *International Journal of Oncology*. https://doi.org/10.3892/ijo.15.6.1133

Kaseb, H., & Aeddula, N. (2022). Bladder Cancer. In *Treasure Island (FL): StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK536923/

Kassouf, W., Traboulsi, S. L., Kulkarni, G. S., Breau, R. H., Zlotta, A., Fairey, A., So, A., Lacombe, L., Rendon, R., Aprikian, A. G., Siemens, D. R., Izawa, J. I., & Black, P. (2015). CUA guidelines on the management of non-muscle invasive bladder cancer. *Canadian Urological Association Journal*, *9*(9–10), 690. https://doi.org/10.5489/cuaj.3320

Kastenhuber, E. R., & Lowe, S. W. (2017). Putting p53 in Context. *Cell*, *170*(6), 1062–1078. https://doi.org/10.1016/j.cell.2017.08.028

Kaushal, S., & Khandakar, H. (2021). Molecular Classification of Bladder Cancer. In F. Ziglioli & U. Maestroni (Eds.), *Modern Approach to Diagnosis and Treatment of Bladder Cancer*. IntechOpen. https://doi.org/10.5772/intechopen.97393

Khan, Md. A., Chen, H., Zhang, D., & Fu, J. (2013). Twist: A molecular target in cancer therapeutics. *Tumor Biology*, *34*(5), 2497–2506. https://doi.org/10.1007/s13277-013-1002-x

Kim, H.-S., Heo, J.-I., Park, S.-H., Shin, J.-Y., Kang, H.-J., Kim, M.-J., Kim, S. C., Kim, J., Park, J.-B., & Lee, J.-Y. (2014). Transcriptional activation of p21(WAF^1/CIP^1) is mediated by increased DNA binding activity and increased interaction between p53 and Sp1 via phosphorylation during replicative senescence of human embryonic fibroblasts. *Molecular Biology Reports*, *41*(4), 2397–2408. https://doi.org/10.1007/s11033-014-3094-9

Kim, L. H. C., & Patel, M. I. (2020). Transurethral resection of bladder tumour (TURBT). *Translational Andrology and Urology*, *9*(6), 3056–3072. https://doi.org/10.21037/tau.2019.09.38

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., & Shay, J. W. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science (New York, N.Y.)*, *266*(5193), 2011–2015. https://doi.org/10.1126/science.7605428

Kitchen, M. O., Bryan, R. T., Emes, R. D., Glossop, J. R., Luscombe, C., Cheng, K. K., Zeegers, M. P., James, N. D., Devall, A. J., Mein, C. A., Gommersall, L., Fryer, A. A., & Farrell, W. E. (2016). Quantitative genome-wide methylation analysis of high-grade non-muscle invasive bladder cancer. Epigenetics, 11(3), 237–246. https://doi.org/10.1080/15592294.2016.1154246

Knowles, M. A., & Hurst, C. D. (2015). Molecular biology of bladder cancer: New insights into pathogenesis and clinical diversity. *Nature Reviews Cancer*, *15*(1), 25–41. https://doi.org/10.1038/nrc3817

Koch, A., Joosten, S. C., Feng, Z., de Ruijter, T. C., Draht, M. X., Melotte, V., Smits, K. M., Veeck, J., Herman, J. G., Van Neste, L., Van Criekinge, W., De Meyer, T., & van Engeland, M. (2018). Analysis of DNA methylation in cancer: Location revisited. *Nature Reviews Clinical Oncology*, *15*(7), 459–466. https://doi.org/10.1038/s41571-018-0004-4

Kohfeldt, E., Sasaki, T., Göhring, W., & Timpl, R. (1998). Nidogen-2: A new basement membrane protein with diverse binding properties. *Journal of Molecular Biology*, *282*(1), 99–109. https://doi.org/10.1006/jmbi.1998.2004

Kompier, L. C., Lurkin, I., van der Aa, M. N. M., van Rhijn, B. W. G., van der Kwast, T. H., & Zwarthoff, E. C. (2010b). FGFR3, HRAS, KRAS, NRAS and PIK3CA mutations in bladder cancer and their potential as biomarkers for surveillance and therapy. *PloS One*, *5*(11), e13821. https://doi.org/10.1371/journal.pone.0013821

Kostine, M., Marabelle, A., Schaeverbeke, T., & Kfoury, M. (2019). Les limites des inhibiteurs de points de contrôle immunitaire et la gestion de leur toxicité. *Médecine/Sciences*, *35*(12), 949–956. https://doi.org/10.1051/medsci/2019191

Kress, M., May, E., Cassingena, R., & May, P. (1979). Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. *Journal of Virology*, *31*(2), 472–483. https://doi.org/10.1128/JVI.31.2.472-483.1979

Lammers, R. J. M., Sylvester, R. J., Lee, C. T., & Witjes, J. A. (2013). NMIBC risk calculators: How useful are they for the practicing urologist and how can their clinical utility be improved? *The Urologic Clinics of North America*, *40*(2), 155–164. https://doi.org/10.1016/j.ucl.2013.01.002

Lang, G. A., Iwakuma, T., Suh, Y.-A., Liu, G., Rao, V. A., Parant, J. M., Valentin-Vega, Y. A., Terzian, T., Caldwell, L. C., Strong, L. C., El-Naggar, A. K., & Lozano, G. (2004). Gain of Function of a p53 Hot Spot Mutation in a Mouse Model of Li-Fraumeni Syndrome. *Cell*, *119*(6), 861–872. https://doi.org/10.1016/j.cell.2004.11.006

Larsen, L. K., Lind, G. E., Guldberg, P., & Dahl, C. (2019). DNA-Methylation-Based Detection of Urological Cancer in Urine: Overview of Biomarkers and Considerations on Biomarker Design, Source of DNA, and Detection Technologies. *International Journal of Molecular Sciences*, *20*(11), E2657. https://doi.org/10.3390/ijms20112657

Lavallee, E., Sfakianos, J. P., & Mulholland, D. J. (2021). Tumor Heterogeneity and Consequences for Bladder Cancer Treatment. Cancers, 13(21), 5297. https://doi.org/10.3390/cancers13215297

Leão, R., Apolónio, J. D., Lee, D., Figueiredo, A., Tabori, U., & Castelo-Branco, P. (2018). Mechanisms of human telomerase reverse transcriptase (hTERT) regulation: Clinical impacts in cancer. *Journal of Biomedical Science*, *25*(1), 22. https://doi.org/10.1186/s12929-018-0422-8

Lee, H.-H., & Kim, S. H. (2020). Review of non-invasive urinary biomarkers in bladder cancer. *Translational Cancer Research*, *9*(10), 6554–6564. https://doi.org/10.21037/tcr-20-1990

Lee, R., & Droller, M. J. (2000). THE NATURAL HISTORY OF BLADDER CANCER. *Urologic Clinics of North America*, *27*(1), 1–13. https://doi.org/10.1016/S0094-0143(05)70229-9

Lenis, A. T., Lec, P. M., Chamie, K., & Mshs, M. (2020). Bladder Cancer: A Review. *JAMA*, *324*(19), 1980. https://doi.org/10.1001/jama.2020.17598

Lenz, D. L., & Harpster, L. E. (2003). Urothelial carcinoma in a man with hereditary nonpolyposis colon cancer. *Reviews in Urology*, *5*(1), 49–53.

