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Exploring the Role of RAS Signaling Pathways in Colorectal Cancer Progression and Targeted Therapeutic Approaches

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Résumé

Le cancer colorectal (CCR) implique des mutations génétiques, principalement KRAS (30-40%) et BRAF (8-15%), qui sont cruciales pour le développement de la maladie et servent de biomarqueurs pour le pronostic et la personnalisation des traitements. Ces mutations activent la voie MAPK, permettant des thérapies ciblées. Notre recherche évalue la prévalence des mutations RAS en Afrique du Nord et au Moyen-Orient, en mettant l'accent sur le Maroc, et démontre l'utilité du pyroséquençage pour les tests de mutation RAS. En Afrique du Nord, 46,4% des patients ont des mutations KRAS, contre 38,1% au Moyen-Orient. La variante G12D de KRAS est la plus commune. Les mutations NRAS et BRAF sont moins fréquentes. Notre cohorte marocaine montre que 57,5% des patients ont des mutations RAS, principalement KRAS avec des mutations au codon 12. Les mutations BRAF sont absentes. Ces résultats soulignent l'importance de traitements adaptés aux profils génétiques régionaux. L'étude confirme le rôle des mutations RAS comme biomarqueurs clés et leur potentiel pour améliorer le diagnostic et les traitements personnalisés, visant à un meilleur pronostic pour les patients atteints de CCR.

Mots-clefs (5): Cancer Colorectal, RAS, KRAS, BRAF, Pyrosequençage

Abstract

Colorectal cancer (CRC) involves abnormal cell growth in the colon or rectum, with KRAS and BRAF mutations being pivotal in disease progression and as biomarkers for treatment customization. This research evaluates RAS mutation prevalence in the MENA region, focusing on Morocco, and validates pyrosequencing for mutation testing. Findings indicate 46.4% of North African CRC patients have KRAS mutations, predominantly the G12D variant, while NRAS and BRAF mutations are rarer. In the Middle East, 38.1% show RAS mutations, with similar trends. Our Moroccan cohort reveals 57.5% with RAS mutations, mainly KRAS at codon 12, suggesting advanced disease stages. The absence of BRAF mutations and the non-significant correlation of NRAS mutations with clinical features call for region-specific therapeutic approaches. The study underscores RAS mutations significance as biomarkers and their role in enhancing personalized medicine for CRC, aiming to improve patient prognosis through better diagnosis and targeted treatment strategies.

Keywords (5): Colorectal Cancer, RAS, KRAS BRAF, Pyrosequencing.

Dedication

This work is dedicated to:

To my dearest Parents: Said and Aicha

Thank you for your love and for teaching me the value of hard work. Your unwavering support has been the bedrock of my journey. You've nurtured my dreams with your sacrifices and celebrated even my smallest victories with the grandest cheers. This thesis is not just my achievement, it is the culmination of the values and perseverance you've instilled in me. May God Almighty preserve you and grant your health, long life, and happiness. I love you dearly.

To my siblings, My lifelong allies: Larbi, Habib, and Jamila

Thank you for the brotherly love you have always shown me; may this work be considered as much yours as it is mine. To those who have never ceased to fill me with joy, your encouragement and support have been invaluable during challenging times.

To my husband my steadfast partner in life: Ali Enes

You've been the quiet strength behind my every endeavor, offering solace in moments of doubt and sharing the burden of my challenges. Your love has been a constant reminder of what truly matters, and this journey would have been incomplete without you by my side.

To my dad: Aiden

Your wisdom and support have been a guiding light. Thank you for your encouragement and belief in my endeavors.

To my dearest friends: RANIA, YOUSSRA, SARA

Thank you for your support and encouragement have been essential during difficult times. May our friendship remain strong and everlasting.

The LRB-P3 team

May they find in this work a tribute of gratitude and appreciation for their support and contribution to the realization of this project.

Foreword

The present work was conducted at the Laboratory of Research and Biosecurity-P3 of the Mohamed V Military Teaching Hospital of Rabat (HMIMV) in collaboration with the Laboratory of Biology of Human Pathologies and Genomic Center of Human Pathologies, Department of Biology, Faculty of Sciences, Mohammed V University, Rabat, Morocco.

This work has been directed by **Pr. DAKKA Nadia**, of the Biology of Human Pathologies and Genomic Center of Human Pathologies, Faculty of Sciences, Rabat, Morocco, and by Pr. **LARAQUI Abdelilah**, of the Royal School of Military Health Services (ERSSM).

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I wish to extend my deepest appreciation and profound gratitude to **Professor DAKKA Nadia**, the former director of Biologie and Humain Pathologies laboratory, for the remarkable influence you have had on my academic pursuits.

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Lastly, I convey my sincere appreciation to **Professor SEKHSOKH Yassine** for your detailed review and enriching contributions to my thesis. Your valuable input has been pivotal in enhancing the quality of my research.

Résumé

Le cancer colorectal est marqué par une prolifération anormale des cellules du côlon ou du rectum. Les mutations génétiques, principalement dans le gène KRAS (30-40 % des cas) et BRAF (8-15 %, dont la mutation V600E), jouent un rôle clé dans l'initiation et la progression de la maladie. Ces mutations, en particulier KRAS, sont également des biomarqueurs importants pour le pronostic et la personnalisation des traitements. Elles activent la voie MAPK, ouvrant la voie à des thérapies ciblées prometteuses, combinées à la chimiothérapie et à l'immunothérapie, pour améliorer les résultats chez les patients atteints de mutations KRAS ou BRAF.

L'objectif principal de ce projet de recherche est d'évaluer la prévalence et la diversité des mutations des gènes RAS chez les patients atteints de CCR dans la région du Moyen-Orient et de l'Afrique du Nord, avec un accent particulier sur le Maroc. De plus, cette étude vise à introduire et à démontrer l'utilité d'une technique moléculaire spécifique, le pyroséquençage, comme méthode viable pour les tests de mutation RAS de routine. En examinant de manière exhaustive le paysage des mutations RAS chez les patients atteints de CCR et en introduisant une méthode de test efficace, nous visons à contribuer à l'identification de biomarqueurs précieux pour le CCR dans la région du MENA, potentiellement améliorer le diagnostic précoce et les stratégies de traitement pour ce cancer prévalent.

Notre analyse des mutations RAS et BRAF chez les patients atteints de CCR en Afrique du Nord et au Moyen-Orient révèle des tendances régionales. En Afrique du Nord, 46,4 % présentent des mutations KRAS, notamment la variante G12D, tandis que les mutations NRAS et BRAF sont moins fréquentes, à 3,2 % et 3,5 % respectivement. Au Moyen-Orient, 38,1 % des cas montrent des mutations RAS, principalement KRAS avec la variante G12D. Les mutations NRAS et BRAF, principalement V600E, sont présentes dans 3,3 % et 2,6 % des cas. Ces profils de mutation distincts soulignent l'importance de traitements personnalisés basés sur le paysage génétique spécifique de chaque région.

Dans notre cohorte marocaine, 57,5 % des patients présentent des mutations RAS, surtout KRAS (56,2 %), avec des mutations au codon 12 liées à des stades avancés de la maladie. Les mutations BRAF sont absentes et les mutations NRAS ne montrent pas de corrélation significative avec les caractéristiques cliniques. Ces découvertes mettent en lumière le besoin de stratégies thérapeutiques adaptées aux profils génétiques régionaux.

Cette étude enrichit la compréhension des mutations RAS dans le CCR, confirmant leur rôle de biomarqueurs clés et leur potentiel pour guider les diagnostics et traitements personnalisés. Nos travaux aspirent à améliorer les soins, le diagnostic précoce et les thérapies ciblées, pour un meilleur pronostic des patients atteints de CCR.

Mots clés: Cancer Colorectal, RAS, KRAS, BRAF, Pyrosequençage.

Abstract

Colorectal cancer (CRC) is characterized by abnormal proliferation of cells in the colon or rectum. Genetic mutations, primarily in the KRAS gene (30-40% of cases) and BRAF (8-15%, including the V600E mutation), play a key role in the initiation and progression of the disease. These mutations, particularly KRAS, are also significant biomarkers for prognosis and treatment personalization. They activate the MAPK pathway, paving the way for promising targeted therapies, combined with chemotherapy and immunotherapy, to improve outcomes in patients with KRAS or BRAF mutations.

The main objective of this research project is to assess the prevalence and spectrum of RAS gene mutations in CRC patients in the Middle East and North Africa region, with a particular focus on Morocco. Furthermore, this study aims to introduce and demonstrate the utility of a specific molecular technique, pyrosequencing, as a viable method for routine RAS mutation testing. By thoroughly examining the RAS mutation landscape in CRC patients and introducing an effective testing method, we aim to contribute to the identification of valuable biomarkers for CRC in the MENA region, potentially improving early diagnosis and treatment strategies for this prevalent cancer.

Our analysis of RAS and BRAF mutations in CRC patients in North Africa and the Middle East reveals regional trends. In North Africa, 46.4% exhibit KRAS mutations, notably the G12D variant, while NRAS and BRAF mutations are less frequent, at 3.2% and 3.5% respectively. In the Middle East, 38.1% of cases show RAS mutations, mainly KRAS with the G12D variant. NRAS and BRAF mutations, primarily V600E, are present in 3.3% and 2.6% of cases. These distinct mutation profiles underscore the importance of personalized treatments based on the specific genetic landscape of each region.

In our Moroccan cohort, 57.5% of patients exhibit RAS mutations, predominantly KRAS (56.2%), with codon 12 mutations linked to advanced stages of the disease. BRAF mutations are absent, and NRAS mutations do not show a significant correlation with clinical features. These findings highlight the need for therapeutic strategies tailored to regional genetic profiles.

This study enriches the understanding of RAS mutations in CRC, confirming their role as key biomarkers and their potential to guide personalized diagnostics and treatments. Our work aspires to improve care, early diagnosis, and targeted therapies, for a better prognosis of CRC patients.

Keywords: Colorectal Cancer, RAS, KRAS BRAF, Pyrosequencing.

Publication List

a. Publications

Published Original Articles:

1. **Benmokhtar, S.**, Laraqui, A., El Boukhrissi, F., Hilali, F., Bajjou, T., Jafari, M., El Zaitouni, S., Baba, W., El Mchichi, B., Elannaz, H., Lahlou, I., Chahdi, H., Oukabli, M., Mahfoud, T., Tanz, R., Ichou, M., Ennibi, K., Dakka, N., & Sekhsokh, Y. (2022). Clinical Significance of Somatic Mutations in RAS/RAF/MAPK Signaling Pathway in Moroccan and North African Colorectal Cancer Patients. *Asian Pacific Journal of Cancer Prevention*, *23*(11), 3725–3733. https://doi.org/10.31557/APJCP.2022.23.11.3725

Published Reviews

1. **Benmokhtar, S**., Laraqui, A., Hilali, F., Bajjou, T., El Zaitouni, S., Jafari, M., Baba, W., Elannaz, H., Lahlou, I. A., Hafsa, C., Oukabli, M., Mahfoud, T., Tanz, R., Ichou, M., Ennibi, K., Dakka, N., & Sekhsokh, Y. (2024). *RAS/RAF/MAPK* Pathway Mutations as Predictive Biomarkers in Middle Eastern Colorectal Cancer: A Systematic Review. *Clinical Medicine Insights: Oncology*, *18*, 11795549241255651. https://doi.org/10.1177/11795549241255651

2. Jafari, M., Laraqui, A., Baba, W., **Benmokhtar, S**., Zaitouni, S. E., Ali, A. A., Bounaim, A., Moujahid, M., Tanz, R., Mahfoud, T., Sbitti, Y., Annaz, H. E., Abi, R., Tagajdid, M. R., Kochri, S. E., Lahlou, I. A., Hsaini, H. E., Belayachi, L., Benjouad, A., Ennibi, K. (2022). Prevalence and patterns of mutations in RAS/RAF/MEK/ERK/MAPK signaling pathway in colorectal cancer in North Africa. *BMC Cancer*, *22*(1), 1142. https://doi.org/10.1186/s12885-022-10235-w

b. Communications

1. Selected Poster at "2nd International Scientific Conference for Research and Ethics, Cancer Research: Monitoring therapeutics and ethical issues". (19 and 20 April 2019). The Polydisciplinary Faculty of Taroudant, Ibn Zohr University.

Poster: "Prevalence of RAS Mutations in Metastatic Colorectal Cancer: A retrospective observational study from Morocco**".**

2. Presented a published poster at the "63rd Edition of the Innovation in Biology Days – E-Congress" (December 8 to 11, 2020) held at the Palais des Congrès in Paris.

Poster: "Mutational status of Full RAS gene in Moroccan colorectal cancer cases**".**

3. Oral Presentation of Experimental Results

Presented the findings of the paper titled **“**Clinical Significance of Somatic Mutations in the RAS/RAF/MAPK Signaling Pathway in Moroccan and North African Colorectal Cancer**”** during the Doctoral Days organized by the Doctoral Center of Science and Technology, from July 19 to 21, 2022, at the Faculty of Sciences in Rabat.

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List of Abbreviations

4E-BP1	4E Binding Protein 1
5-FU	5-Fluorouracil
AJCC	American Joint Committee On Cancer
AKT=PKB	Protein Kinase B
AML	Acute Myelogenous Leukemia
APC	Adenomatous Polyposis Coli
APS	Adenosine 5'-Phosphosulfate
ASR	Age-Standardized Rate
ATP	Adenosine Triphosphate
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B
CCR	Cancer Colorectal
CD	Crohn's Disease
CH	Constant Heavy Chain
CIMP	Cpg Island Methylator Phenotype
CIN	Chromosomal Instability
CL	Constant Light Chain
CMS	Consensus Molecular Subtypes
COSMIC	Catalogue Of Somatic Mutations In Cancer
CpG	Cytosine-Phosphate-Guanine
CRC	Colorectal Cancer
CRCSC	Colorectal Cancer Subtyping Consortium
CRM	Circumferential Resection Margin
CRT	Chemoradiation Therapy
CT	Computed Tomography
DACH	1,2-Diaminocyclohexane
DFS	Disease-Free Survival
dMMR	Deficient Mismatch Repair
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside Triphosphates

EGFR	Epidermal Growth Factor Receptor
eIF4E	Eukaryotic Initiation Factor 4E
ERK	Extracellular Signal-Regulated Kinase
EtBr	Ethidium Bromide
Fabs	Fragment, Antigen-Binding
FAP	Familial Adenomatous Polyposis
FFPE	Formalin-Fixed Paraffin-Embedded
FIT	Fecal Immunochemical Test
FOBT	Fecal Occult Blood Test
FOXO	Forkhead Box O
GAPs	Gtpase-Activating Proteins
GDP	Guanosine Diphosphate
GEFs	Guanine Nucleotide Exchange Factors
gFOBT	Guaiac-Based Fecal Occult Blood Test
Grb2	Growth Factor Receptor-Bound Protein 2
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine Triphosphate
GTPases	Guanosine Triphosphatases
HDI	Human Development Index
HER2	Human Epidermal Growth Factor Receptor 2
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HNSCC:	Head And Neck Squamous Cell Carcinoma.
HRAS	Harvey Rat Sarcoma Viral Oncogene Homolog
HVR	Hypervariable Region
IARC	International Agency For Research On Cancer
IBD	Inflammatory Bowel Disease
IGF-1	Insulin-Like Growth Factor 1
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-7	Interleukin-7

IL-8	Interleukin-8
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LAC	Lung Adenocarcinoma
LOD	Limit Of Detection
LOH+	Loss Of Heterozygosity Positive
MAPK	Mitogen-Activated Protein Kinase
MBW	Molecular Biology Water
mCRC	Metastatic Colorectal Cancer
ME	Middle East
MEK	Mitogen-Activated Protein Kinase Kinase
MINT1	Methylated In Tumors 1
MINT2	Methylated In Tumors 2
MINT31	Methylated In Tumors 31
MLH1	Mult Homolog 1
MMR	Mismatch Repair
moAbs	Monoclonal Antibodies
MoH	Ministry Of Health
MRC	Magnetic Resonance Colonography
MRI	Magnetic Resonance Imaging
MSH2	Muts Protein Homolog 2
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability-High
MSS	Microsatellite Stable
mTOR	Mammalian Target Of Rapamycin
MT-sDNA	Multi-Target Stool DNA Test
NA	North Africa
NIH	National Institutes Of Health
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
NSCLC	Non-Small Cell Lung Cancer
NTC	No Template Control

ORF	Open Reading Frame
OS	Overall Survival
p16	Cyclin-Dependent Kinase Inhibitor 2a (Cdkn2a)
PAHs	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein 1
PDAC	Pancreatic Ductal Adenocarcinoma
PDK1	Phosphoinositide-Dependent Kinase-1.
PD-L1	Programmed Death Ligand 1
PFS	Progression-Free Survival
PI3K	Phosphatidylinositol 3-Kinase.
PI3K	Phosphoinositide 3-Kinase.
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha.
PIP2	Phosphatidylinositol-(4,5)-Biphosphate
PIP3	Phosphatidylinositol-(3,4,5)-Biphosphate
P-loop	Phosphate-Binding Loops
PPAP	Polymerase Proofreading-Associated Polyposis
PPi	Pyrophosphate
PTEN	Phosphatase And Tensin Homolog
RAF	Rapidly Accelerated Fibrosarcoma.
RAS	Rat Sarcoma
RTK	Receptor Tyrosine Kinase
SCNA	Somatic Copy Number Alteration
SH2	Src Homology 2
SOS	Son Of Seven-Less
SR	Surgical Resection
TBE	Tris, Borate, Edta
TGF-β	Transforming Growth Factor-Beta
TKIs	Tyrosine Kinase Inhibitors
TNF-α	Tumor Necrosis Factor Alpha

TNM	Tumor-Node-Metastasis
Tp53	Tumor Proteine P53
UC	Ulcerative Colitis
UICC	Union For International Cancer Control
VEGF	Vascular Endothelial Growth Factor
VH	Variable Heavy Chain
VL	Variable Light Chain
VPT	Vacuum Preparation Tool
Wnt	Wingless-Related Integration Site
WT	Wild-Type

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General introduction

Colorectal cancer (CRC) is a highly prevalent and life-threatening malignancy, imposing a substantial burden on global public health systems. Its complex etiology and multifactorial nature have been extensively studied, revealing the significant role of genetic alterations, such as mutations in the RAS and BRAF genes, in colorectal carcinogenesis. These genetic abnormalities not only influence disease progression but also impact therapeutic response and patient prognosis. CRC is a complex disease characterized by histological, morphological, and genetic changes, originating as a malignant tumor in the colon and rectum *(Simon 2016)*. The adenoma-carcinoma sequence, a progressive model of CRC development, highlights the role of genetic heterogeneity, leading to various alterations in oncogenes, tumor suppressor genes, and DNA damage recognition and repair genes *(Saetang and Sangkhathat 2017)*. Consequently, distinct tumor categories arise, each driving specific oncogenic signaling pathways. To effectively address the multifaceted factors contributing to CRC pathogenesis, a comprehensive approach encompassing diagnosis, treatment, and targeted therapies is crucial *(Miele et al., 2020)*.

CRC is a significant global public health concern, ranking as the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide. In 2020, there were an estimated 1.9 million new cases of CRC and 935,000 deaths globally (*Globocan 2020, Xi and Xu 2021*). These alarming statistics emphasize the urgency of addressing CRC as a major public health issue. Furthermore, projections suggest a 60% increase in new cases and deaths by 2030, with over 2.2 million new cases and 1.1 million deaths anticipated (*Arnold et al., 2017)*.

In Morocco, CRC poses a significant health concern. According to Globocan 2020, there were approximately 4,324 newly diagnosed cases and 2,374 reported deaths, accounting for 7.3% of all cancer cases and 2.8% of cancer-related deaths *(Ferlay et al., 2020)*. The incidence rate of CRC in Morocco is 7.1 per 100,000 population, with age-standardized rates (ASR) ranging from 3.8 to 8.4 per 100,000 in males and 2.6 to 7.4 per 100,000 in females. Although both men and women are affected by this disease, men tend to have a slightly higher incidence rate *(Fondation Lalla Salma Pévention et traitement des cancers, 2012)*. Within the group of individuals who underwent screening, the frequency of identifying advanced adenomas was 4.0 cases per 1000 individuals, while the detection rate for CRC stood at 0.5 cases per 1000 individuals *(Selmouni et al., 2022)*.

CRC is influenced by various risk factors, both environmental and genetic in nature. Environmental factors such as diet, physical inactivity, obesity, smoking, and excessive alcohol consumption have been associated with an increased risk of developing CRC. Additionally, genetic factors play a crucial role in the development and progression of CRC.

The RAS-MAPK pathway is dysregulated in CRC due to frequent mutations in the KRAS gene. KRAS and NRAS are oncogenes that control vital cellular processes. They interact with downstream effectors in the MAPK pathway, including RAF, MEK, and extracellular-signal-regulated kinase (Kano et al. 2016). Additionally, BRAF, a key mediator downstream of KRAS, influences intracellular signaling and cell growth. Mutations in the BRAF gene contribute to cancer development and progression in CRC *(Karimi et al., 2021; Bashir et al., 2022)*.

In the context of mCRC, the choice of treatment is often influenced by the mutational profile of the KRAS and NRAS genes. Specifically, mutations occurring in exon 2, exon 3, and exon 4 of these genes have been correlated with resistance to anti-EGFR therapies, including cetuximab and panitumumab. Understanding the mutational status of KRAS and NRAS is crucial for determining the most effective treatment approach in mCRC patients *(Allegra et al., 2016)*.

In the context of this study, the objectives are:

- Investigate the role of the RAS gene in colorectal cancer (CRC) and its impact on disease progression, therapeutic response, and patient prognosis.
- Analyze the genetic heterogeneity and molecular characteristics associated with RAS gene mutations in CRC to understand the underlying mechanisms of colorectal carcinogenesis.
- Determine the clinical significance of RAS gene mutations in mCRC and their influence on treatment decisions and response to anti-EGFR therapies.

I. Colorectal Cancer

I.1 Incidence and Mortality of CRC

I.1.1 In the World

Colorectal cancer (CRC) is a major public health concern worldwide. It is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths globally. The incidence and mortality rates of CRC vary widely across different regions of the world, with the highest rates observed in developed countries.

According to the latest data, the incidence of CRC is highest in European regions, Australia and New Zealand, Europe, and North America. In contrast, the incidence rates are relatively low in Africa and Asia. The highest mortality rates are observed in Central and Eastern Europe, followed by Australia and New Zealand, and North America. The lowest mortality rates are observed in Africa and Asia *(Sung et al., 2021)*.

The Global Cancer Statistics 2020 report projects that the global number of new CRC cases will reach 3.2 million in 2040, based on the projection of aging, population growth, and human development **(Table 1)** *(Xi and Xu 2021)*.

Table 1: Projected Incidence of CRC Cases from 2020 to 2040 *(Xi and Xu 2021)*.

Cancer sites	**2020**	**2040**
Colon	1,148,515	1,916,781
Rectum	732,210	1,160,296
Anus	50,865	77,597
Total	1,931,590	3,154,674

In 2020, CRC accounted for approximately 1.93 million new cases and 935,000 deaths globally, representing 10% of all cancer cases and 9.4% of cancer-related deaths worldwide. The gender and geographic distribution of CRC incidence and mortality also show disparities, with China, the United States, and Japan having the highest number of cases and deaths *(Xi and Xu 2021)* **(Figure 1)**.

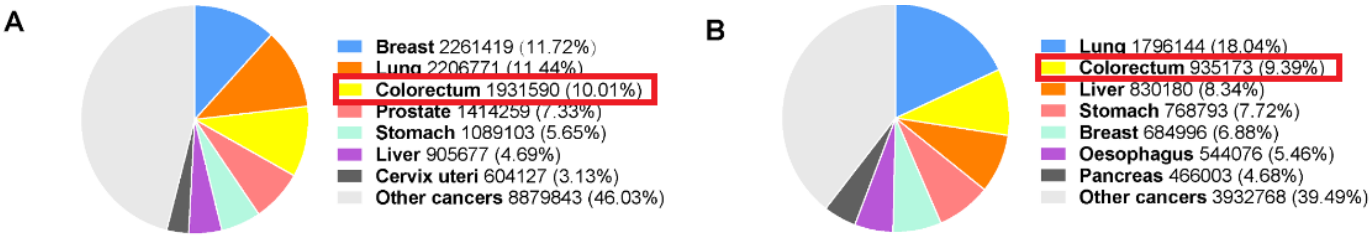


Figure 1: Global Cancer Burden in 2020: Incidence and Mortality of Major Cancer Types in Both Sexes *(Xi and Xu 2021)*.

(A) Estimated number of new cancer cases in 2020 worldwide.

(B) Estimated number of cancer-related deaths in 2020 worldwide.

Source: GLOBOCAN 2020.

The incidence and mortality rates were approximately 25% lower in women compared to men. CRC ranked third in terms of incidence but second in terms of mortality for both genders. Among women, it was the second most diagnosed cancer with 865,630 cases (9.4% of all cancers), and the second most deadly with 419,536 deaths (9.5% of all cancers). Among men, CRC was the third most common cancer with 1,065,960 cases (10.6% of all cancers), and the third most deadly with 515,637 deaths (10.3% of all cancers) **(Figure 2)**.

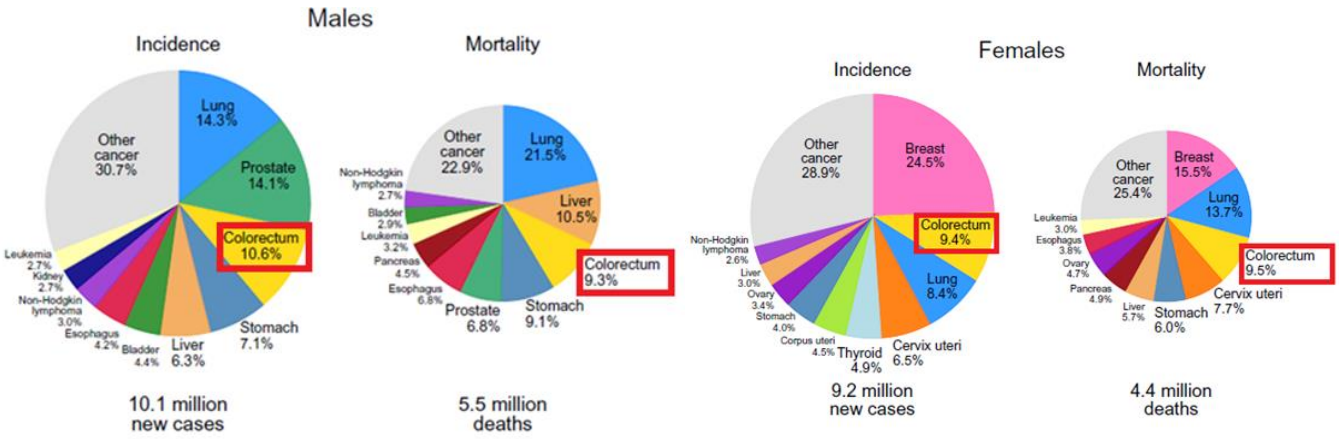


Figure 2 : Global incidence and mortality rates by gender in GLOBOCAN 2020 *(Sung et al., 2021)*.

The occurrence of CRC shows a direct association with the Human Development Index (HDI), a composite measure reflecting life expectancy, education, and income. Hence, countries with higher HDI generally experience higher rates of CRC compared to those with lower HDI. However, when considering age-standardized incidence rates, regions with high HDI have a higher occurrence of CRC, while countries with medium HDI exhibit the lowest rates. Age-standardized incidence rates are used to account for age differences between populations, enabling a more accurate comparison of CRC occurrence across different regions. Therefore, the link between HDI and CRC incidence may be partially explained by variations in age

distribution among populations. It's crucial to acknowledge that socioeconomic status is a multifaceted concept encompassing factors beyond HDI, such as occupation, income, and social support *(Xi and Xu 2021)* **(Figure 3)**.

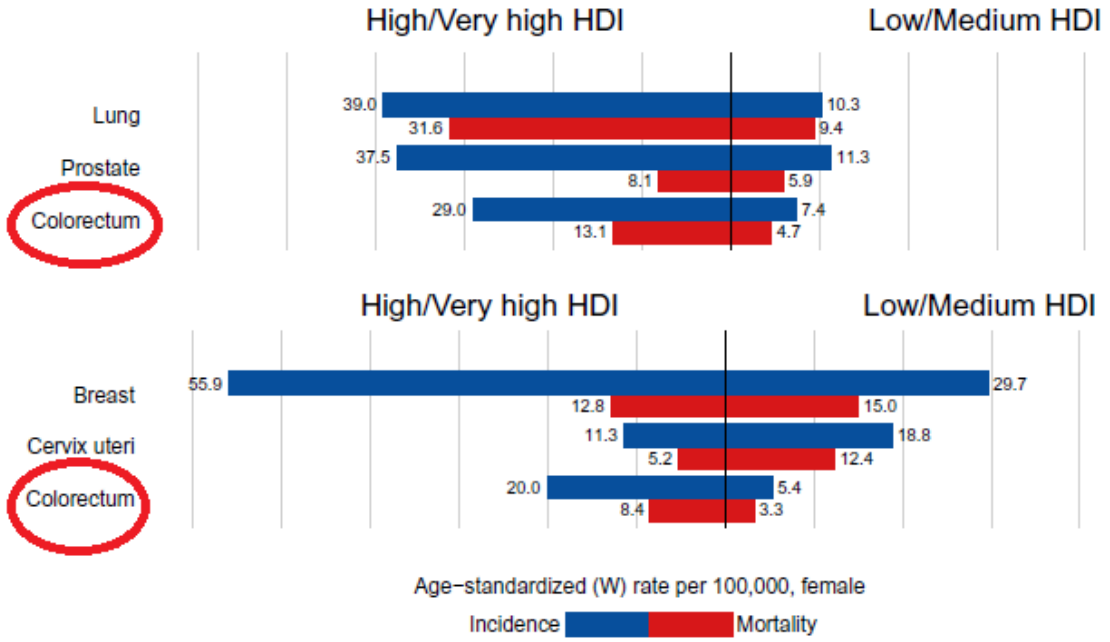


Figure 3*:* Comparison of CRC Incidence and Mortality ASR in High/Very High vs. Low/Medium Human Development Index (HDI) Countries in 2020 *(Sung et al., 2021)*.

The incidence rates of colon and rectal cancers show significant variations across different regions worldwide. According to available data, there is a 9-fold difference in colon cancer incidence rates among world regions, with the highest rates observed in European regions, Australia/New Zealand, and Northern America. Hungary has the highest incidence rate of colon cancer in men, while Norway has the highest rate in women. Similarly, the regional distribution of rectal cancer incidence rates is comparable, with Eastern Asia having the highest rates. However, most regions in Africa and South Central Asia experience low rates of both colon and rectal cancer incidence. These findings underscore the importance of implementing region-specific screening and prevention strategies to reduce the occurrence of CRC *(Sung et al.,2021)* **(Figure 4)**.