Li, H., Lu, H., Cui, W., Huang, Y., & Jin, X. (2021). A TP53-based immune prognostic model for muscle-invasive bladder cancer. *Aging*, *13*(2), 1929–1946. https://doi.org/10.18632/aging.202150

Li Hong-Tao., Duymich, C. E., Weisenberger, D. J., & Liang, G. (2016). Genetic and Epigenetic Alterations in Bladder Cancer. *International Neurourology Journal*, *20*(Suppl 2), S84-94. https://doi.org/10.5213/inj.1632752.376

Li Raymund, & Leslie, S. (2022). Cystitis. In *Treasure Island (FL): StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK482435/

Li, X.-L., Zhou, J., Chen, Z.-R., & Chng, W.-J. (2015). P53 mutations in colorectal cancer—Molecular pathogenesis and pharmacological reactivation. *World Journal of Gastroenterology*, *21*(1), 84–93. https://doi.org/10.3748/wjg.v21.i1.84

Li, Y., Sun, L., Guo, X., Mo, N., Zhang, J., & Li, C. (2021). Frontiers in Bladder Cancer Genomic Research. *Frontiers in Oncology*, *11*, 670729. https://doi.org/10.3389/fonc.2021.670729

Ling, X., & Arlinghaus, R. B. (2005). Knockdown of STAT3 Expression by RNA Interference Inhibits the Induction of Breast Tumors in Immunocompetent Mice. *Cancer Research*, *65*(7), 2532–2536. https://doi.org/10.1158/0008-5472.CAN-04-2425

Linn, J. F., Sesterhenn, I., Mostofi, F. K., & Schoenberg, M. (1998). THE MOLECULAR CHARACTERISTICS OF BLADDER CANCER IN YOUNG PATIENTS. *Journal of Urology*, *159*(5), 1493–1496. https://doi.org/10.1097/00005392-199805000-00022

Liu, J., Zhang, C., & Feng, Z. (2014). Tumor suppressor p53 and its gain-of-function mutants in cancer. *Acta Biochimica Et Biophysica Sinica*, *46*(3), 170–179. https://doi.org/10.1093/abbs/gmt144

Liu, Y., Snow, B. E., Hande, M. P., Baerlocher, G., Kickhoefer, V. A., Yeung, D., Wakeham, A., Itie, A., Siderovski, D. P., Lansdorp, P. M., Robinson, M. O., & Harrington, L. (2000). Telomerase-associated protein TEP1 is not essential for telomerase activity or telomere length maintenance in vivo. *Molecular and Cellular Biology*, *20*(21), 8178–8184. https://doi.org/10.1128/MCB.20.21.8178-8184.2000

Lobo, N., Shariat, S. F., Guo, C. C., Fernandez, M. I., Kassouf, W., Choudhury, A., Gao, J., Williams, S. B., Galsky, M. D., Taylor, J. A., Roupret, M., & Kamat, A. M. (2020). What Is the Significance of Variant Histology in Urothelial Carcinoma? *European Urology Focus*, *6*(4), 653–663. https://doi.org/10.1016/j.euf.2019.09.003

Lokeshwar, V. B., & Soloway, M. S. (2001). CURRENT BLADDER TUMOR TESTS: DOES THEIR PROJECTED UTILITY FULFILL CLINICAL NECESSITY? *Journal of Urology*, *165*(4), 1067–1077. https://doi.org/10.1016/S0022-5347(05)66428-2

Lopez-Beltran, A., Cimadamore, A., Blanca, A., Massari, F., Vau, N., Scarpelli, M., Cheng, L., & Montironi, R. (2021). Immune Checkpoint Inhibitors for the Treatment of Bladder Cancer. *Cancers*, *13*(1), E131. https://doi.org/10.3390/cancers13010131

López-Knowles, E., Hernández, S., Kogevinas, M., Lloreta, J., Amorós, A., Tardón, A., Carrato, A., Kishore, S., Serra, C., Malats, N., Real, F. X., & on behalf of the EPICURO Study Investigators. (2006). The p53 Pathway and Outcome among Patients with T1G3 Bladder Tumors. *Clinical Cancer Research*, *12*(20), 6029–6036. https://doi.org/10.1158/1078-0432.CCR-06-0206

Lotan, Y., & Roehrborn, C. G. (2003). Sensitivity and specificity of commonly available bladder tumor markers versus cytology: Results of a comprehensive literature review and meta-analyses. *Urology*, *61*(1), 109–118. https://doi.org/10.1016/S0090-4295(02)02136-2

Lowder, C. Y., Dhir, T., Goetz, A. B., Thomsett, H. L., Bender, J., Tatarian, T., Madhavan, S., Petricoin, E. F., Blais, E., Lavu, H., Winter, J. M., Posey, J., Brody, J. R., Pishvaian, M. J., & Yeo, C. J. (2020). A step towards personalizing next line therapy for resected pancreatic and related cancer patients: A single institution's experience. *Surgical Oncology*, *33*, 118–125. https://doi.org/10.1016/j.suronc.2020.02.003

Lu, D., Ni, Z., Liu, X., Feng, S., Dong, X., Shi, X., Zhai, J., Mai, S., Jiang, J., Wang, Z., Wu, H., & Cai, K. (2019). Beyond T Cells: Understanding the Role of PD-1/PD-L1 in Tumor-Associated Macrophages. *Journal of Immunology Research*, *2019*, 1–7. https://doi.org/10.1155/2019/1919082

Lukacz, E. S., Sampselle, C., Gray, M., MacDiarmid, S., Rosenberg, M., Ellsworth, P., & Palmer, M. H. (2011). A healthy bladder: A consensus statement: Consensus statement - a healthy bladder. *International Journal of Clinical Practice*, *65*(10), 1026–1036. https://doi.org/10.1111/j.1742-1241.2011.02763.x

Maeng, C. H., Lee, J., van Hummelen, P., Park, S. H., Palescandolo, E., Jang, J., Park, H. Y., Kang, S. Y., MacConaill, L., Kim, K.-M., & Shim, Y.-M. (2012). High-Throughput Genotyping in Metastatic Esophageal Squamous Cell Carcinoma Identifies Phosphoinositide-3-Kinase and BRAF Mutations. *PLoS ONE*, *7*(8), e41655. https://doi.org/10.1371/journal.pone.0041655

Mantica, G., Terrone, C., & Der Merwe, A. V. (2021). Bladder Cancer and Associated Risk Factors: The African Panorama. *European Urology*, *79*(5), 568–570. https://doi.org/10.1016/j.eururo.2020.11.041

Muraki, K., Nyhan, K., Han, L., & Murnane, J. P. (2012). Mechanisms of telomere loss and their consequences for chromosome instability. Frontiers in Oncology, 2. https://doi.org/10.3389/fonc.2012.00135

Marteijn, J. A., Lans, H., Vermeulen, W., & Hoeijmakers, J. H. J. (2014). Understanding nucleotide excision repair and its roles in cancer and ageing. *Nature Reviews. Molecular Cell Biology*, *15*(7), 465–481. https://doi.org/10.1038/nrm3822

Martin, A. C. R., Facchiano, A. M., Cuff, A. L., Hernandez-Boussard, T., Olivier, M., Hainaut, P., & Thornton, J. M. (2002). Integrating mutation data and structural analysis of the TP53 tumor-suppressor protein. *Human Mutation*, *19*(2), 149–164. https://doi.org/10.1002/humu.10032

Martínez, P., & Blasco, M. A. (2011). Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nature Reviews. Cancer*, *11*(3), 161–176. https://doi.org/10.1038/nrc3025

Masutomi, K., & Hahn, W. C. (2003). Telomerase and tumorigenesis. *Cancer Letters*, *194*(2), 163–172. https://doi.org/10.1016/s0304-3835(02)00703-6

Matau, A., & Roy, C. (2014). Tumeurs de la vessie et de l'urètre. In F. Boudghène, *IRM du pelvis de l'homme et de la femme* (pp. 93–111). Springer Paris. https://doi.org/10.1007/978-2-8178-0428-6_7

Matulewicz, R. S., & Steinberg, G. D. (2020). Non-muscle-invasive Bladder Cancer: Overview and Contemporary Treatment Landscape of Neoadjuvant Chemoablative Therapies. *Reviews in Urology*, *22*(2), 43–51.