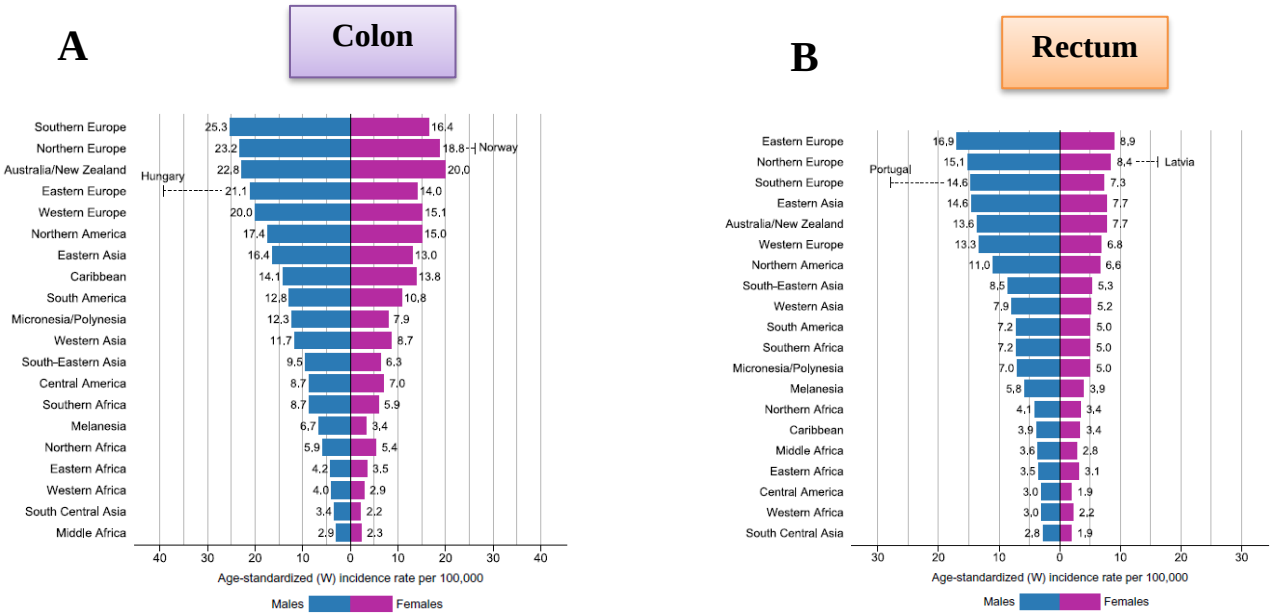


Figure 4: Global Disparities in Age-Standardized Incidence Rates of Colon and Rectal Cancers by Sex in 2020. **Source:** Globocan 2020 *(Sung et al., 2021)*.

I.1.2 **In the Middle East and North Africa**

CRC is becoming a significant health issue in the Middle East, with rising incidence and mortality rates in recent years. Although the region has relatively lower ASR for CRC incidence and mortality compared to other regions, the incidence of early-onset CRC is increasing in the Middle East (ME). In fact, around 20% of all early-onset CRC cases worldwide are found in Asia. The incidence and mortality rates of CRC vary among countries in the Middle East. For instance, Iran has an incidence rate of 8.5 per 100,000 and a mortality rate of 5.2 per 100,000, while Saudi Arabia has an incidence rate of 6.5 per 100,000 and a mortality rate of 3.5 per 100,000. Jordan has an ASR for CRC incidence before age 50 years of 6.2 per 100,000 and an overall incidence rate of 10.3 per 100,000. The United Arab Emirates (UAE) has an incidence rate of 10.3 per 100,000 and a mortality rate of 4.5 per 100,000 *(Shamseddine et al., 2023)*.

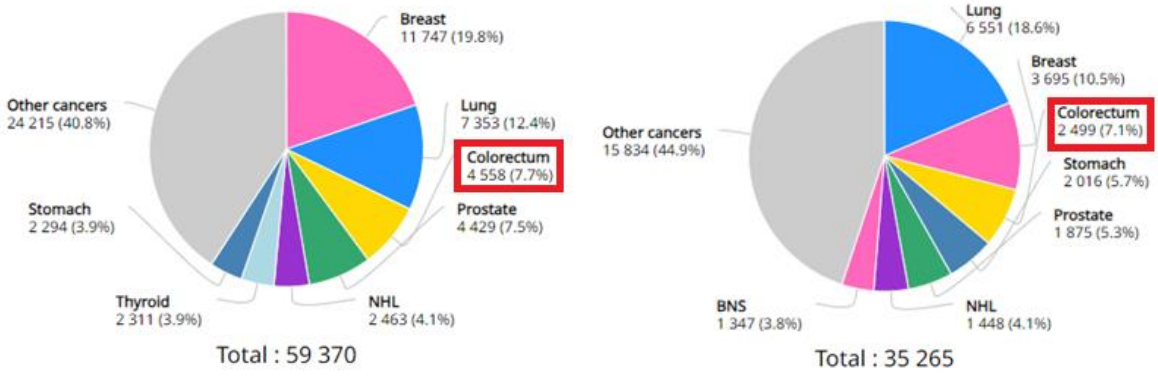
In North Africa (NA), the ASR of CRC is higher than in sub-Saharan Africa (SSA), with Libya reporting the highest ASR for CRC in the region. Notably, the incidence of CRC has been on the rise in Northern Tunisia, increasing from 6.4 per 100,000 in 1994 to 12.4 per 100,000 in 2009. Interestingly, CRC in young patients is more prevalent in North African countries, accounting for 11% of cases in Morocco and even higher in the Middle East, with 46% in the UAE and 25% in Egypt. Male patients dominate the reported cases across all studies, making up 56.6% of those diagnosed with CRC. The average age of patients at diagnosis is 52.7 years old, with 45.1% of patients being diagnosed above the age of 50 years *(Jafari et al., 2022)*.

I.1.3 **In Morocco**

Morocco faces a significant public health challenge with CRC, which is the third most common cancer among both men and women. In 2020, there were 4558 new cases of CRC detected in the country, with most cases being diagnosed at advanced stages. The incidence rates of CRC were estimated to be 12.9 and 9.9 per 100,000 person-years among men and women, respectively. CRC ranks third among cancer deaths in both sexes combined, with 2499 CRC deaths estimated in 2020. The mortality rates of CRC were 7.2 and 5.4 per 100,000 person-years in men and women, respectively *(Selmouni et al., 2022)* **(Figure 5 & Figure 6)**.

The International Agency for Research on Cancer (IARC) has projected that the incidence and mortality rates of colorectal cancer in Morocco will increase significantly over the next two decades. This trend is consistent with what has been observed in other low- and middle-income countries, where there has been a documented 300% rise in the incidence of colorectal cancer over the past 20 years.

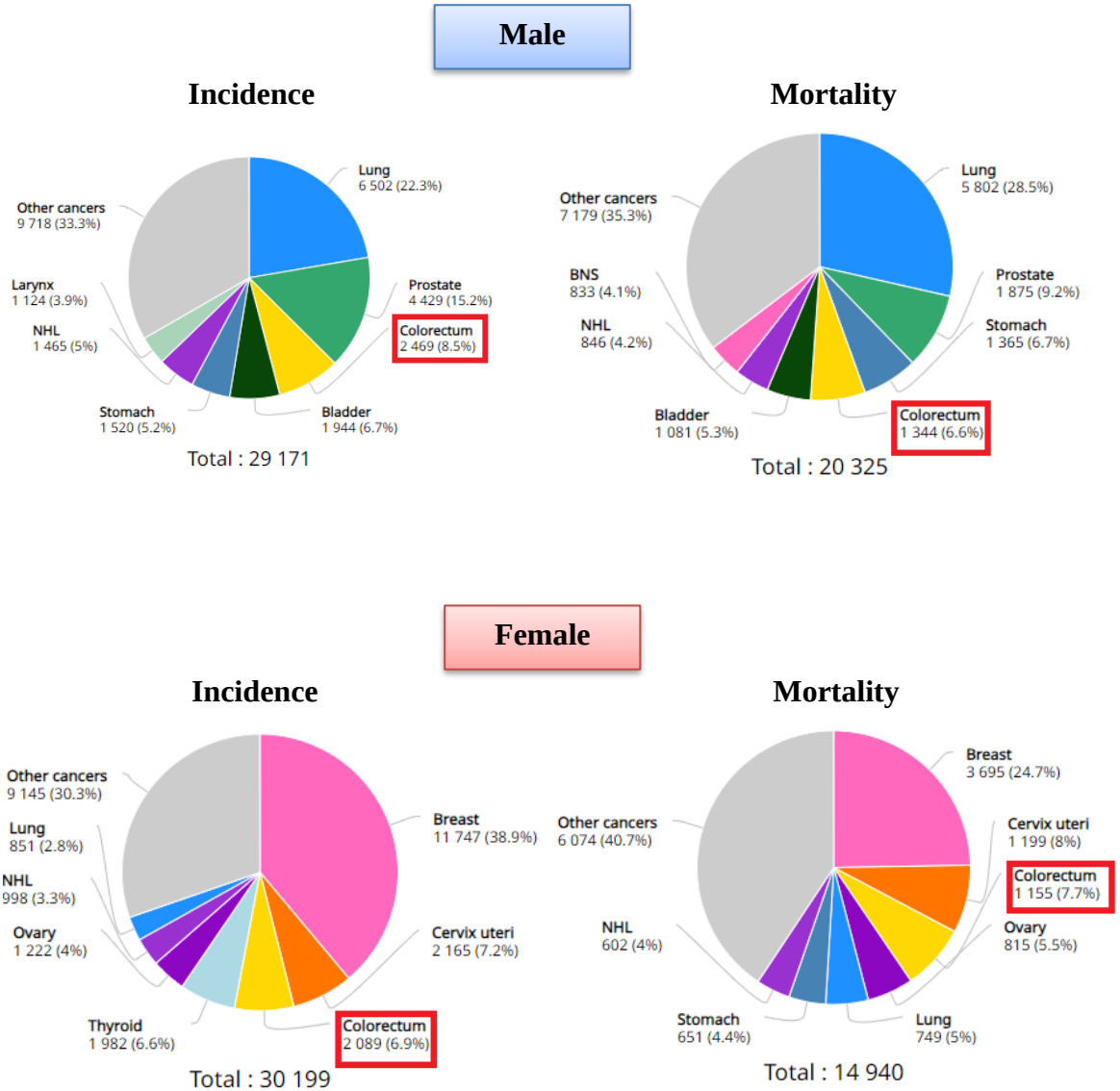
Due to the increasing burden of CRC in Morocco, the Ministry of Health (MoH) has explored the possibility of introducing a CRC screening program. In other countries, organized, population-based CRC screening programs have been shown to significantly reduce CRC incidence and mortality rates. To make an informed decision, the MoH conducted a demonstration project to evaluate the feasibility and effectiveness of integrating CRC screening with primary health care services in Morocco.



Estimated number of new cases in 2020, MOROCCO, both sexes, all ages

Estimated number of deaths in 2020, MOROCCO, both sexes, all ages

Figure 5: Global Cancer Burden in 2020, MOROCCO: Incidence and Mortality of Major Cancer Types in Both Sexes. **Source**: Globocan 2020



Available at: Global Cancer Observatory (https://gco.iarc.fr/)

Figure 6: Global incidence and mortality rates by gender in 2020, MOROCCO Source: Globocan 2020

I.1.4 Risk Factors Associated with CRC

CRC development is influenced by multiple factors, which can be broadly categorized into two groups: modifiable and non-modifiable risk factors. Modifiable risk factors refer to those elements that can be changed or controlled, while non-modifiable risk factors are beyond our influence. Gaining a comprehensive understanding of these risk factors is of paramount importance for early detection and effective prevention of CRC.

I.1.4.1 Modifiable Risk Factors

❖ **Overweight and Obesity**

CRC is influenced by multiple risk factors, and obesity is a significant contributor. Numerous studies have shown a strong association between excess body weight and the

development of CRC. Both overweight and obese individuals, regardless of gender, have a significantly higher risk of CRC compared to those with a normal weight. For every five kilograms of weight gain, there is a 3% increase in the overall risk of CRC *(Rawla et al., 2019)*.

The relationship between obesity and CRC is complex and not fully understood, leading to ongoing research. One important area of focus is adipose tissue, which acts as an endocrine organ and plays a role in regulating energy intake and inflammation. Abnormal or excessive fat accumulation can lead to changes in the secretion of hormones and cytokines from adipose tissue. Overweight and obese individuals tend to have higher levels of certain factors (such as leptin, resistin, TNF-α, IL-1, IL-6, IL-7, and IL-8) that can affect cell growth, suppress apoptosis, induce oxidative stress, weaken immune responses, and inhibit the activity of the IGF-1 axis all factors associated with cancer development and progression *(Sawicki et al ., 2021)*.

Maintaining a healthy weight through a balanced diet and regular physical activity is crucial in reducing the risk of developing CRC. Health education focused on CRC risk factors and nutrition, with an emphasis on a balanced diet, plays an essential role in secondary prevention.

- **Physical inactivity**

Physical inactivity is a significant risk factor for developing CRC. Studies have consistently shown that individuals who engage in regular physical activity have a significantly lower risk of CRC compared to those who lead sedentary lifestyles. Regular physical exercise not only helps in reducing the risk of CRC but also offers numerous other health benefits. It has been demonstrated to improve immune system function, regulate hormone levels, and prevent obesity, all of which contribute to cancer prevention *(Sninsky et al., 2022)*.

Conversely, leading a sedentary lifestyle, characterized by prolonged sitting or lying down and lack of exercise, is associated with an increased risk of CRC. Sedentary behavior can lead to weight gain, poor glucose control, and heightened inflammation in the body, all of which promote the development of CRC.

Health education that emphasizes the importance of physical activity and encourages individuals to engage in regular exercise is crucial for secondary prevention.

- **Dietary Patterns**

Diet plays a significant role in the development of CRC. A diet high in red and processed meat is a crucial risk factor, while a diet low in fiber, calcium, vitamin D, and dairy products is

also linked to an increased risk. Dietary recommendations suggest consuming a diet low in red and processed meat and high in fiber, fruits, vegetables, calcium, vitamin D, and dairy products. Consuming a diet that is high in fiber, fruits, and vegetables provides a large number of bioactive compounds that have potent antioxidant and anti-inflammatory properties capable of inhibiting DNA and cellular damage. Furthermore, high consumption of dairy products, particularly milk, has a probable inverse association with the risk of developing CRC. Therefore, maintaining a healthy diet that is low in red and processed meat and high in fiber, fruits, vegetables, calcium, vitamin D, and dairy products is crucial to reduce the risk of developing CRC. Health education emphasizing the importance of healthy eating habits and a balanced diet is essential for secondary prevention of CRC *(Sawicki et al ., 2021)*.

- **Alcohol Consumption**

Numerous scientific investigations have consistently established a robust association between alcohol consumption and a heightened risk of CRC. Engaging in the habit of consuming more than one alcoholic beverage daily has been unequivocally linked to the development of both colon polyps and CRC. In comparison to non-drinkers, heavy alcohol drinkers face a significantly higher risk of developing both conventional adenomas and serrated polyps. For example, a comprehensive Korean study reported that substantial alcohol consumption increased the risk of adenomas on surveillance colonoscopy by approximately 86% compared to individuals who abstained from alcohol *(Yang et al., 2019)*. Likewise, another study demonstrated an elevated risk of large adenomas among heavy drinkers when compared to a control group of non-drinkers *(Bardou et al., 2002)*. Given these findings, it is highly advisable to adopt measures to curtail alcohol consumption, as doing so serves as an effective strategy for mitigating the risk of developing CRC.