McBride, O. W., Merry, D., & Givol, D. (1986). The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). *Proceedings of the National Academy of Sciences of the United States of America*, *83*(1), 130–134. https://doi.org/10.1073/pnas.83.1.130

McEachern, M. J., Krauskopf, A., & Blackburn, E. H. (2000). Telomeres and their control. *Annual Review of Genetics*, *34*, 331–358. https://doi.org/10.1146/annurev.genet.34.1.331

Menon, V., & Povirk, L. (2014). Involvement of p53 in the repair of DNA double strand breaks: Multifaceted Roles of p53 in homologous recombination repair (HRR) and non-homologous end joining (NHEJ). *Sub-Cellular Biochemistry*, *85*, 321–336. https://doi.org/10.1007/978-94-017-9211-0_17

Miyamoto, H., Miller, J. S., Fajardo, D. A., Lee, T. K., Netto, G. J., & Epstein, J. I. (2010). Non-invasive papillary urothelial neoplasms: The 2004 WHO/ISUP classification system. *Pathology International*, *60*(1), 1–8. https://doi.org/10.1111/j.1440-1827.2009.02477.x

Mizukoshi, E., & Kaneko, S. (2019). Telomerase-Targeted Cancer Immunotherapy. *International Journal of Molecular Sciences*, *20*(8), 1823. https://doi.org/10.3390/ijms20081823

Mokkapati, S., Bechtel, M., Reibetanz, M., Miosge, N., & Nischt, R. (2012). Absence of the basement membrane component nidogen 2, but not of nidogen 1, results in increased lung metastasis in mice. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*, *60*(4), 280–289. https://doi.org/10.1369/0022155412436586

Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *38*(1), 23–38. https://doi.org/10.1038/npp.2012.112

Morgan, T., Koreckij, T., & Corey, E. (2009). Targeted Therapy for Advanced Prostate Cancer: Inhibition of the PI3K/Akt/mTOR Pathway. *Current Cancer Drug Targets*, *9*(2), 237–249. https://doi.org/10.2174/156800909787580999

Mostofi, F. K., Davis, C. J., Sesterhenn, I. A., & Sobin, L. H. (1999). *Histological Typing of Urinary Bladder Tumours*. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-59871-5

Mowatt, G., Zhu, S., Kilonzo, M., Boachie, C., Fraser, C., Griffiths, T., N'Dow, J., Nabi, G., Cook, J., & Vale, L. (2010). Systematic review of the clinical effectiveness and cost-effectiveness of photodynamic diagnosis and urine biomarkers (FISH, ImmunoCyt, NMP22) and cytology for the detection and follow-up of bladder cancer. *Health Technology Assessment*, *14*(4). https://doi.org/10.3310/hta14040

Mrakovcic, M., & Fröhlich, L. (2018). P53-Mediated Molecular Control of Autophagy in Tumor Cells. Biomolecules, 8(2), 14. https://doi.org/10.3390/biom8020014

Mukhtar, S., & Perry, M. J. A. (2011). FUTURE PROSPECTS FOR BLADDER CANCER BIOMARKERS: COMMENTS. *BJU International*, *108*(10), 1541–1543. https://doi.org/10.1111/j.1464-410X.2011.10642.x

Muller, M., Bhattacharya, S. S., Moore, T., Prescott, Q., Wedig, T., Herrmann, H., & Magin, T. M. (2009). Dominant cataract formation in association with a vimentin assembly disrupting mutation. *Human Molecular Genetics*, *18*(6), 1052–1057. https://doi.org/10.1093/hmg/ddn440

Muller, P. A. J., & Vousden, K. H. (2013). P53 mutations in cancer. *Nature Cell Biology*, *15*(1), 2–8. https://doi.org/10.1038/ncb2641

Muller, P. A. J., & Vousden, K. H. (2014). Mutant p53 in cancer: New functions and therapeutic opportunities. *Cancer Cell*, *25*(3), 304–317. https://doi.org/10.1016/j.ccr.2014.01.021

Murez, T., Savoie, P.-H., Fléchon, A., Rocher, L., Camparo, P., Morel-Journel, N., Ferretti, L., & Méjean, A. (2020). [French ccAFU guidelines - update 2020-2022: Retroperitoneal sarcoma]. *Progres En Urologie: Journal De l'Association Francaise D'urologie Et De La Societe Francaise D'urologie*, *30*(12S), S314–S330. https://doi.org/10.1016/S1166-7087(20)30755-7

Neuzillet, Y., & Lebret, T. (2010). Les vraies contre-indications du BCG dans le traitement des tumeurs de vessie. *Progrès en Urologie*, *20*, S41–S45. https://doi.org/10.1016/S1166-7087(10)70025-7

Niedworok, C., & Gratzke, C. (2015). [Alternatives to cystectomy]. *Der Urologe. Ausg. A*, *54*(4), 504–509. https://doi.org/10.1007/s00120-015-3775-6

Offer, H., Wolkowicz, R., Matas, D., Blumenstein, S., Livneh, Z., & Rotter, V. (1999). Direct involvement of p53 in the base excision repair pathway of the DNA repair machinery. *FEBS Letters*, *450*(3), 197–204. https://doi.org/10.1016/S0014-5793(99)00505-0

Oganesian, L., & Karlseder, J. (2009). Telomeric armor: The layers of end protection. *Journal of Cell Science*, *122*(Pt 22), 4013–4025. https://doi.org/10.1242/jcs.050567

Okorokov, A. L., & Orlova, E. V. (2009). Structural biology of the p53 tumour suppressor. *Current Opinion in Structural Biology*, *19*(2), 197–202. https://doi.org/10.1016/j.sbi.2009.02.003

Olszewski, M., Pruszko, M., Snaar-Jagalska, E., Zylicz, A., & Zylicz, M. (2019). Diverse and cancer type-specific roles of the p53 R248Q gain-of-function mutation in cancer migration and invasiveness. *International Journal of Oncology*. https://doi.org/10.3892/ijo.2019.4723

Ozaki, T., & Nakagawara, A. (2011). Role of p53 in Cell Death and Human Cancers. *Cancers*, *3*(1), 994–1013. https://doi.org/10.3390/cancers3010994

Parry, D. A. D. (2006). Hendecad repeat in segment 2A and linker L2 of intermediate filament chains implies the possibility of a right-handed coiled-coil structure. *Journal of Structural Biology*, *155*(2), 370–374. https://doi.org/10.1016/j.jsb.2006.03.017

Passoni, N., Gayed, B., Kapur, P., Sagalowsky, A. I., Shariat, S. F., & Lotan, Y. (2016). Cell-cycle markers do not improve discrimination of EORTC and CUETO risk models in predicting recurrence and progression of non–muscle-invasive high-grade bladder cancer. *Urologic Oncology: Seminars and Original Investigations*, *34*(11), 485.e7-485.e14. https://doi.org/10.1016/j.urolonc.2016.05.014

Pei, H., Li, Y., Liu, M., & Chen, Y. (2017). Targeting Twist expression with small molecules. *MedChemComm*, *8*(2), 268–275. https://doi.org/10.1039/C6MD00561F