Furthermore, it is essential to emphasize that alcohol's potential role in carcinogenesis involves various mechanisms. These mechanisms include the generation of reactive oxygen species and nitrogen species, production of mutagenic acetaldehyde, depletion of S-adenosylmethionine, inactivation of tumor suppressor genes, hormonal imbalances, reduction in folate concentration, and impairment of retinoic acid metabolism *(Sawicki et al ., 2021)*. The intricate interplay of these factors underscores the significance of understanding the multifaceted impact of alcohol consumption on CRC development.

❖ **Smoking**

CRC is strongly linked to smoking, which is considered a significant risk factor. Several studies have shown a clear association between smoking and an increased risk of developing CRC. The harmful substances present in tobacco smoke can have detrimental effects on the digestive system, including the colon and rectum.

The harmful substances present in tobacco smoke, such as polycyclic aromatic hydrocarbons (PAHs), nitrosamines, aromatic amines, and benzene, can have detrimental effects on the digestive system, including the colon and rectum. These toxic compounds are absorbed into the bloodstream after being inhaled, and then they are distributed throughout the body, leading to inflammation, oxidative stress, and damage to the cells lining the gastrointestinal tract *(Gui et al.,2021)*.

Additionally, smoking can adversely affect the gut microbiome, the complex community of microorganisms residing in the intestines. Studies have found that smoking alters the composition of the gut microbiome, potentially promoting the growth of harmful bacteria while reducing beneficial bacteria. Such disruptions in the gut microbiota have been implicated in various health conditions, including CRC *(Gui et al.,2021)*.

Smoking not only increases the risk of CRC but also affects its progression and prognosis. Individuals who continue smoking after a CRC diagnosis face a higher risk of cancer recurrence, treatment complications, and poorer outcomes than those who quit smoking. However, quitting smoking, even after years of tobacco use, can significantly reduce the risk of developing CRC, highlighting the importance of early cessation as a preventive measure *(Alwers et al., 2021)*.

I.1.4.2 **Non-Modifiable Risk Factors**

❖ **Age**

Age is a well-established risk factor for CRC. The incidence of CRC increases with age, with the majority of cases occurring in individuals over the age of 50. This is due to a variety of factors, including changes in the body's cells and tissues over time, exposure to environmental toxins, and a decrease in the body's ability to repair damaged DNA. As individuals age, their risk of developing precancerous polyps in the colon or rectum also increases. These polyps can develop into cancer over time if left untreated. While age is a non-modifiable risk factor, individuals can take steps to reduce their risk of developing CRC, such as undergoing regular screenings and adopting a healthy lifestyle. It is important for individuals

over the age of 50 to undergo regular screenings for CRC, as early detection and treatment can significantly improve outcomes and increase the chances of survival *(Wele et al ., 2022)*.

- **Sex**

Sex is a well-established factor in the risk of developing CRC. Studies have shown that men are more likely to develop CRC than women, with some estimates suggesting that men have a 20-30% higher risk of developing the disease. The reasons for this difference are not entirely clear, but it is thought to be related to hormonal differences between men and women. Specifically, estrogen has been shown to have a protective effect against CRC, and women have higher levels of this hormone than men. Additionally, lifestyle factors such as diet, physical activity, and smoking may also play a role in the sex differences in CRC risk. Overall, while the exact mechanisms underlying the sex differences in CRC risk are not fully understood, it is clear that sex is an important factor that should be taken into account when assessing an individual's risk of developing this disease *(Wele et al ., 2022)*.

- **Race and Ethnicity**

CRC incidence and mortality rates exhibit notable variations based on race and ethnicity. Some ethnic groups, including African Americans and Ashkenazi Jews, face a higher risk of developing CRC compared to other populations. Conversely, Asian Americans and Hispanics tend to have a lower risk. Interestingly, there are differences in the prevalence of large polyps on screening colonoscopy until age 65, at which point the prevalence between blacks and whites becomes equivalent. Moreover, African Americans have a higher prevalence of proximal colon polyps and villous histology compared to other groups. In contrast, Hispanic individuals have a 25% lower risk of large adenomas than non-Hispanic whites between ages 50-79, and Asian Americans show an adenoma prevalence similar to whites *(Carethers 2021)*.

The reasons for these disparities are complex and likely involve a combination of genetic, environmental, and lifestyle factors. Healthcare providers should consider these factors when assessing an individual's risk of developing CRC and tailor appropriate screening and preventive strategies accordingly.

- **High-Risk Genetic Syndromes**

Certain genetic syndromes significantly increase the risk of developing CRC. Examples of these syndromes include Lynch syndrome (hereditary non-polyposis colorectal cancer or HNPCC) and familial adenomatous polyposis (FAP). However, it is important to note that there are other genetic syndromes that also elevate the risk of CRC. These include Peutz-Jeghers

syndrome, juvenile polyposis syndrome, polymerase proofreading-associated polyposis (PPAP), and serrated polyposis syndrome. Individuals with these syndromes carry specific gene mutations that greatly raise their susceptibility to developing CRC, and in some cases, other types of cancer as well *(Chen et al.,2022)*. To effectively manage the risk associated with these genetic syndromes, genetic counseling, and testing are crucial for individuals with a family history of these conditions. This allows for the identification of potential gene mutations and enables the implementation of appropriate risk management strategies to reduce the likelihood of developing CRC and associated cancers.

- **Inflammatory Bowel Disease (IBD)**

Inflammatory bowel diseases, like ulcerative colitis (UC) and Crohn's disease (CD), are chronic conditions that lead to inflammation in the digestive tract. Individuals with long-standing and extensive IBD face a heightened risk of developing CRC when compared to the general population. To detect any precancerous changes early, it is crucial for these patients to undergo regular monitoring and surveillance. The risk of CRC in IBD is influenced by factors such as the duration and extent of inflammation, the presence of dysplasia, and other risk factors, including a family history of CRC. Therefore, regular surveillance colonoscopy is of utmost importance in identifying dysplasia or early cancer and reducing the CRC risk in IBD patients. It is noteworthy that most CRC in IBD follows a different path than the traditional sporadic adenoma-to-carcinoma sequence, instead characterized by non-polypoid dysplasia with late APC mutations *(Lucafò et al., 2021)*.

- **Diabetes**

Numerous studies have demonstrated a potential association between type 2 diabetes and an elevated risk of CRC. Although the precise mechanisms connecting diabetes and CRC remain not entirely elucidated, factors like insulin resistance, chronic inflammation, and shared lifestyle elements are suspected to contribute. Managing diabetes through a healthy lifestyle and appropriate medical care holds promise in mitigating this risk. Patients with diabetes face an increased likelihood of developing colorectal adenomas due to hyperglycemia and hyperinsulinemia, both prevalent in diabetes. These conditions may play a role in heightening CRC risk by inducing chronic inflammation and oxidative stress, potentially damaging DNA. Consequently, individuals with diabetes are advised to undergo regular screening for colorectal adenomas and collaborate closely with their healthcare providers to manage blood sugar levels effectively, thereby reducing their risk of developing diabetes-related complications, including CRC. Proactive monitoring and targeted intervention strategies can significantly benefit

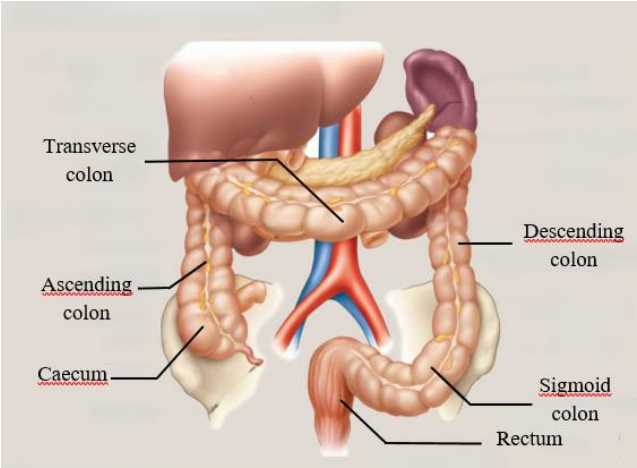
individuals at risk, promoting early detection and timely management of potential CRC development *(Yu et al., 2022)*.

I.2 Colorectal Pathophysiology

I.2.1 Structure and Function of the Colon

I.2.1.1 Anatomy of the Colon

The colon, also known as the large intestine, is a tubular organ that stretches from the cecum to the rectum. It has a diameter of about 150 to 180 cm and spans a length of approximately 1.5 meters (Siri et al., 2020). The colon can be categorized into three primary segments: the cecum, colon (consisting of ascending, transverse, descending, and sigmoid portions), and rectum, which connects to the anal canal *(Bazira 2023)* (**Figure 7)**.



Source: From Figure 65.1, page 1186 in Standring, S. (2021). Gray's Anatomy (42nd ed.) Philadelphia, PA: Elsevier. *(Bazira 2023)*. **Adapted**

Figure 7:Anatomy of the Colon

I.2.1.2 Histology of the Colon

From a histological perspective, the colon is structured with four concentric layers, arranged from the innermost to the outermost (**Figure 8)**:

- Mucosa: This layer includes an epithelium (epithelial layer), a lamina propria, and a muscular layer of the mucosa (muscularis mucosae).
- Submucosa: Comprised of loose connective tissue.
- Muscular propria: Consisting of an inner circular layer and an outer longitudinal layer.
- Serosa: The outermost layer.

The colonic mucosa is primarily composed of glandular epithelium resting on a basement membrane, which overlays a connective tissue called the chorion. Positioned above

the chorion is a thin layer of muscle known as the muscularis mucosae. Within the epithelium, there are straight, tightly packed tubular glands called crypts of Lieberkühn, which accommodate various types of cells *(Siri et al., 2020)*.

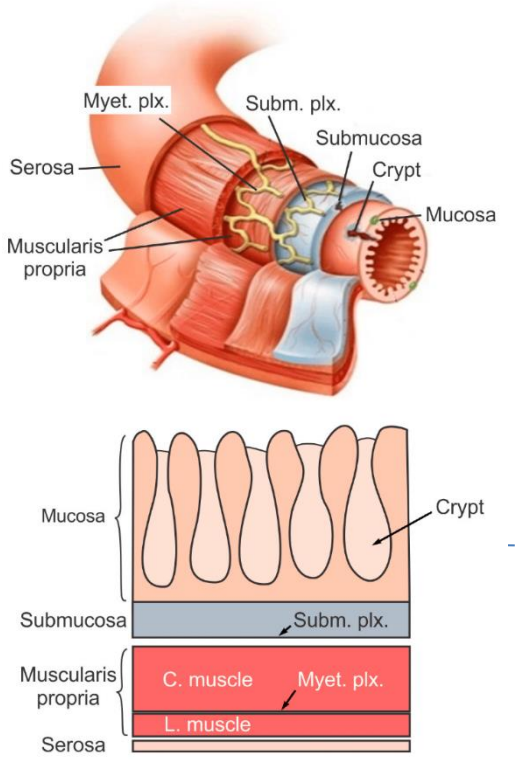


Figure 8: Illustration of the Structural Layers in the Large Intestine *(Siri et al., 2020)*.

I.2.1.3 Functions of the colon

The colon performs three primary functions that are essential for proper digestion and overall digestive health: water absorption, feces storage, and nutrient absorption. Additionally, it plays a vital role in mucus production and acts as a defense mechanism against harmful bacteria *(Kahai et al., 2023)*.

- **Water Absorption**: The colon facilitates the extraction of water from digested food within the small intestine, thereby contributing to the formation of solid feces.
- **Feces Storage**: Acting as a reservoir, the colon temporarily holds feces until they can be expelled from the body.
- **Nutrient Absorption**: In addition to the small intestine, the colon absorbs specific nutrients like electrolytes and short-chain fatty acids that were not previously absorbed.
- **Mucus Production**: A vital function of the colon involves the secretion of mucus to facilitate the smooth passage of feces through the colon and rectum.

- **Defense Against Harmful Bacteria**: The colon hosts a substantial population of beneficial bacteria, which play a crucial role in safeguarding against harmful bacteria and viruses.

I.2.1.4 The rectum

The rectum, located in the anal canal between the rectosigmoid junction and the dentate line, has a cylindrical shape and measures around 12 to 15 cm in length. Its diameter changes, with a narrower portion at the junction with the sigmoid colon and a wider section beyond. Its primary function is to store fecal matter before it is eliminated through the anus. Similar to the colon, the rectal wall is composed of four distinct layers that overlap each other: the mucosa, deep mucosa, submucosa, muscularis propria, and serosa *(Wang and Wiseman 2023)* **(Figure 9)**.

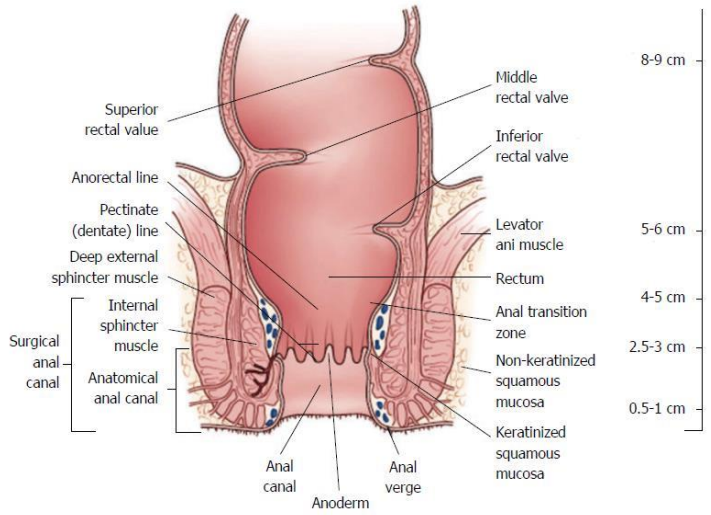


Figure 9: Schematic coronal section of the rectum and anal canal *(Gaertner et al., 2015)*.

I.2.2 Carcinogenesis Phases

The development of colorectal cancer is a complex process that occurs over several years. It involves several stages, including initiation, promotion, and progression *(Siddiqui et al.2015)* **(Figure 10)**.

- **Initiation**

Initiation is the first stage of colorectal carcinogenesis. It occurs due to exposure to certain carcinogens, which may be present in the diet, environment, or lifestyle. These carcinogens cause damage to the DNA in the cells lining the colon or rectum, leading to mutations that alter the genetic code. These mutations can interfere with the normal cell cycle, resulting in uncontrolled cell growth. While initiation can result in the formation of a pre-cancerous lesion, it does not necessarily guarantee progression to cancer.

- **Promotion**

The second stage of colorectal carcinogenesis is known as promotion. During this stage, the mutated cells in the colon or rectum start to divide more rapidly. This is due to changes in the surrounding tissue and microenvironment, such as chronic inflammation or the release of growth factors. This increase in cell division can lead to the formation of a polyp, which is a small, benign growth in the colon or rectum. Some polyps can remain benign for years, while others may transform into cancerous growths.

- **Progression**

The final stage of colorectal carcinogenesis is progression. During this stage, the cancerous cells in the colon or rectum continue to grow and invade adjacent tissues. They may also enter the bloodstream and metastasize to other parts of the body. The severity of cancer at this stage is determined by factors such as the size and location of the tumor, as well as whether it has spread to other organs.

The transformation of a normal cell into a cancerous cell involves acquiring new characteristics, including:

- Independence from normal cell growth signals.
- Evasion of programmed cell death.
- Ability to divide indefinitely.

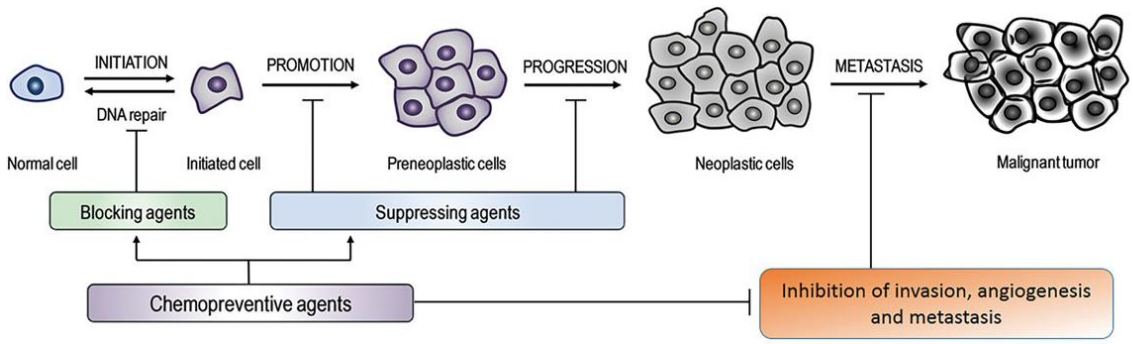


Figure 10: The Cascade of Carcinogenesis: Initiation, Promotion, and Progression *(Siddiqui et al.2015).*

I.2.2.1 Adenoma to Carcinoma Sequence

Colorectal tumorigenesis typically starts in the epithelial cells of the mucosa. The majority of colorectal cancers stem from an initial benign growth known as an adenoma. Over time, these adenomas can progress and transform into cancerous cells **(Figure 11)**. In advanced stages, colorectal cancer has the potential to spread to distant sites, with the liver, lungs, and peritoneum being the most common locations for metastasis *(Ares and Estevez-Boullosa 2011)*.

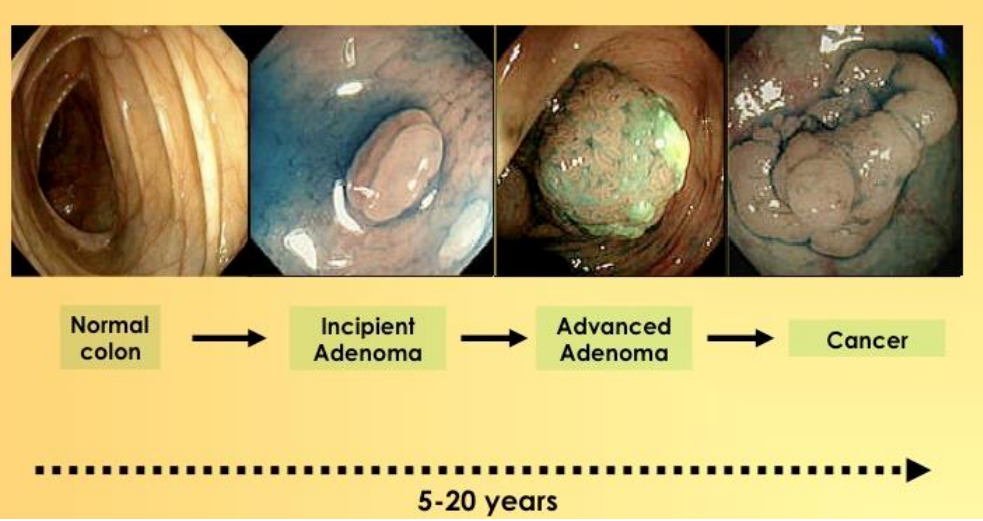


Figure 11: Progression of Colorectal Carcinomas: From Normal Colon to Invasive Carcinoma *(Ares and Estevez-Boullosa 2011)*.

From a morphological perspective, CRC is a multi-step process characterized by the development of several lesions described below *(Simon 2016)* **(Figure 12)**:

- **Aberrant Crypt Foci**: These are early microscopic lesions characterized by clusters of abnormal crypts in the colon or rectum. They are considered potential precursors to adenomas and carcinomas *(Wargovich et al., 2010)*.
- **Adenomatous Polyps**: Most CCRs develop from pre-existing polyps, which are potentially serious precancerous conditions. Polyps are localized growths in the intestinal lining. The risk of CCR depends on the number and size of the polyps *(Simon 2016)*.

  There are two main types of polyps with malignant potential:

  - **Adenomas**: These can be further classified into three types - villous, tubular, and tubulovillous. Adenomas have a greater potential for harboring cancerous cells, especially those with a villous component of 25% or more.
  - **Serrated sessile polyps**: These are flat polyps with serrated or saw-toothed glands. They account for 25-35% of all CCRs.

- **Dysplasia**: Dysplasia is a term used to describe the presence of abnormal cells in the lining of the colon or rectum. These cells have the potential to become cancerous and are therefore considered precancerous. Dysplasia is identified by changes in the appearance of the cells, including their size, shape, and organization. Additionally, dysplastic cells exhibit increased cell division and may have abnormal nuclei *(Pulusu and Lawrance 2017)*.
- **Adenocarcinoma**: Adenocarcinoma is the most common type of CRC, accounting for about 90% of cases. It can be classified into two main types based on their progression. The first type is in situ adenocarcinoma, which develops from severe dysplasia and remains confined to the surface of the colon or rectum without invading the muscular mucosa. The second type is invasive adenocarcinoma, where cancer cells invade not only the muscular mucosa but also the deeper layers of the colon and rectum, increasing the risk of metastasis *(Fleming et al., 2012)*.
- **Metastases**: Metastasis is common in CRC and can spread through the bloodstream or lymphatic system. Approximately 21% of patients have metastases at the time of diagnosis, which can occur in various sites such as lymph nodes, liver, lung, peritoneum, brain, and bone *(Chakedis and Schmidt 2017)*.

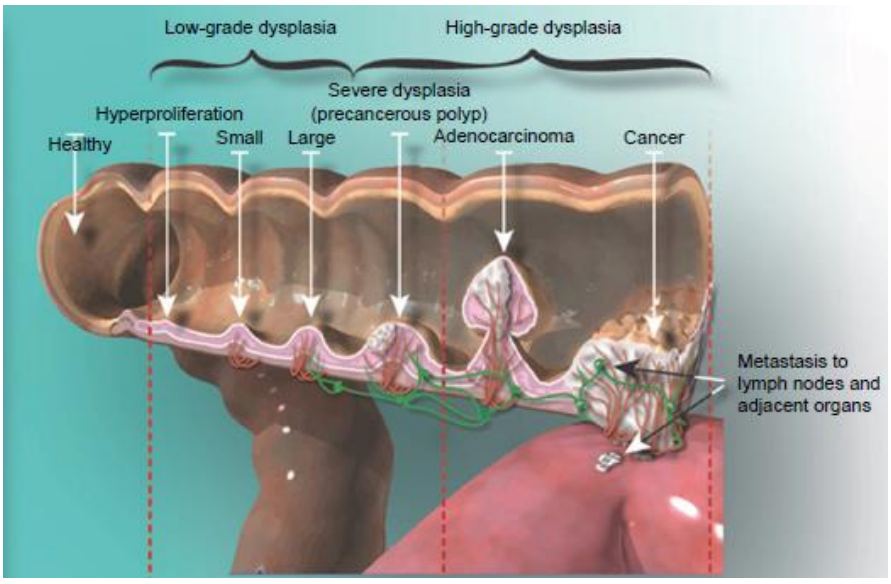


Figure 12: From Healthy Colon to Invasive Carcinoma: An Illustrated Journey of CRC Development *(Simon 2016)*.

I.3 TNM Staging System for CRC: Classification and Staging

Accurate tumor classification plays a pivotal role in clinical practice as it guides the selection of optimal treatment and management strategies for patients. In the case of CRC detection, the tumor is initially assessed anatomically and histologically, enabling the assignment of a stage and grade to the tumor, respectively.

I.3.1 Anatomical Stage or TNM Stage

The American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging system is the most widely used prognostic standard for CRC. It has undergone multiple revisions over the past few decades, with the latest edition being the eighth edition published by the Union for International Cancer Control (UICC) in 2017. This internationally recognized pTNM classification system provides comprehensive information about the anatomical extension of CRC, including the local invasion (T), involvement of regional lymph nodes (N), and distant metastasis (M). By accurately categorizing tumors, the TNM staging system assists in determining prognosis and guiding treatment decisions for patients with CRC *(Weiser 2018)*. The classification system consists of three components **(Table 2)** *(Amin et al., 2017)*:

- The T (**Tumor**) classification, represented by a number ranging from 0 to 4, denotes the depth of tumor infiltration. Higher T numbers indicate larger tumors that have extended into adjacent tissues.
- The N (**Node**) classification, followed by a number from 0 to 3, indicates the involvement of lymph nodes near the colon. A higher N number suggests a greater extent of lymph node metastasis.
- The M (**Metastasis**) classification, indicated by either 0 or 1, signifies the presence or absence of distant organ metastasis or lymph node involvement in locations other than those near the colon, such as the lungs or bones.

Table 2: Staging of CRC according to the 8th edition of the AJCC Cancer Staging Manual *(Amin et al., 2017).*

Tumor = T	Node = N	Metastasis = M
T0: Clinically undetectable tumor.	**N0**: No regional lymph node metastasis.	**M0**: No distant metastases.
Tis: Intraepithelial or intramucosal carcinoma (in situ cancer).	**N1**: 1 to 3 regional lymph nodes.	**M1a**: Localized metastasis to a single organ, excluding regional lymph nodes, such as the liver, lung, ovary, or other specific organs.
T1: Tumor invades submucosa	**N1a**: 1 regional lymph node.	**M1b**: Metastases in multiple organs or peritoneal.
T2: Tumor invades muscularis propria	**N1b**: 2-3 regional lymph nodes.	
T3: Tumor invades through muscularis propria into subserosa or non-peritonealised pericolic tissues	**N1c**: Tumor satellites in the subserosa without metastatic lymph nodes.	
T4: Tumour directly invades other organs or structures and/or perforates visceral peritoneum	**N2**: ≥ 4 regional lymph nodes.	
T4a: Penetration of the visceral peritoneum.	**N2a**: 4-6 regional lymph nodes.	
T4b: Invasion of a neighboring structure.	**N2b**: ≥ 7 regional lymph nodes.	

I.3.2 Histological Grade

The histological grade of a tumor, determined through examination of a biopsy, reflects the degree of dysplasia and differentiation rate of tumor cells, indicating its aggressiveness. There are four histological grades: G1 (well-differentiated), G2 (moderately differentiated), G3 (poorly differentiated), and G4 (undifferentiated) **(Figure 13)**. The TNM characteristics assigned to cancer can be grouped into five stages, designated by Roman numerals I to IV. Stage 0 corresponds to in situ cancers. The TNM staging system, referred to as pTNM, is based on microscopic examination of surgical specimens or biopsies and provides a more accurate prognostic estimate *(Amin et al., 2017)* **(Table 3) (Figure 13 & Figure 14)**.

Table 3: TNM-Based Staging of CRC according to the 8th Edition of the AJCC Cancer Staging Manual, 2017 *(Amin et al., 2017).*

Stage	Tumor = T	Node = N	Metastasis = M
0	pTis		
I	pT1-2		
IIA	pT3	N0	
IIB	pT4a		M0
IIC	pT4b		
IIIA	pT1-T2 et pT1	N1/N1c - N2a	
IIIB	pT3-T4a, pT2-T3, pT1-T2	N1/N1c - N2a -N2b	
IIIC	pT4a ; p T3-T4a; pT4b	N2a - N2b - N1 -N2	
IVA			M1a
IVB	Any T	Any N	M1b
IVC			M1c

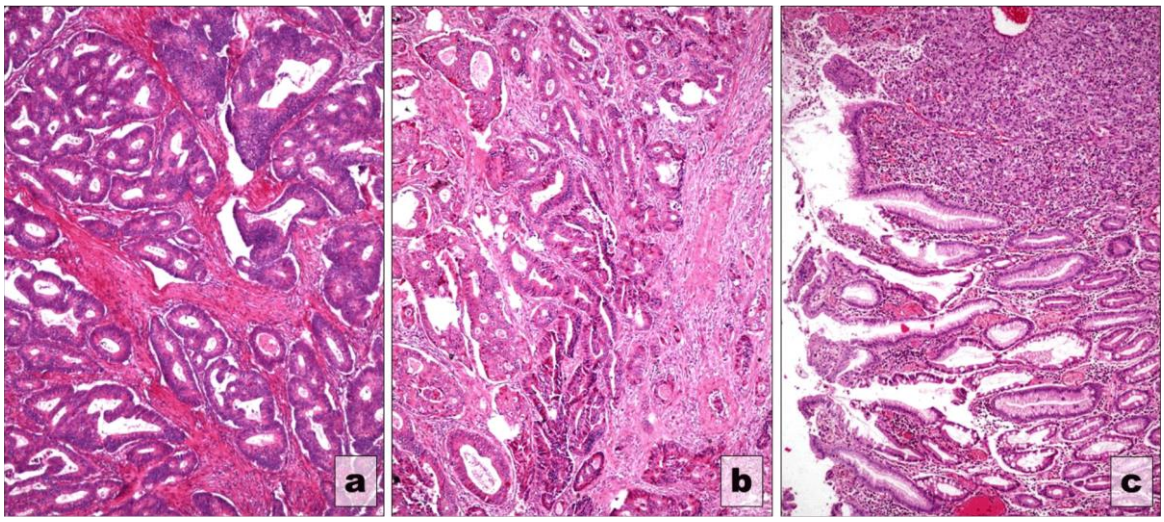


Figure 13:Tumor grading: (a) Well-differentiated adenocarcinoma; (b) Moderately differentiated adenocarcinoma; (c) Poorly differentiated adenocarcinoma *(Dumitrescu et al.,2015).*

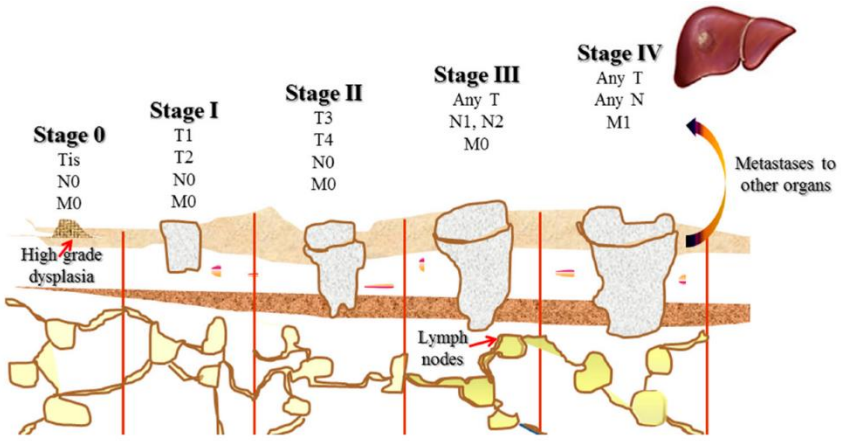


Figure 14: Colorectal Cancer Classification by the American Joint Committee on Cancer *(Lin et al., 2015).*

I.4 Molecular Aspects: Characteristic Genetic Alterations in Colorectal Cancers

CRC is a complex disease that arises from the accumulation of genetic and epigenetic alterations in the colonic epithelium. Several key genetic pathways have been implicated in the development and progression of CRC, including chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP). These pathways are characterized by specific genetic alterations that affect critical genes involved in cell cycle regulation, DNA repair, and signaling pathways. For example, mutations in the APC, KRAS, and TP53 genes are commonly found in CRC and are associated with the initiation and progression of the disease. Other genetic alterations, such as mutations in the PIK3CA gene and inactivation of the TGF-β receptor, have also been implicated in the development of CRC. Understanding the molecular basis of CRC is critical for the development of effective diagnostic and therapeutic strategies for this disease *(Dey et al., 2023)*.