Penzo, M., Ludovini, V., Treré, D., Siggillino, A., Vannucci, J., Bellezza, G., Crinò, L., & Montanaro, L. (2015). Dyskerin and TERC expression may condition survival in lung cancer patients. *Oncotarget*, *6*(25), 21755–21760. https://doi.org/10.18632/oncotarget.4580

Perera, T. P. S., Jovcheva, E., Mevellec, L., Vialard, J., De Lange, D., Verhulst, T., Paulussen, C., Van De Ven, K., King, P., Freyne, E., Rees, D. C., Squires, M., Saxty, G., Page, M., Murray, C. W., Gilissen, R., Ward, G., Thompson, N. T., Newell, D. R., … Lorenzi, M. V. (2017). Discovery and Pharmacological Characterization of JNJ-42756493 (Erdafitinib), a Functionally Selective Small-Molecule FGFR Family Inhibitor. *Molecular Cancer Therapeutics*, *16*(6), 1010–1020. https://doi.org/10.1158/1535-7163.MCT-16-0589

Perreau, J., Lilienbaum, A., Vasseur, M., & Paulin, D. (1988). Nucleotide sequence of the human vimentin gene and regulation of its transcription in tissues and cultured cells. *Gene*, *62*(1), 7–16. https://doi.org/10.1016/0378-1119(88)90575-6

Pestana, A., Vinagre, J., Sobrinho-Simões, M., & Soares, P. (2017). TERT biology and function in cancer: Beyond immortalisation. *Journal of Molecular Endocrinology*, *58*(2), R129–R146. https://doi.org/10.1530/JME-16-0195

Piccart-Gebhart, M. J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I., Gianni, L., Baselga, J., Bell, R., Jackisch, C., Cameron, D., Dowsett, M., Barrios, C. H., Steger, G., Huang, C.-S., Andersson, M., Inbar, M., Lichinitser, M., Láng, I., … Gelber, R. D. (2005). Trastuzumab after Adjuvant Chemotherapy in HER2-Positive Breast Cancer. *New England Journal of Medicine*, *353*(16), 1659–1672. https://doi.org/10.1056/NEJMoa052306

Pike, A. M., Strong, M. A., Ouyang, J. P. T., & Greider, C. W. (2019). TIN2 Functions with TPP1/POT1 To Stimulate Telomerase Processivity. *Molecular and Cellular Biology*, *39*(21), e00593-18. https://doi.org/10.1128/MCB.00593-18

Plna, K., & Hemminki, K. (2001). Familial bladder cancer in the National Swedish Family Cancer Database. *The Journal of Urology*, *166*(6), 2129–2133.

Porten, S. P. (2018). Epigenetic Alterations in Bladder Cancer. *Current Urology Reports*, *19*(12), 102. https://doi.org/10.1007/s11934-018-0861-5

Puisieux, A., Valsesia-Wittmann, S., & Ansieau, S. (2006). A twist for survival and cancer progression. *British Journal of Cancer*, *94*(1), 13–17. https://doi.org/10.1038/sj.bjc.6602876

Puzio-Kuter, A. M., Castillo-Martin, M., Kinkade, C. W., Wang, X., Shen, T. H., Matos, T., Shen, M. M., Cordon-Cardo, C., & Abate-Shen, C. (2009). Inactivation of p53 and Pten promotes invasive bladder cancer. *Genes & Development*, *23*(6), 675–680. https://doi.org/10.1101/gad.1772909

Qin, Q., Xu, Y., He, T., Qin, C., & Xu, J. (2012). Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. *Cell Research*, *22*(1), 90–106. https://doi.org/10.1038/cr.2011.144

Quax, W., Egberts, W. V., Hendriks, W., Quax-Jeuken, Y., & Bloemendal, H. (1983). The structure of the vimentin gene. *Cell*, *35*(1), 215–223. https://doi.org/10.1016/0092-8674(83)90224-6

Raitanen, M. P., Leppilahti, M., Tuhkanen, K., Forssel, T., Nylund, P., Tammela, T., & FinnBladder Group. (2001). Routine follow-up cystoscopy in detection of recurrence in patients being monitored for bladder cancer. *Annales Chirurgiae Et Gynaecologiae*, *90*(4), 261–265.

Raitanen, M.-P., Aine, R., Rintala, E., Kallio, J., Rajala, P., Juusela, H., Tammela, T. L. J., & FinnBladder Group. (2002). Differences between local and review urinary cytology in diagnosis of bladder cancer. An interobserver multicenter analysis. *European Urology*, *41*(3), 284–289. https://doi.org/10.1016/s0302-2838(02)00006-4

Raj, N., & Attardi, L. D. (2017). The Transactivation Domains of the p53 Protein. *Cold Spring Harbor Perspectives in Medicine*, *7*(1), a026047. https://doi.org/10.1101/cshperspect.a026047

Rambau, P. F., Chalya, P. L., & Jackson, K. (2013). Schistosomiasis and urinary bladder cancer in North Western Tanzania: A retrospective review of 185 patients. *Infectious Agents and Cancer*, *8*(1), 19. https://doi.org/10.1186/1750-9378-8-19

Ravi, K., Kumar, T., Bakshi, H., Desai, J., Sen, S., & Yadav, V. (2013). Non urothelial bladder cancers: A case series. *Indian Journal of Surgical Oncology*, *4*(1), 2–8. https://doi.org/10.1007/s13193-012-0176-9

Reinert, T., Borre, M., Christiansen, A., Hermann, G. G., Ørntoft, T. F., & Dyrskjøt, L. (2012). Diagnosis of Bladder Cancer Recurrence Based on Urinary Levels of EOMES, HOXA9, POU4F2, TWIST1, VIM, and ZNF154 Hypermethylation. *PLoS ONE*, *7*(10), e46297. https://doi.org/10.1371/journal.pone.0046297

Reisman, D., Greenberg, M., & Rotter, V. (1988). Human p53 oncogene contains one promoter upstream of exon 1 and a second, stronger promoter within intron 1. *Proceedings of the National Academy of Sciences of the United States of America*, *85*(14), 5146–5150. https://doi.org/10.1073/pnas.85.14.5146

Renard, I., Joniau, S., van Cleynenbreugel, B., Collette, C., Naômé, C., Vlassenbroeck, I., Nicolas, H., de Leval, J., Straub, J., Van Criekinge, W., Hamida, W., Hellel, M., Thomas, A., de Leval, L.,

Bierau, K., & Waltregny, D. (2010). Identification and Validation of the Methylated TWIST1 and NID2 Genes through Real-Time Methylation-Specific Polymerase Chain Reaction Assays for the Noninvasive Detection of Primary Bladder Cancer in Urine Samples. *European Urology*, *58*(1), 96–104. https://doi.org/10.1016/j.eururo.2009.07.041

Rentsch, C. A., Müller, D. C., Ruiz, C., & Bubendorf, L. (2017). Comprehensive Molecular Characterization of Urothelial Bladder Carcinoma: A Step Closer to Clinical Translation? *European Urology*, *72*(6), 960–961. https://doi.org/10.1016/j.eururo.2017.06.022

Rizzo, A., Mollica, V., Cimadamore, A., Santoni, M., Scarpelli, M., Schiavina, R., Cheng, L., Lopez-Beltran, A., Brunocilla, E., Montironi, R., & Massari, F. (2021). TNM staging towards a personalized approach in metastatic urothelial carcinoma: What will the future be like?—a narrative review. *Translational Andrology and Urology*, *10*(3), 1541–1552. https://doi.org/10.21037/tau-20-1109

Rödel, C. (2004). Current status of radiation therapy and combined-modality treatment for bladder cancer. *Strahlentherapie Und Onkologie: Organ Der Deutschen Rontgengesellschaft ... [et Al]*, *180*(11), 701–709. https://doi.org/10.1007/s00066-004-9195-y

Rodriguez Pena, M. D. C., Springer, S. U., Taheri, D., Li, L., Tregnago, A. C., Eich, M.-L., Eltoum, I.-E. A., VandenBussche, C. J., Papadopoulos, N., Kinzler, K. W., Vogelstein, B., & Netto, G. J. (2020). Performance of novel non-invasive urine assay UroSEEK in cohorts of equivocal urine cytology. *Virchows Archiv*, *476*(3), 423–429. https://doi.org/10.1007/s00428-019-02654-1

Rouprêt, M., Pignot, G., Masson-Lecomte, A., Compérat, E., Audenet, F., Roumiguié, M., Houédé, N., Larré, S., Brunelle, S., Xylinas, E., Neuzillet, Y., & Méjean, A. (2020). [French ccAFU guidelines - update 2020-2022: Bladder cancer]. *Progres En Urologie: Journal De l'Association Francaise D'urologie Et De La Societe Francaise D'urologie*, *30*(12S), S78–S135. https://doi.org/10.1016/S1166-7087(20)30751-X

Ru, Yuanbin., Dancik, G. M., & Theodorescu, D. (2011). Biomarkers for prognosis and treatment selection in advanced bladder cancer patients. *Current Opinion in Urology*, *21*(5), 420–427. https://doi.org/10.1097/MOU.0b013e32834956d6

Russell, C. M., Lebastchi, A. H., Borza, T., Spratt, D. E., & Morgan, T. M. (2016). The Role of Transurethral Resection in Trimodal Therapy for Muscle-Invasive Bladder Cancer. *Bladder Cancer*, *2*(4), 381–394. https://doi.org/10.3233/BLC-160076

Saginala, K., Barsouk, A., Aluru, J. S., Rawla, P., Padala, S. A., & Barsouk, A. (2020). Epidemiology of Bladder Cancer. *Medical Sciences*, *8*(1), 15. https://doi.org/10.3390/medsci8010015

Saleem, M., & Hamawy, K. (2022). Hematuria. In *Treasure Island (FL): StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK534213/

Sarris, E., Saif, M., & Syrigos, K. (2012). The Biological Role of PI3K Pathway in Lung Cancer. *Pharmaceuticals*, *5*(11), 1236–1264. https://doi.org/10.3390/ph5111236

Satelli, A., & Li, S. (2011). Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cellular and Molecular Life Sciences: CMLS*, *68*(18), 3033–3046. https://doi.org/10.1007/s00018-011-0735-1

Satyal, U., Sikder, R. K., McConkey, D., Plimack, E. R., & Abbosh, P. H. (2019). Clinical implications of molecular subtyping in bladder cancer. *Current Opinion in Urology*, *29*(4), 350–356. https://doi.org/10.1097/MOU.0000000000000641

Schlichtholz, B. (2004). Clinical implications of p53 mutation analysis in bladder cancer tissue and urine sediment by functional assay in yeast. *Carcinogenesis*, *25*(12), 2319–2323. https://doi.org/10.1093/carcin/bgh256

Schmitz-Dräger, C., Bonberg, N., Pesch, B., Todenhöfer, T., Sahin, S., Behrens, T., Brüning, T., & Schmitz-Dräger, B. J. (2016). Replacing cystoscopy by urine markers in the follow-up of patients with low-risk non–muscle-invasive bladder cancer?—An International Bladder Cancer

Network project. *Urologic Oncology: Seminars and Original Investigations*, *34*(10), 452–459. https://doi.org/10.1016/j.urolonc.2016.06.001

Shang, D., Liu, Y., Matsui, Y., Ito, N., Nishiyama, H., Kamoto, T., & Ogawa, O. (2008). Demethylating Agent 5-Aza-2′-Deoxycytidine Enhances Susceptibility of Bladder Transitional Cell Carcinoma to Cisplatin. *Urology*, *71*(6), 1220–1225. https://doi.org/10.1016/j.urology.2007.11.029

Shariat, S. F., Canto, E. I., Kattan, M. W., & Slawin, K. M. (2004). Beyond prostate-specific antigen: New serologic biomarkers for improved diagnosis and management of prostate cancer. *Reviews in Urology*, *6*(2), 58–72.

Shariat, S. F., Sfakianos, J. P., Droller, M. J., Karakiewicz, P. I., Meryn, S., & Bochner, B. H. (2010). The effect of age and gender on bladder cancer: A critical review of the literature. *BJU International*, *105*(3), 300–308. https://doi.org/10.1111/j.1464-410X.2009.09076.x

Shermadou, E. S., Rahman, S., & Leslie, S. W. (2022). Anatomy, Abdomen and Pelvis, Bladder. In *Treasure Island (FL): StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK531465/

Shirahata, A., Sakata, M., Sakuraba, K., Goto, T., Mizukami, H., Saito, M., Ishibashi, K., Kigawa, G., Nemoto, H., Sanada, Y., & Hibi, K. (2009). Vimentin methylation as a marker for advanced colorectal carcinoma. *Anticancer Research*, *29*(1), 279–281.

Shull, A. Y., Latham-Schwark, A., Ramasamy, P., Leskoske, K., Oroian, D., Birtwistle, M. R., & Buckhaults, P. J. (2012). Novel somatic mutations to PI3K pathway genes in metastatic melanoma. *PloS One*, *7*(8), e43369. https://doi.org/10.1371/journal.pone.0043369

Sidransky, D., Von Eschenbach, A., Tsai, Y., Jones, P., Summerhayes, I., Marshall, F., Paul, M., Green, P., Hamilton, Frost, P., & et, al. (1991). Identification of p53 gene mutations in bladder cancers and urine samples. *Science*, *252*(5006), 706–709. https://doi.org/10.1126/science.2024123

Siroky, M. B. (2004). The aging bladder. *Reviews in Urology*, *6 Suppl 1*, S3-7.

Sjödahl, G., Lauss, M., Lövgren, K., Chebil, G., Gudjonsson, S., Veerla, S., Patschan, O., Aine, M., Fernö, M., Ringnér, M., Månsson, W., Liedberg, F., Lindgren, D., & Höglund, M. (2012). A molecular taxonomy for urothelial carcinoma. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *18*(12), 3377–3386. https://doi.org/10.1158/1078-0432.CCR-12-0077-T

Smal, M. P., Rolevich, A. I., Polyakov, S. L., Krasny, S. A., & Goncharova, R. I. (2014). FGFR3 and TP53 mutations in a prospective cohort of Belarusian bladder cancer patients. *Experimental Oncology*, *36*(4), 246–251.