I.4.1 Genomic and Epigenomic Instability

The genomic and epigenomic instability of CRC is a complex phenomenon that involves a wide range of genetic and epigenetic alterations **(Figure 15)**.

I.4.1.1 Chromosomal Instability (CIN)

CRC is often characterized by chromosomal instability (CIN), resulting in changes in chromosome number and structure, including deletions, gains, translocations, and other rearrangements. CIN is the primary cause of sporadic CRC, accounting for approximately 84% of cases *(Müller et al., 2016)*.

The generation of CIN in CRC can be attributed to several factors, such as the loss of heterozygosity (LOH+), telomere dysfunction, abnormalities in chromosomal segregation, and modifications in the DNA damage response system (Pino and Chung 2010). Within the CIN pathway, the most commonly affected genes are APC, p53, and KRAS, accounting for around 84% of cases *(Aran et al., 2016)*.

I.4.1.2 Microsatellite Instability (MSI)

Microsatellites are short, repetitive sequences of DNA composed of 1-6 base pairs. During DNA replication, errors can occur in these sequences, which are normally identified and repaired by the mismatch repair (MMR) genes. However, when the MMR genes are defective, cells may exhibit abnormal lengths of microsatellite repeats, leading to a condition known as microsatellite instability (MSI) *(Pačínková and Popovici 2019)*.

MSI was initially observed in patients with Lynch syndrome or hereditary non-polyposis colorectal cancer (HNPCC). This syndrome is caused by a genetic mutation in two genes: MSH2 (Muts Protein Homolog 2) and MLH1 (MulT Homolog 1), which are involved in the DNA mismatch repair system. In sporadic cases of CRC, MSI can occur due to either hypermethylation of the MLH1 promoter or a mutation in the MSH2 gene. Approximately 15% of sporadic colon cancer cases exhibit MSI. These abnormalities in the MMR system result in replication errors, particularly in microsatellite regions of DNA, contributing to the development of CRC (Dey et al., 2023). The prevalence of MSI-H CRC decreases in advanced stages and represents around 20% of cases in stage I-II, 12% in stage III, and 4% in stage IV CRC *(Mondaca and Yaeger 2019)*.

I.4.1.3 CpG Island Methylator Phenotype (CIMP)

The CpG island methylator phenotype (CIMP) is a pathway of genomic instability present in approximately 20% of CRC cases. It is frequently associated with mutations in the BRAF, KRAS genes, and microsatellite instability. CIMP is characterized by excessive methylation of CpG islands in the promoter regions of tumor suppressor genes, leading to the silencing of gene transcription and reduced protein expression. In CIMP-positive tumors, several markers, including MINT1, MINT31, MINT2, p16, and MLN1, are commonly hypermethylated *(Dey et al., 2023)*. Among these markers, the MLH1 gene's promoter region is frequently hypermethylated, resulting in decreased MLH1 protein expression. This methylation of MLH1 contributes to microsatellite instability in CIMP-positive tumors, following the distinct CIMP pathway. Recognizing the specific characteristics and markers associated with CIMP-positive tumors is crucial for distinguishing them from other types of

colorectal cancer. Notably, CIMP is more frequently observed in the proximal colon, while its occurrence in rectal tumors is relatively uncommon *(Mondaca and Yaeger 2019)*.

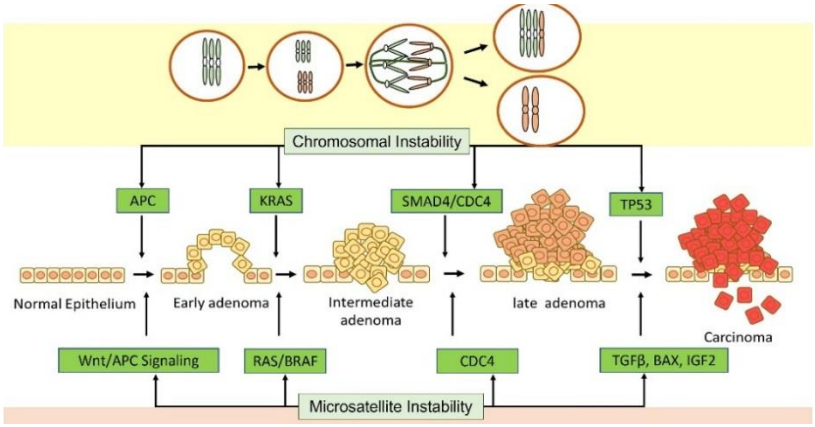


Figure 15: Illustration of the Interplay between CIN and MSI Pathways in the Progression of CRC *(Dey et al., 2023).*

I.5 The Landscape of Somatic Point Mutations in Colorectal Tumors

Somatic point mutations are frequently observed genetic alterations in CRC. They can be categorized into two sections: passenger mutations and driver mutations. In CRC, driver mutations are commonly identified in genes like APC, KRAS, and TP53, which play a crucial role in regulating cell growth and division **(Figure 16)**. The accumulation of somatic mutations over time can lead to genomic instability and the emergence of more aggressive cancer subtypes. Understanding the significance of somatic point mutations in CRC is crucial for the development of targeted therapies and enhancing patient outcomes.

Passenger mutations are genetic alterations that do not contribute to the development or progression of cancer. They are typically harmless and do not affect the functioning of genes or proteins within the cell. On the other hand, driver mutations actively promote the growth and progression of tumors by triggering uncontrolled cell proliferation and the formation of tumors. Driver mutations are often found in oncogenes, which stimulate cell growth, or tumor suppressor genes that normally prevent cells from becoming cancerous *(Aparisi et al., 2019)*.

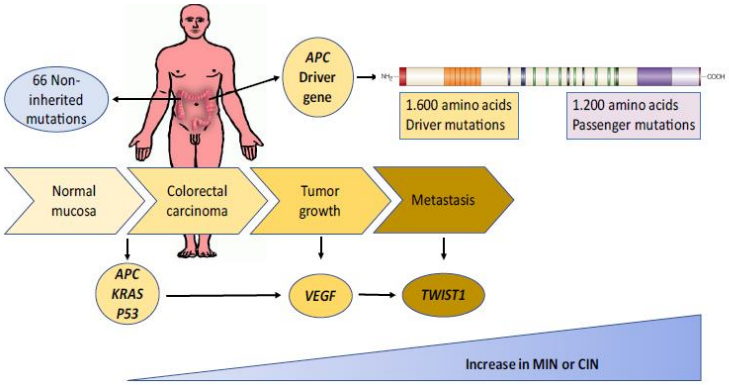


Figure 16: Genomic evolution of colorectal cancer: the impact of somatic mutations *(Aparisi et al., 2019).*

The cellular signaling pathways that regulate cellular processes such as cell survival, proliferation, apoptosis, adhesion, differentiation, and angiogenesis have been linked to the development of CRC. These pathways often intersect and include the RAS/EGFR/MAPK pathway, the Notch pathway, the PI3K pathway, the TGF-β pathway, and the Wnt pathway **(Figure 17)** (Koveitypour et al., 2019).

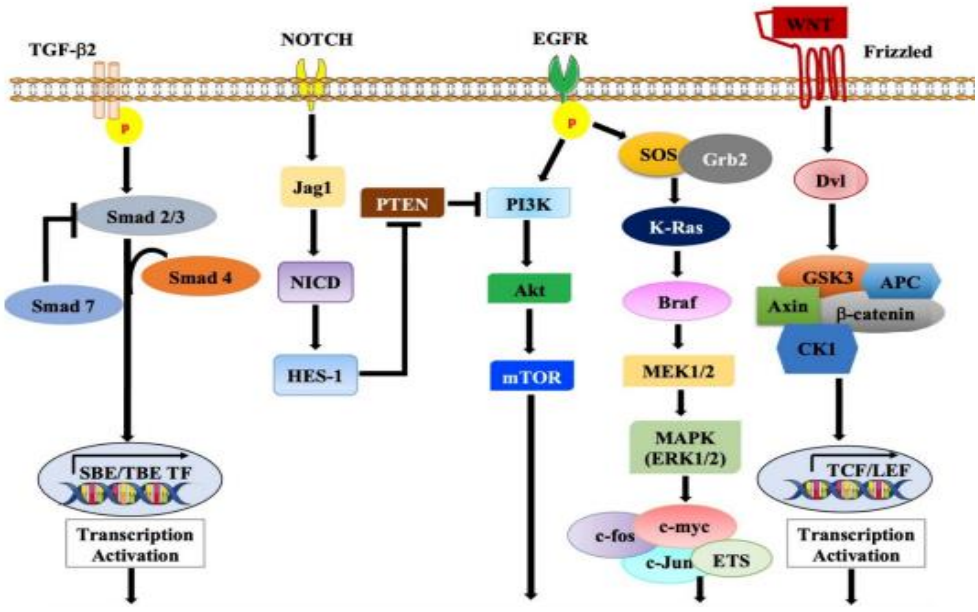


Figure 17: A comprehensive illustration of signaling pathways in CRC *(Malki et al., 2020).*

I.6 Consensus Molecular Subtypes (CMS) of CRC

The ColoRectal Cancer Subtyping Consortium (CRCSC) analyzed gene expression data from thousands of CRC patients to develop the consensus molecular subtypes (CMS) of CRC. Proposed by Guinney et al. in 2015, CMS categorizes CRC into four distinct subtypes based on gene expression patterns *(Guinney et al., 2015)* **(Table 4)**. The four subtypes are characterized by different biological features, clinical outcomes, and responses to treatment. CMS1, also known as the immune-mediated subtype, is associated with immune activation. CMS2, also known as the canonical subtype, is characterized by canonical Wnt signaling. CMS3, also known as the metabolic subtype, is associated with metabolic dysregulation. CMS4, also known as the mesenchymal subtype, is characterized by stromal invasion. CMS has been shown to be a useful tool for predicting patient outcomes and guiding treatment decisions *(Malki et al., 2020)*.

Table 4: Comparison of Clinicopathological Features and Molecular Characteristics of Colorectal Cancer Subtypes *(Guinney et al., 2015).*

	CMS1	**CMS2**	**CMS3**	**CMS4**
Alternate name	MSI immune	Canonical	Metabolic	Mesenchymal
Incidence	14%	37%	13%	23%
Molecular Features	MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
Mutations	**BRAF mutations**		**KRAS mutations**	
Signatures	Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Clinical Features	Worse survival after relapse			Worse relapse-free and overall survival (OS)

I.7 Diagnosis

Early diagnosis is crucial for the effective management of CRC, as it significantly improves the prognosis and enhances the chances of successful treatment. Stool tests, endoscopy, imaging, and other relevant approaches are some of the diagnostic methods used to identify CRC. These methods play a vital role in the early detection and management of CRC.

I.7.1 Stool Tests

I.7.1.1 Fecal Occult Blood Test (FOBT)

FOBT is a non-invasive screening test that can detect small amounts of blood in the stool, which may indicate the presence of CRC or other gastrointestinal disorders. FOBT can be either guaiac-based (gFOBT) or immunochemical (FIT). Both types of tests are designed to detect hidden blood in the stool. The gFOBT uses a chemical reaction to detect the presence of heme in the stool, while the FIT uses antibodies to detect human hemoglobin *(Ramdzan et al., 2019)*. If the FOBT results are positive, further investigation through colonoscopy is necessary, as it is the gold standard for CRC screening and diagnosis. FOBT is an economical and simple test that can be performed at home or in a clinical setting, making it a convenient option for many individuals.

I.7.1.2 Fecal Immunochemical Test (FIT)

FIT is another type of stool test that detects the presence of human hemoglobin in the stool. FIT is more specific than gFOBT and has a higher sensitivity for detecting CRC. FIT is also a non-invasive test that can be performed at home or in a clinical setting. If the FIT results are positive, further investigation through colonoscopy is necessary *(Lee and Lee 2018)*.

I.7.1.3 Multi-target Stool DNA test (MT-sDNA)

The multi-target stool DNA test (MT-sDNA), also known as the Stool DNA test, is a newer screening method for CRC. Unlike other tests that require patients to collect multiple stool samples or undergo bowel preparation, MT-sDNA uses a single random stool sample that patients can collect at home without any special preparation or dietary restrictions. The test identifies specific genetic alterations and abnormal DNA associated with CRC and can also detect DNA mutations linked to pre-cancerous polyps. MT-sDNA provides a higher sensitivity rate than traditional FOBT, which means it can detect more cases of CRC and pre-cancerous polyps *(Berger et al., 2016)*.

I.7.2 Endoscopic Procedures

I.7.2.1 Colonoscopie

Colonoscopy is a highly effective tool for the diagnosis and prevention of CRC, as it allows for the visualization of the entire large bowel and the distal part of the small bowel using a flexible, long endoscope. This screening technique involves the use of a flexible tube with a camera and light at the end to thoroughly examine the colon and rectum, enabling physicians to identify and remove any polyps or abnormal tissue that may be present. Despite some limitations, such as patient adherence and operator-dependence, colonoscopy is widely recognized as the gold standard for CRC screening and prevention in many regions and countries *(Hadjipetrou et al.,2017)*.

I.7.2.2 Flexible Sigmoidoscopy (FS)

Similar to colonoscopy, flexible sigmoidoscopy examines the rectum and the lower part of the colon. However, it does not reach the entire colon as colonoscopy does. This procedure is often used as a screening tool, and if any abnormalities are detected, a full colonoscopy is recommended *(Hadjipetrou et al.,2017)*.

I.7.3 Imaging Techniques

I.7.3.1 Computed Tomography (CT) Scan

Computed Tomography (CT) scan is a medical imaging test that utilizes X-rays and computer technology to produce detailed images of the body. CT scans can provide detailed

images of the colon and surrounding structures, which can help detect large tumors and any potential spread to nearby lymph nodes or other organs. Although CT scans can be used to detect CRC, they are not recommended as a routine screening test for the general population. Instead, CT scans are more commonly used for preoperative assessment and postoperative surveillance for recurrence of CRC. CT scans can help determine the location and size of the tumor, as well as whether the cancer has spread to other parts of the body. However, CT scans do have some risks, such as exposure to radiation and the use of contrast material, which can cause allergic reactions in some people *(Tan and Iyer 2010)*.

I.7.3.2 Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) screening, also referred to as Magnetic Resonance Colonography (MRC), presents a non-invasive alternative to colonoscopy for CRC screening. This approach proves particularly valuable for high-risk patients, such as individuals with Lynch syndrome gene mutation. Utilizing a potent magnet and radio waves, MRC produces highly detailed images of the colon and rectum without the need for sedation or analgesics. It represents a less invasive option for CRC screening; however, additional research is imperative to establish its comparative effectiveness against colonoscopy *(Lim et al., 2010)*.

I.7.4 Biopsie and Pathology

Biopsy and pathology are critical components of the diagnostic process for CRC. When abnormalities are detected, biopsies are performed to obtain tissue samples for examination by pathologists. Through histopathological evaluation, pathologists identify cancer cells and determine the cancer type and grade. Staging, using the TNM system, assesses the extent of cancer spread. Molecular pathology plays an increasingly important role in identifying biomarkers that guide treatment decisions, such as KRAS and BRAF mutations, MSI, and MMR status. These biomarkers can help predict response to chemotherapy and immunotherapy and inform decisions about adjuvant therapy. Biopsy and pathology are essential for confirming CRC, providing critical information for treatment planning, and contributing to improved personalized approaches for CRC patients *(Fleming et al., 2012)*.