Smeby, J., Sveen, A., Eilertsen, I. A., Danielsen, S. A., Hoff, A. M., Eide, P. W., Johannessen, B., Hektoen, M., Skotheim, R. I., Guren, M. G., Nesbakken, A., & Lothe, R. A. (2019). Transcriptional and functional consequences of TP53 splice mutations in colorectal cancer. *Oncogenesis*, *8*(6), 35. https://doi.org/10.1038/s41389-019-0141-3

Sobin, L. H. (1974). Histological Classification of Tumours. In E. Grundmann (Ed.), *Geschwülste · Tumors I: Vol. 6 / 5* (pp. 327–350). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-65895-2_4

Soder, A. I., Hoare, S. F., Muir, S., Going, J. J., Parkinson, E. K., & Keith, W. N. (1997). Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer. *Oncogene*, *14*(9), 1013–1021. https://doi.org/10.1038/sj.onc.1201066

Song, S., Vuai, M. S., & Zhong, M. (2018). The role of bacteria in cancer therapy—Enemies in the past, but allies at present. *Infectious Agents and Cancer*, *13*, 9. https://doi.org/10.1186/s13027-018-0180-y

Spring, J., Yanze, N., Middel, A. M., Stierwald, M., Gröger, H., & Schmid, V. (2000). The Mesoderm Specification Factor Twist in the Life Cycle of Jellyfish. *Developmental Biology*, *228*(2), 363–375. https://doi.org/10.1006/dbio.2000.9956

Springer, S. U., Chen, C.-H., Rodriguez Pena, M. D. C., Li, L., Douville, C., Wang, Y., Cohen, J. D., Taheri, D., Silliman, N., Schaefer, J., Ptak, J., Dobbyn, L., Papoli, M., Kinde, I., Afsari, B., Tregnago, A. C., Bezerra, S. M., VandenBussche, C., Fujita, K., … Netto, G. J. (2018). Non-invasive detection of urothelial cancer through the analysis of driver gene mutations and aneuploidy. *ELife*, *7*, e32143. https://doi.org/10.7554/eLife.32143

Srivastava, P., Jaiswal, P. K., Singh, V., & Mittal, R. D. (2011). Role of p53 gene polymorphism and bladder cancer predisposition in northern India. *Cancer Biomarkers*, *8*(1), 21–28. https://doi.org/10.3233/DMA-2011-0816

Staal, S. P., Huebner, K., Croce, C. M., Parsa, N. Z., & Testa, J. R. (1988). The AKT1 proto-oncogene maps to human chromosome 14, band q32. *Genomics*, *2*(1), 96–98. https://doi.org/10.1016/0888-7543(88)90114-0

Sugeeta, S. S., Sharma, A., Ng, K., Nayak, A., & Vasdev, N. (2021). Biomarkers in Bladder Cancer Surveillance. *Frontiers in Surgery*, *8*, 735868. https://doi.org/10.3389/fsurg.2021.735868

Sui, X., Kong, N., Wang, Z., & Pan, H. (2013). Epigenetic regulation of the human telomerase reverse transciptase gene: A potential therapeutic target for the treatment of leukemia (Review). *Oncology Letters*, *6*(2), 317–322. https://doi.org/10.3892/ol.2013.1367

Sundquist, W. I., & Klug, A. (1989). Telomeric DNA dimerizes by formation of guanine tetrads between hairpin loops. *Nature*, *342*(6251), 825–829. https://doi.org/10.1038/342825a0

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209–249. https://doi.org/10.3322/caac.21660

Sweeney, P., Kursh, E. D., & Resnick, M. I. (1992). Partial cystectomy. *The Urologic Clinics of North America*, *19*(4), 701–711.

Sylvester, R. J., Rodríguez, O., Hernández, V., Turturica, D., Bauerová, L., Bruins, H. M., Bründl, J., van der Kwast, T. H., Brisuda, A., Rubio-Briones, J., Seles, M., Hentschel, A. E., Kusuma, V. R. M., Huebner, N., Cotte, J., Mertens, L. S., Volanis, D., Cussenot, O., Subiela Henríquez, J. D., … van Rhijn, B. W. G. (2021). European Association of Urology (EAU) Prognostic Factor Risk Groups for Non–muscle-invasive Bladder Cancer (NMIBC) Incorporating the WHO 2004/2016 and WHO 1973 Classification Systems for Grade: An Update from the EAU NMIBC Guidelines Panel. *European Urology*, *79*(4), 480–488. https://doi.org/10.1016/j.eururo.2020.12.033

Sylvester, R. J., van der Meijden, A. P. M., Oosterlinck, W., Witjes, J. A., Bouffioux, C., Denis, L., Newling, D. W. W., & Kurth, K. (2006). Predicting Recurrence and Progression in Individual Patients with Stage Ta T1 Bladder Cancer Using EORTC Risk Tables: A Combined Analysis of 2596 Patients from Seven EORTC Trials. *European Urology*, *49*(3), 466–477. https://doi.org/10.1016/j.eururo.2005.12.031

Tazi, M., Er-Raki, A., & Benjaafar, N. (2013). Cancer incidence in Rabat, Morocco: 2006-2008. *Ecancermedicalscience*, 7:338.

Têtu, B. (2009). Diagnosis of urothelial carcinoma from urine. *Modern Pathology*, *22*(S2), S53–S59. https://doi.org/10.1038/modpathol.2008.193

Thanasoula, M., Escandell, J. M., Suwaki, N., & Tarsounas, M. (2012). ATM/ATR checkpoint activation downregulates CDC25C to prevent mitotic entry with uncapped telomeres. *The EMBO Journal*, *31*(16), 3398–3410. https://doi.org/10.1038/emboj.2012.191

The Cancer Genome Atlas Research Network. (2014). Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature*, *507*(7492), 315–322. https://doi.org/10.1038/nature12965

The Oxford-Illumina WGS500 Consortium, Cazier, J.-B., Rao, S. R., McLean, C. M., Walker, A. K., Wright, B. J., Jaeger, E. E. M., Kartsonaki, C., Marsden, L., Yau, C., Camps, C., Kaisaki, P., Taylor, J., Catto, J. W., Tomlinson, I. P. M., Kiltie, A. E., & Hamdy, F. C. (2014). Whole-genome sequencing of bladder cancers reveals somatic CDKN1A mutations and clinicopathological associations with mutation burden. *Nature Communications*, *5*(1), 3756. https://doi.org/10.1038/ncomms4756

Thiery, J. P. (2002). Epithelial–mesenchymal transitions in tumour progression. *Nature Reviews Cancer*, *2*(6), 442–454. https://doi.org/10.1038/nrc822

Thun, M. J., Lally, C. A., Calle, E. E., Heath, C. W., Flannery, J. T., & Flanders, W. D. (1997). Cigarette Smoking and Changes in the Histopathology of Lung Cancer. *JNCI Journal of the National Cancer Institute*, *89*(21), 1580–1586. https://doi.org/10.1093/jnci/89.21.1580

Tran, L., & Puckett, Y. (2022). Urinary Incontinence. In *Treasure Island (FL): StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK559095/

Turati, F., Bosetti, C., Polesel, J., Serraino, D., Montella, M., Libra, M., Facchini, G., Ferraroni, M., Tavani, A., La Vecchia, C., & Negri, E. (2017). Family history of cancer and the risk of bladder cancer: A case–control study from Italy. Cancer Epidemiology, 48, 29–35. https://doi.org/10.1016/j.canep.2017.03.003

Ulazzi, L., Sabbioni, S., Miotto, E., Veronese, A., Angusti, A., Gafà, R., Manfredini, S., Farinati, F., Sasaki, T., Lanza, G., & Negrini, M. (2007). Nidogen 1 and 2 gene promoters are aberrantly methylated in human gastrointestinal cancer. *Molecular Cancer*, *6*(1), 17. https://doi.org/10.1186/1476-4598-6-17

Urakami, S., Shiina, H., Enokida, H., Hirata, H., Kawamoto, K., Kawakami, T., Kikuno, N., Tanaka, Y., Majid, S., Nakagawa, M., Igawa, M., & Dahiya, R. (2006). Wnt Antagonist Family Genes as Biomarkers for Diagnosis, Staging, and Prognosis of Renal Cell Carcinoma Using Tumor and Serum DNA. *Clinical Cancer Research*, *12*(23), 6989–6997. https://doi.org/10.1158/1078-0432.CCR-06-1194

Vafaei, S., Zekiy, A. O., Khanamir, R. A., Zaman, B. A., Ghayourvahdat, A., Azimizonuzi, H., & Zamani, M. (2022). Combination therapy with immune checkpoint inhibitors (ICIs); a new frontier. Cancer Cell International, 22(1), 2. https://doi.org/10.1186/s12935-021-02407-8

Van Cutsem, E., Köhne, C.-H., Hitre, E., Zaluski, J., Chang Chien, C.-R., Makhson, A., D'Haens, G., Pintér, T., Lim, R., Bodoky, G., Roh, J. K., Folprecht, G., Ruff, P., Stroh, C., Tejpar, S., Schlichting, M., Nippgen, J., & Rougier, P. (2009). Cetuximab and Chemotherapy as Initial Treatment for Metastatic Colorectal Cancer. *New England Journal of Medicine*, *360*(14), 1408–1417. https://doi.org/10.1056/NEJMoa0805019

Van der Auwera, I., Bovie, C., Svensson, C., Trinh, X. B., Limame, R., van Dam, P., van Laere, S. J., van Marck, E. A., Dirix, L. Y., & Vermeulen, P. B. (2010). Quantitative methylation profiling in tumor and matched morphologically normal tissues from breast cancer patients. *BMC Cancer*, *10*, 97.

van Kessel, K. E. M., Beukers, W., Lurkin, I., Ziel-van der Made, A., van der Keur, K. A., Boormans, J. L., Dyrskjøt, L., Márquez, M., Ørntoft, T. F., Real, F. X., Segersten, U., Malats, N., Malmström, P.-U., Van Criekinge, W., & Zwarthoff, E. C. (2017). Validation of a DNA Methylation-Mutation Urine Assay to Select Patients with Hematuria for Cystoscopy. *Journal of Urology*, *197*(3 Part 1), 590–595. https://doi.org/10.1016/j.juro.2016.09.118

van Rhijn, B. W. G., Burger, M., Lotan, Y., Solsona, E., Stief, C. G., Sylvester, R. J., Witjes, J. A., & Zlotta, A. R. (2009). Recurrence and Progression of Disease in Non–Muscle-Invasive Bladder Cancer: From Epidemiology to Treatment Strategy. *European Urology*, *56*(3), 430–442. https://doi.org/10.1016/j.eururo.2009.06.028

van Rhijn, B. W. G., Hentschel, A. E., Bründl, J., Compérat, E. M., Hernández, V., Čapoun, O., Bruins, H. M., Cohen, D., Rouprêt, M., Shariat, S. F., Mostafid, A. H., Zigeuner, R., Dominguez-Escrig, J. L., Burger, M., Soukup, V., Gontero, P., Palou, J., van der Kwast, T. H., Babjuk, M., … Multicenter EAU Non-Muscle-Invasive Bladder Cancer Guidelines Panel Study Consortium on the WHO1973 WHO 2004 2016 Classification Systems for Grade. (2021). Prognostic Value of the WHO1973 and WHO2004/2016 Classification Systems for Grade in Primary Ta/T1 Non-muscle-invasive Bladder Cancer: A Multicenter European Association of Urology Non-muscle-invasive Bladder Cancer Guidelines Panel Study. *European Urology Oncology*, *4*(2), 182–191. https://doi.org/10.1016/j.euo.2020.12.002

van Rhijn, B. W. G., Zuiverloon, T. C. M., Vis, A. N., Radvanyi, F., van Leenders, G. J. L. H., Ooms, B. C. M., Kirkels, W. J., Lockwood, G. A., Boevé, E. R., Jöbsis, A. C., Zwarthoff, E. C., & van der Kwast, T. H. (2010). Molecular Grade (FGFR3/MIB-1) and EORTC Risk Scores Are Predictive in Primary Non–Muscle-Invasive Bladder Cancer. *European Urology*, *58*(3), 433–441. https://doi.org/10.1016/j.eururo.2010.05.043

Végran, F., Rebucci, M., Chevrier, S., Cadouot, M., Boidot, R., & Lizard-Nacol, S. (2013). Only missense mutations affecting the DNA binding domain of p53 influence outcomes in patients with breast carcinoma. *PloS One*, *8*(1), e55103. https://doi.org/10.1371/journal.pone.0055103

Vinagre, J., Almeida, A., Pópulo, H., Batista, R., Lyra, J., Pinto, V., Coelho, R., Celestino, R., Prazeres, H., Lima, L., Melo, M., da Rocha, A. G., Preto, A., Castro, P., Castro, L., Pardal, F., Lopes, J. M., Santos, L. L., Reis, R. M., … Soares, P. (2013). Frequency of TERT promoter mutations in human cancers. *Nature Communications*, *4*, 2185. https://doi.org/10.1038/ncomms3185

Vonderheide, R. H. (2002). Telomerase as a universal tumor-associated antigen for cancer immunotherapy. *Oncogene*, *21*(4), 674–679. https://doi.org/10.1038/sj.onc.1205074

Wallace, K. M., & Drake, M. J. (2015). Overactive bladder. *F1000Research*, *4*, 1406. https://doi.org/10.12688/f1000research.7131.1

Wasserman, J. D., Novokmet, A., Eichler-Jonsson, C., Ribeiro, R. C., Rodriguez-Galindo, C., Zambetti, G. P., & Malkin, D. (2015). Prevalence and Functional Consequence of *TP53* Mutations in Pediatric Adrenocortical Carcinoma: A Children's Oncology Group Study. *Journal of Clinical Oncology*, *33*(6), 602–609. https://doi.org/10.1200/JCO.2013.52.6863

Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichtsteiner, S., Kim, N. W., Trager, J. B., Taylor, R. D., Carlos, R., Andrews, W. H., Wright, W. E., Shay, J. W., Harley, C. B., & Morin, G. B. (1997). Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nature Genetics*, *17*(4), 498–502. https://doi.org/10.1038/ng1297-498

Wick, M., Zubov, D., & Hagen, G. (1999). Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). *Gene*, *232*(1), 97–106. https://doi.org/10.1016/s0378-1119(99)00108-0

Witjes, J. A., Bruins, H. M., Cathomas, R., Compérat, E. M., Cowan, N. C., Gakis, G., Hernández, V., Linares Espinós, E., Lorch, A., Neuzillet, Y., Rouanne, M., Thalmann, G. N., Veskimäe, E., Ribal, M. J., & van der Heijden, A. G. (2021). European Association of Urology Guidelines on Muscle-invasive and Metastatic Bladder Cancer: Summary of the 2020 Guidelines. *European Urology*, *79*(1), 82–104. https://doi.org/10.1016/j.eururo.2020.03.055

Wright, J. D. (2002). Factors governing loss and rescue of DNA binding upon single and double mutations in the p53 core domain. *Nucleic Acids Research*, *30*(7), 1563–1574. https://doi.org/10.1093/nar/30.7.1563

Wu, Y., Ma, J., Sun, Y., Tang, M., & Kong, L. (2020). Effect and mechanism of PI3K/AKT/mTOR signaling pathway in the apoptosis of GC-1 cells induced by nickel nanoparticles. Chemosphere, 255, 126913. https://doi.org/10.1016/j.chemosphere.2020.126913

Xing, X., Yuan, X., Liu, T., Dai, M., Fan, Y., Liu, C., Strååt, K., Björkholm, M., & Xu, D. (2021). Regulatory region mutations of TERT, PLEKHS1 and GPR126 genes as urinary biomarkers in upper tract urothelial carcinomas. *Journal of Cancer*, *12*(13), 3853–3861. https://doi.org/10.7150/jca.56779