II. Exploring the Molecular Mechanisms of the *RAS* Signaling Pathway

The RAS signaling pathway continues to stand as a captivating subject of research, offering profound insights into cellular communication and wielding considerable influence over cell growth, differentiation, and survival. As scientific exploration progressed, the revelation of RAS oncogene activation due to DNA and protein mutations catapulted the field into a realm

of heightened fascination, propelling researchers to delve deeper into the molecular underpinnings of cancer etiology. Through relentless investigation, scientists unveiled the intricate interplay of oncogene activation and tumor suppressor gene inactivation, both orchestrated by intricate changes in protein structure and function. At its core, the RAS signaling pathway presents itself as an intricate web of proteins orchestrating a symphony of cellular processes, underscoring its pivotal role. Intriguingly, dysregulation of this pathway has emerged as a linchpin in the genesis and progression of diverse human cancers, with CRC featuring prominently among them. Hence, a profound grasp of the intricate molecular tapestry that constitutes the RAS signaling pathway remains indispensable, forming the bedrock upon which the edifice of effective cancer therapies can be erected. Armed with this knowledge, researchers and clinicians alike are primed to forge innovative therapeutic avenues that could potentially tame the aberrant RAS pathway and revolutionize the landscape of cancer treatment *(Fernández-Medarde et al., 2021)*

II.1 RAS gene

The RAS gene family stands as a pivotal constellation of related genes, orchestrating a complex symphony of cellular signaling pathways. These genes encode proteins that wield remarkable influence over a multitude of fundamental cellular processes. At the heart of this gene family lies the RAS oncogene, a linchpin in the intricate web of cell growth and regulation. Its protein product serves as a nexus for myriad cellular functions, orchestrating a harmonious interplay that encompasses not only cell proliferation, but also apoptosis, migration, fate specification, and differentiation. The intricate choreography conducted by the RAS gene family extends across a diverse array of cellular contexts, underscoring its role as a master regulator *(Shah et al., 2019)*. Through its ability to mediate cellular responses to external cues, internal conditions, and developmental cues, the RAS gene family effectively bridges the realms of extracellular stimuli and intracellular processes. This unique positioning allows these genes to exert a profound impact on cellular fate, exerting their influence from the earliest stages of embryogenesis to the intricacies of adult tissue maintenance. The intricate involvement of the RAS gene family in such fundamental aspects of cellular life illuminates its significance as a focal point of scientific inquiry. A deeper understanding of the mechanisms underlying the activity of the RAS gene family holds vast promise, not only for unraveling the intricacies of cellular behavior, but also for inspiring novel therapeutic strategies that could potentially be harnessed to intervene in diseases where its dysregulation plays a decisive role *(Arrington et al., 2012)*.

II.1.1 The History and Significance of the RAS Gene Family

For decades, the RAS gene family has remained a focal point of intensive investigation within the realm of cancer biology. In 1975, a pivotal milestone was reached when Scolnick and colleagues at the National Institutes of Health (NIH) identified the initial RAS gene. This discovery emerged from their groundbreaking research on the Harvey sarcoma virus and the Kirsten sarcoma virus, both notorious for inducing sarcoma formation in animals and triggering cell transformation in culture settings. A monumental leap transpired in 1982 with the identification of human RAS genes exhibiting mutationally activated traits, substantially advancing our comprehension of this family's implications **(Figure 18)** *(Cox and Der 2010)*.

The RAS gene family comprises three closely related genes: KRAS, NRAS, and HRAS. While KRAS (Kirsten rat sarcoma viral oncogene homolog) and NRAS (neuroblastoma RAS viral oncogene homolog) mutations recurrently surface in human cancers, HRAS (Harvey rat sarcoma viral oncogene homolog) held sway during the early annals of RAS exploration. Nonetheless, a perceptible shift has lately unfurled, focusing more attention on KRAS, deemed vital for mouse development but not shared by NRAS or HRAS. A glance at the COSMIC database reveals HRAS as the least frequently mutated gene in human cancers, whereas KRAS mutations dominate, closely pursued by NRAS mutations *(Fernández-Medarde et al., 2021)*.

Subsequent to these pivotal revelations, researchers have ardently devoted themselves to unraveling the intricate structure, biochemistry, and biological implications of RAS proteins. Operating as small GTPases, RAS proteins undertake the role of molecular switches in signaling pathways that intricately govern cell growth, differentiation, and survival. Their significance transcends various cellular processes, encompassing proliferation, migration, and apoptosis. Perturbations stemming from mutations in RAS genes can trigger aberrant pathway activation, thereby intricately contributing to the onset and advancement of diverse cancer types *(Han et al., 2017)*.

Yet, even after more than four decades since their discovery, RAS proteins have proven a formidable challenge as targets for cancer therapy. This is largely attributed to their strong affinity for GTP and intricate interactions with downstream effectors. However, recent strides in understanding their underlying structure and functions have kindled renewed optimism for the potential development of efficacious anti-RAS therapies *(Khan et al., 2020)*.

In summation, the saga of the RAS gene family chronicles a narrative of discovery, innovation, and unwavering determination in the face of substantial challenges. The unflagging

endeavors of researchers worldwide persistently expand the boundaries of comprehension, holding the promise of novel treatments and transformative cures on the horizon.

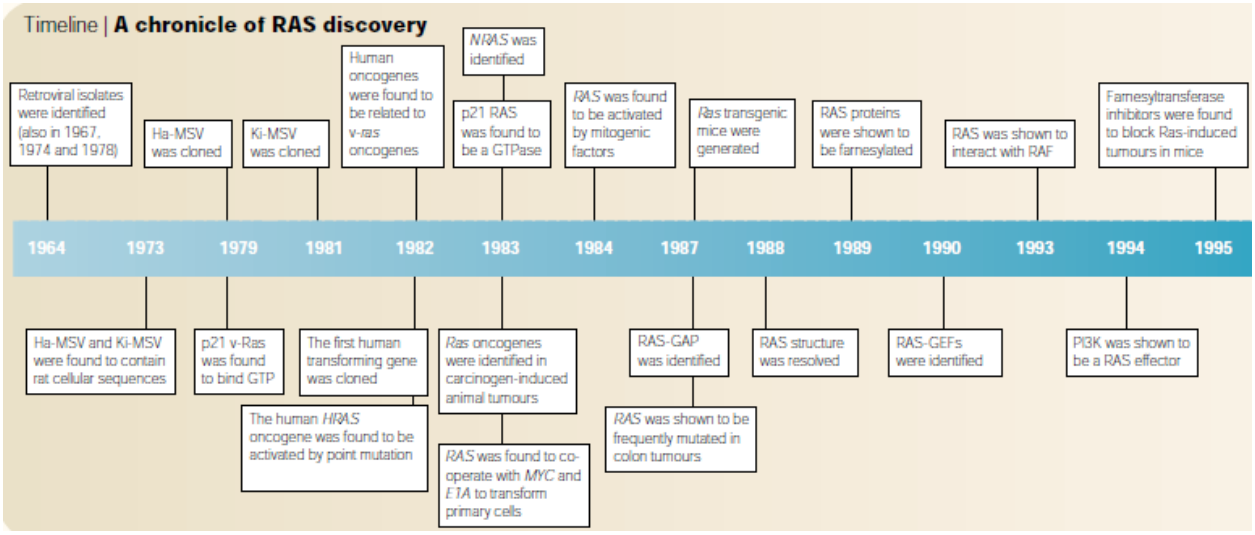


Figure 18: The Evolution of RAS Research: A Historical Overview *(Malumbres and Barbacid 2003).*

II.1.2 Oncogenic Potential and Heterogeneity of RAS Gene Mutations in Cancer

The RAS gene family, acknowledged for its prominent oncogenic role, frequently bears mutations across diverse human cancers. Missense mutations encompassing HRAS, NRAS, and KRAS genes collectively feature in approximately 27% of all cancer instances *(Hobbs et al., 2016)*. However, the incidence of these mutations varies contingent on the specific cancer type under scrutiny. Within the spectrum of RAS genes, KRAS mutations emerge as the most prevalent, constituting 86% of all RAS mutations in cancer occurrences. In contrast, NRAS mutations manifest less frequently, contributing to 11% of cases, while HRAS mutations, being the rarest, are detectable in a mere 3% of cases.

Forefronting this landscape, KRAS mutations manifest heightened oncogenicity, entangling themselves in the genesis of diverse cancers including pancreatic, colorectal, and lung malignancies. With pancreatic cancers, KRAS mutations attain a striking prevalence, encompassing around 90% of cases. A parallel scenario unfolds in CRC, where KRAS mutations figure prominently, accounting for 52% of cases. Notably, in CRC, KRAS assumes the mantle of the predominant mutated isoform, reigning at 86%. In contrast, NRAS mutations are a minority at 14%, and HRAS mutations have not yet been observed **(Figure 19)**.

The weight of these revelations situates KRAS as an alluring target for anti-cancer therapies, spotlighting its pivotal relevance *(Cox et al., 2014)*. This intricate web of RAS gene

mutations, varying prevalences, and their potent oncogenic contributions underscore the complexity and individuality characterizing the cancer landscape.

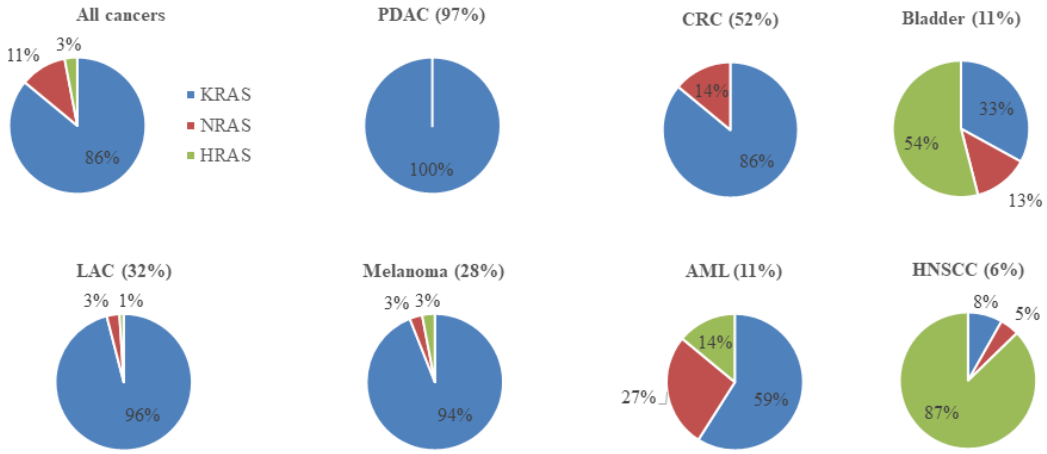


Figure 19: Frequency of RAS Mutations in Different Types of Cancer *(Cox et al., 2014)*

Abbreviations: PDAC: Pancreatic Ductal Adenocarcinoma; CRC: Colorectal Cancer; LAC: Lung Adenocarcinoma; AML: Acute Myelogenous Leukemia; HNSCC: Head and Neck Squamous Cell Carcinoma.

II.1.3 The Structure of the KRAS Gene

The KRAS gene, situated on the short arm of chromosome 12 (12p11.1-12p12.1) *(Parikh et al., 2022)* **(Figure 20)**, encodes the Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) protein. This protein holds membership within the RAS family of GTPase proteins, pivotal in the encoding of GTP/GDP-binding proteins. Spanning approximately 30 kb, the gene is constructed from six exons. Exons 1 and 2 serve as non-coding regions, while exons 3, 4, 5, and 6 assume roles as coding regions **(Figure 21)**. Remarkably, exon 2 emerges with the highest mutation rate, directly associating with adverse prognoses and drug resistance in CRC *(Meng et al., 2021)*.

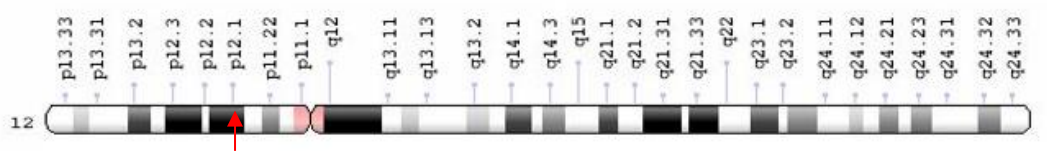


Figure 20:Genomic Location of KRAS gene

Source: KRAS Gene - KRAS Proto-Oncogene, GTPase. 2023

Available at: https://www.genecards.org/cgi-bin/carddisp.pl?gene=KRAS

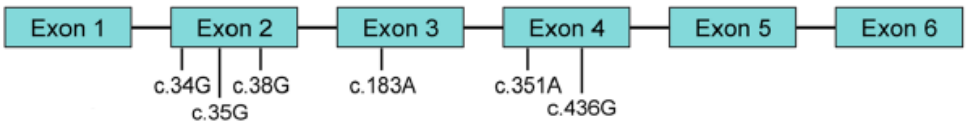


Figure 21: KRAS Gene Structure with Common Mutations Hostpots *(Parikh et al., 2022).*

Within the KRAS gene lies a distinctive characteristic—two splice variants, KRAS 4A and KRAS 4B, each emerging from distinct utilization of exons 4. Predominantly, KRAS 4B takes the spotlight as the principal isoform expressed in human cancers, boasting expansive and elevated expression levels. Notably, this prominence notwithstanding, KRAS 4A holds its ground by finding expression across various cancer types. Moreover, its presence assumes added significance, contributing to heightened adaptability of tumor cells when confronted with stressors **(Figure 22)** *(ZHU et al., 2021)*. This intricate interplay between splice variants unveils a facet of regulatory complexity inherent to the KRAS gene, hinting at its multifaceted contributions to cancer biology.

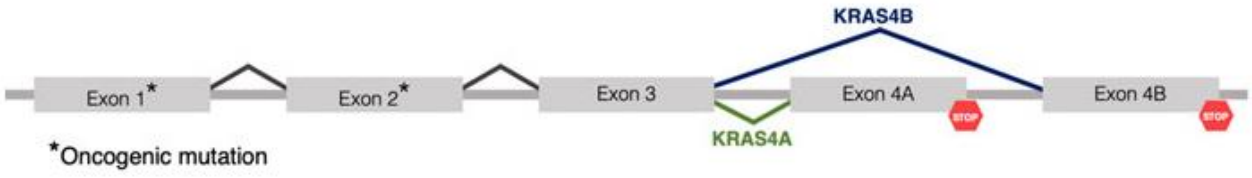


Figure 22: KRAS Splice Variants in Cancer: 4A and 4B Expression
(*Nuevo-Tapioles and Philips 2022).*

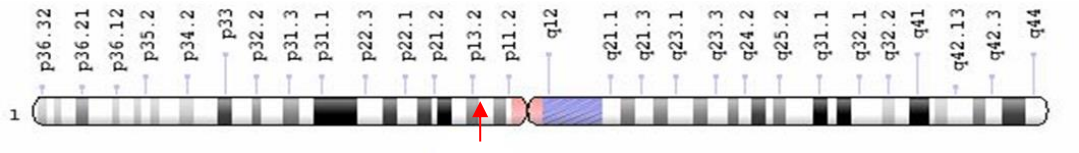
II.1.4 Structure of the *NRAS* Gene

As an integral member of the RAS gene family, the NRAS gene assumes a role of significant import, encoding a diminutive GTPase pivotal in steering cell signaling pathways. Anchored on the short arm of chromosome 1 (1p13.2) **(Figure 23)**, this gene stretches across a span of approximately 33 kb of DNA. Orchestrating its intricate symphony are seven exons, with their coding realms orchestrating the synthesis of the NRAS protein. Concurrently, six non-coding introns elegantly interplay, serving as segments excised during the gene's expression journey *(Eisfeld et al., 2014)*.

At its core, NRAS thrives as a molecular switch, orchestrating an oscillation between two distinctive states: the active (GTP-bound) and the quiescent (GDP-bound) configuration. This orchestrated oscillation serves as a conduit for the transmission of signals from the cell's surface receptors, ultimately orchestrating their delivery to the nucleus. This intricate relay, in turn, stands pivotal in calibrating a multitude of indispensable cellular processes, encompassing cell growth, division, and differentiation.