Xu, Yan., Sato, H., Shinohara, K. -i., Komiyama, M., & Sugiyama, H. (2007). T-loop formation by human telomeric G-quadruplex. *Nucleic Acids Symposium Series*, *51*(1), 243–244. https://doi.org/10.1093/nass/nrm122

Xylinas, E., Kent, M., Kluth, L., Pycha, A., Comploj, E., Svatek, R. S., Lotan, Y., Trinh, Q.-D., Karakiewicz, P. I., Holmang, S., Scherr, D. S., Zerbib, M., Vickers, A. J., & Shariat, S. F. (2013). Accuracy of the EORTC risk tables and of the CUETO scoring model to predict outcomes in non-muscle-invasive urothelial carcinoma of the bladder. *British Journal of Cancer*, *109*(6), 1460–1466. https://doi.org/10.1038/bjc.2013.372

Yafi, F. A., Brimo, F., Steinberg, J., Aprikian, A. G., Tanguay, S., & Kassouf, W. (2015). Prospective analysis of sensitivity and specificity of urinary cytology and other urinary biomarkers for bladder cancer. *Urologic Oncology: Seminars and Original Investigations*, *33*(2), 66.e25-66.e31. https://doi.org/10.1016/j.urolonc.2014.06.008

Yao, Y., Bellon, M., Shelton, S. N., & Nicot, C. (2012). Tumor suppressors p53, p63TAα, p63TAγ, p73α, and p73β use distinct pathways to repress telomerase expression. *The Journal of Biological Chemistry*, *287*(24), 20737–20747. https://doi.org/10.1074/jbc.M111.319236

Yegin, Z., Gunes, S., & Buyukalpelli, R. (2013). Hypermethylation of *TWIST1* and *NID2* in Tumor Tissues and Voided Urine in Urinary Bladder Cancer Patients. *DNA and Cell Biology*, *32*(7), 386–392. https://doi.org/10.1089/dna.2013.2030

Yu, Q., Zhang, K., Wang, X., Liu, X., & Zhang, Z. (2010). Expression of transcription factors snail, slug, and twist in human bladder carcinoma. *Journal of Experimental & Clinical Cancer Research*, *29*(1), 119. https://doi.org/10.1186/1756-9966-29-119

Yu, Z., Wang, Y., Jiang, Y., Ma, S., Zhong, Q., Wan, Y., & Wang, X. (2019). NID2 can serve as a potential prognosis prediction biomarker and promotes the invasion and migration of gastric cancer. *Pathology - Research and Practice*, *215*(10), 152553. https://doi.org/10.1016/j.prp.2019.152553

Yue Sitong., Li, Y., Chen, X., Wang, J., Li, M., Chen, Y., & Wu, D. (2021). FGFR-TKI resistance in cancer: Current status and perspectives. *Journal of Hematology & Oncology*, *14*(1), 23. https://doi.org/10.1186/s13045-021-01040-2

Zeegers, M. P. A. (2001). Occupational risk factors for male bladder cancer: Results from a population based case cohort study in the Netherlands. *Occupational and Environmental Medicine*, *58*(9), 590–596. https://doi.org/10.1136/oem.58.9.590

Zerdoumi, Y., Lanos, R., Raad, S., Flaman, J.-M., Bougeard, G., Frebourg, T., & Tournier, I. (2017). Germline TP53 mutations result into a constitutive defect of p53 DNA binding and transcriptional response to DNA damage. *Human Molecular Genetics*, *26*(14), 2591–2602. https://doi.org/10.1093/hmg/ddx106

Zhang, Q., Kim, N.-K., & Feigon, J. (2011). Architecture of human telomerase RNA. *Proceedings of the National Academy of Sciences*, *108*(51), 20325–20332. https://doi.org/10.1073/pnas.1100279108

Zhang, T., Xu, Z., Liu, G., Jiang, B., de Bock, G. H., Groen, H. J. M., Vliegenthart, R., & Xie, X. (2021). Simultaneous Identification of EGFR,KRAS,ERBB2, and TP53 Mutations in Patients with

Non-Small Cell Lung Cancer by Machine Learning-Derived Three-Dimensional Radiomics. *Cancers*, *13*(8), 1814. https://doi.org/10.3390/cancers13081814

Zhang, X., & Zhang, Y. (2015). Bladder Cancer and Genetic Mutations. *Cell Biochemistry and Biophysics*, *73*(1), 65–69. https://doi.org/10.1007/s12013-015-0574-z

Zhang, Z., Xie, D., Li, X., Wong, Y.-C., Xin, D., Guan, X.-Y., Chua, C. W., Leung, S. C. L., Na, Y., & Wang, X. (2007). Significance of TWIST expression and its association with E-cadherin in bladder cancer. *Human Pathology*, *38*(4), 598–606. https://doi.org/10.1016/j.humpath.2006.10.004

Zhao, X., Wang, yuanli, & Liang, C. (2022). Cigarette smoking and risk of bladder cancer: A dose–response meta-analysis. *International Urology and Nephrology*, *54*(6), 1169–1185. https://doi.org/10.1007/s11255-022-03173-w

Zhao, Z., Rahman, M. A., Chen, Z. G., & Shin, D. M. (2017). Multiple biological functions of Twist1 in various cancers. *Oncotarget*, *8*(12), 20380–20393. https://doi.org/10.18632/oncotarget.14608

Zhou, G.-L., Tucker, D. F., Bae, S. S., Bhatheja, K., Birnbaum, M. J., & Field, J. (2006). Opposing Roles for Akt1 and Akt2 in Rac/Pak Signaling and Cell Migration. *Journal of Biological Chemistry*, *281*(47), 36443–36453. https://doi.org/10.1074/jbc.M600788200

Zhu, C.-Z., Ting, H.-N., Ng, K.-H., & Ong, T.-A. (2019). A review on the accuracy of bladder cancer detection methods. *Journal of Cancer*, *10*(17), 4038–4044. https://doi.org/10.7150/jca.28989

Zhu, F., Zhang, Y., Shi, L., Wu, C.-L., Chen, S.-Q., Zheng, H., & Song, D.-K. (2019). Gene mutation detection of urinary sediment cells for NMIBC early diagnose and prediction of NMIBC relapse after surgery. *Medicine*, *98*(32), e16451. https://doi.org/10.1097/MD.0000000000016451

Zhu, L., Sun, J., Wang, L., Li, Z., Wang, L., & Li, Z. (2019). Prognostic and Clinicopathological Significance of PD-L1 in Patients With Bladder Cancer: A Meta-Analysis. *Frontiers in Pharmacology*, *10*, 962. https://doi.org/10.3389/fphar.2019.00962

Zhu, Q.-S., Rosenblatt, K., Huang, K.-L., Lahat, G., Brobey, R., Bolshakov, S., Nguyen, T., Ding, Z., Belousov, R., Bill, K., Luo, X., Lazar, A., Dicker, A., Mills, G. B., Hung, M.-C., & Lev, D. (2011). Vimentin is a novel AKT1 target mediating motility and invasion. *Oncogene*, *30*(4), 457–470. https://doi.org/10.1038/onc.2010.421

Zimmermann, K., Hoischen, S., Hafner, M., & Nischt, R. (1995). Genomic Sequences and Structural Organization of the Human Nidogen Gene (NID). *Genomics*, *27*(2), 245–250. https://doi.org/10.1006/geno.1995.1038

Zink, D., Mayr, C., Janz, C., & Wiesmüller, L. (2002). Association of p53 and MSH2 with recombinative repair complexes during S phase. *Oncogene*, *21*(31), 4788–4800. https://doi.org/10.1038/sj.onc.1205614