Unfolding upstream of the exons lies the promoter region—a realm steeped in significance. Laden with specific DNA sequences, this domain enters into intricate interactions with a cavalcade of transcription factors and regulatory proteins. These interactions, in a choreographed symphony, steer the intricate ballet of NRAS gene transcription and expression

(Eisfeld et al., 2014). In essence, the anatomy of the NRAS gene encapsulates not just a blueprint for protein synthesis but an orchestration of regulatory elements that navigate the cellular narrative



Available at: https://www.genecards.org/cgi-bin/carddisp.pl?gene=NRAS

Figure 23: Genomic Location of NRAS gene
Source: NRAS Gene - NRAS Proto-Oncogene, GTPase. 2023
Available at: https://www.genecards.org/cgi-bin/carddisp.pl?gene=NRAS

II.1.5 Structure of the *HRAS* Gene

Positioned on chromosome 11's abbreviated arm at location 5 (11p15.5) **(Figure 24)**, the HRAS gene extends its influence over 6.5 kb of the human genome canvas. With six exons as its building blocks, it crafts a tapestry of two alternate splice variants. These genetic orchestrations pave the way for a petite GTPase protein, comprised of 189 amino acids. This protein, adorned with distinct domains, dons the role of orchestrator across a multitude of functions spanning from membrane localization to engaging downstream effectors, all while shepherding downstream signaling events.

As HRAS mutations establish connections with a range of cancer types, it's crucial to emphasize the lack of specific HRAS mutations identified in CRC thus far *(Rajasekharan and Thiagarajan 2013)*. Within this mosaic of the HRAS gene, the narrative unfolds beyond mere protein coding, evolving into a symphony of regulatory mastery that profoundly shapes the rhythm and pattern of cellular dynamics.

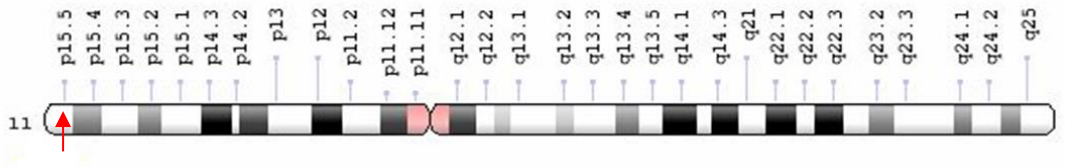


Figure 24: Genomic Location of HRAS gene
Source: HRAS Gene - HRAS Proto-Oncogene, GTPase. 2023
Available at: https://www.genecards.org/cgi-bin/carddisp.pl?gene=HRAS

II.2 RAS protein

II.2.1 Structure and Function of *RAS* Proteins

The RAS gene comprises three isoforms: KRAS, NRAS, and HRAS. Each isoform encodes a protein consisting of 188-189 amino acids with a molecular mass of 21 kDa *(Cox and Der 2010)*. These proteins are primarily localized on the inner side of the plasma membrane and within the Golgi apparatus *(Fehrenbacher et al., 2009)*.

RAS proteins function as molecular switches that alternate between an activated state, where they bind to GTP, and an inactivated state, where they bind to GDP. This switching mechanism is regulated by the binding and hydrolysis of GTP, catalyzed by RAS-specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) respectively. In the inactive state, RAS is bound to GDP and cannot interact with downstream effectors. Upon activation by a GEF, GDP is replaced by GTP, inducing a conformational change in RAS that enables interaction with downstream effectors, initiating signaling cascades. The active state of RAS is transient, as the intrinsic GTPase activity of RAS hydrolyzes GTP to GDP, leading to deactivation. This deactivation process is accelerated by GAPs, which enhance the GTPase activity of RAS, promoting the transition back to the inactive state. Maintaining the proper balance between GEFs and GAPs is critical for controlling RAS activity and downstream signaling output.

RAS proteins play a pivotal role in signal transduction through multiple pathways, including PI3K-AKT-mTOR and RAS/MAPK, to regulate fundamental cellular processes such as proliferation, differentiation, survival, and apoptosis.

Mutations in RAS genes that hinder GTP hydrolysis or amplify GEF-mediated activation can result in constant RAS activation, contributing to the development of cancer. A comprehensive understanding of the mechanisms governing RAS activity and the implications of RAS mutations is essential for the development of effective therapies targeting this vital signaling pathway **(Figure 25)** *(Cox and Der 2010)*.

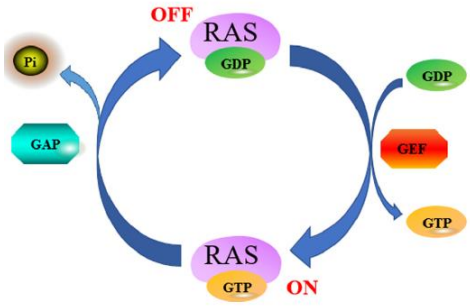


Figure 25: Regulation of the RAS GTP/GDP Cycle by GEFs and GAPs *(Meng et al., 2021).*

II.2.2 Structure and Function of K*RAS* Proteins

The KRAS protein, a member of the RAS family of small GTPases, consists of 188 amino acid residues and possesses a molecular mass of 21 kDa *(Jančík et al., 2010)*. It is structured into three distinct domains: the N-terminal G-domain, Switch I and Switch II regions, and the C-terminal hypervariable region (HVR) **(Figure 26)**.

The G-domain, spanning 80 amino acids, encompasses the GTP-binding pocket responsible for interactions with the β-phosphate and γ-phosphate of GTP via P-loop-phosphate binding loops. Specific residues within positions 116–119 and 152–165 are involved in interactions with the guanine base. Essential for engaging downstream effectors and GAPs, the core effector region, spanning amino acids 32 to 40, collectively contributes to KRAS's signaling function.

Switch regions I and II, situated between the G-domain and the HVR, play critical roles in binding regulators and effectors. The phosphate binding pocket (P-loop) permits transient binding of GTP to KRAS, also serving as the site of GTPase activity that negatively regulates KRAS by facilitating RAS-GTP hydrolysis and guanosine diphosphate binding.

The HVR, located at the C-terminus of the KRAS protein, assumes a vital role in directing posttranslational modifications and determining anchoring to the plasma membrane. Its substantial influence on the biological activity of KRAS establishes it as a pivotal regulatory region governing the protein's function *(Kim et al., 2021)*.

Figure 26: KRAS Protein: Structural Domains and Functions *(Kim et al., 2021).*

II.2.3 Structure and Function of *NRAS* Proteins

The NRAS gene encodes a small GTPase protein encompassing five naturally occurring isoforms, each exhibiting a slightly distinct amino acid sequence. Comprising approximately 189-190 amino acids, contingent upon the isoform, the NRAS protein incorporates several conserved domains, including the G1, G2, G3, G4, and G5 boxes, integral to GTP binding and hydrolysis. Isoforms 1, 2, and 4 reside entirely within the cytoplasm, while isoforms 3 and 5 manifest presence within the nucleus as well. Among these, isoform 1, the canonical variant, is composed of exons 1-7, housing an open reading frame (ORF) spanning from exon 2 to exon 5, and embracing all five conserved domains **(Figure 27)**. Other isoforms diverge from isoform 1 in their exon configuration, yielding truncated or modified protein products. Despite these disparities, all RAS isoforms share an invariant effector domain, facilitating interaction with downstream signaling molecules such as RAF and PI3K *(Eisfeld et al., 2014)*.

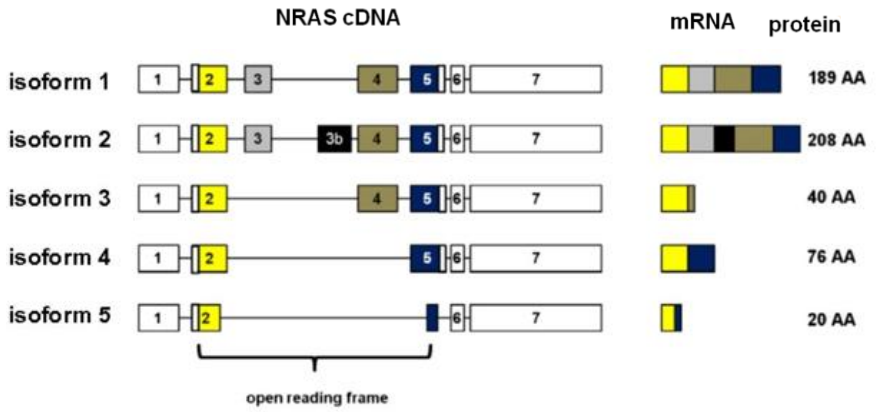


Figure 27: Structure of NRAS isoform *(Eisfeld et al., 2014).*

II.2.4 Activation of *KRAS* Mutations in CRC

Mutations in the KRAS gene are frequent occurrences in CRC and are primarily concentrated in exon 2. These mutations predominantly involve specific points within the gene sequence, with a notable prevalence at glycine 12 (G12), glycine 13 (G13), and glutamine 61 (Q61). Remarkably, G12 mutations exhibit the most extensive occurrence and diversity, comprising 15 distinct point mutations such as G12A, G12D, G12F, G12K, G12N, G12S, G12V, G12Y, G12C, G12E, G12I, G12L, G12R, G12T, and G12W. Among these, G12D stands as the most prevalent, constituting approximately 41% of all G12 mutations, while G12V and G12C closely follow at approximately 28% and 14%, respectively. These mutations lead to a constitutively active KRAS protein, resulting in the disruption of downstream signaling pathways that control cell growth, differentiation, and survival *(Meng et al., 2021)* **(Figure 28)**.

Further reinforcement of the significance of these mutations is found in the COSMIC database. It underscores that mutations at codon 12, including G12A, G12V, G12S, G12R, G12C, and G12D, reign as the most significant, collectively accounting for more than 90% of all KRAS mutations. Likewise, mutations affecting codon 13 (G13D and G13C), codon 61 (Q61L, Q61R, Q61H), codon 146 (A146T, A146V, A146P), and codon 117 (K117N) also contribute to the spectrum of KRAS mutations, although with lower frequencies. These mutations lead to the activation of KRAS, initiating uncontrolled signaling through pathways such as the PI3K-AKT-mTOR and RAS/MAPK cascades, driving crucial processes including cell proliferation, survival, and evasion of apoptosis *(Serebriiskii et al., 2019)*.

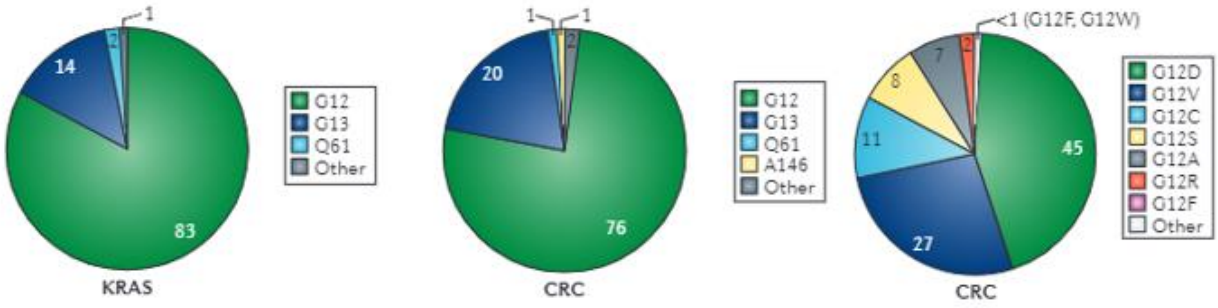


Figure 28: Frequency of KRAS gene mutations *(Cox et al., 2014).*

II.2.5 Activation of *NRAS* Mutations in CRC

NRAS mutations emerge as a relatively common occurrence in CRC, accounting for approximately 5%-10% of cases and showcasing a distribution mirroring that of KRAS mutations. What sets NRAS mutations apart is their concentration within the same codon locations as KRAS mutations, establishing them as true hot-spot mutations. These mutations predominantly manifest within exon 2, present in roughly 3%-5% of CRC cases, and also within exon 3, detected in approximately 2%-6% of CRC cases.

The implications of NRAS mutations are significant, as they incite a cascade of events leading to the anomalous activation of the RAS pathway. In the context of CRC, this activation becomes a pivotal event, contributing substantially to the disease's progression. The underlying mechanism driving this activation primarily involves point mutations situated within hotspot codons found in exon 2 (codons 12 and 13) and exon 3 (codon 61). These specific alterations have been pinpointed as key drivers of the aberrant RAS pathway activity observed in CRC cases influenced by NRAS mutations *(Hecht et al., 2015)*.

II.3 Signaling Pathways Involved in Colorectal Carcinogenesis

Signaling pathways play a crucial role in the initiation and progression of CRC by regulating various cellular processes, including cell proliferation, differentiation, survival, apoptosis, and migration *(Zenonos and Kyprianou 2013)*.

Several pathways have been identified as playing a significant role in CRC, including the Wnt, TGF-β, p53, and receptor tyrosine kinase (RTK) pathways. Of these, the EGFR is a member of the RTK family and is particularly important in the development of CRC. EGFR is overexpressed in 60-80% of CRC cases and is responsible for activating two principal cascades.

- The RAS/MAPK pathway, responsible for cell proliferation, differentiation, survival, angiogenesis, and metastasis

- The PI3K-AKT-mTOR pathway, is critical for cell growth, proliferation, and survival.

Dysregulation of these pathways due to genetic and epigenetic alterations can lead to uncontrolled cell growth and survival, contributing to CRC development and progression *(Zenonos and Kyprianou 2013)*.

II.3.1 EGFR/MAPK Signaling Pathway in CRC

The EGFR signaling pathway forms a multifaceted tapestry of molecular interactions that plays an imperative role in steering the intricate journey of CRC development and progression. This intricate web is ignited when specific ligands such as EGF, TGFα, amphiregulin, and epiregulin engage one of the four distinct EGFR variants (EGFR/ErbB1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4). Upon ligand engagement, a remarkable sequence unfurls: the receptor embarks on dimerization, and the intrinsic kinase activity is roused from dormancy. This inception of kinase activity acts as the clarion call, summoning a cadre of Src homology 2 (SH2) proteins. These SH2 proteins, akin to skilled performers, choreograph an intricate ballet by embracing specific phosphotyrosine residues on the activated receptor, inaugurating a symphony of downstream signaling pathways into motion.

Among the pathways summoned into action are the PI3K/AKT/mTOR and MAPK/ERK cascades, each carrying its distinct message within the cellular landscape. The PI3K/AKT/mTOR pathway navigates the ship of cell proliferation, differentiation, and survival, while the MAPK/ERK pathway steers the course of similar pursuits, orchestrating cellular behaviors that collectively contribute to the vibrant dance of life itself *(Sforza et al., 2016)*.

However, the intricacy of this arrangement can falter when discord creeps in. Dysregulation of the EGFR signaling pathway has been implicated in the pathogenesis of CRC, and aberrant expression of this pathway has been identified as a potential target for CRC treatment. The EGFR signaling cascade has an adaptor protein complex containing the growth factor receptor-bound protein 2 (Grb2) and the son of seven-less (SOS). This complex activates RAS-GTP by binding to phosphorylated tyrosine residues. After RAS activating, there is a cascade of activating RAF MEK and ERK through phosphorylation. It has been suggested that the RAS–RAF–ERK signaling pathway contributes to the control of cell growth, differentiation, and survival. When this pathway is dysregulated, it can lead to malignant

transformation and tumor progression through increased cell proliferation, prolonged survival, angiogenesis, anti-apoptosis, invasion, and metastasis *(Koveitypour et al., 2019)* **(Figure 29)**.

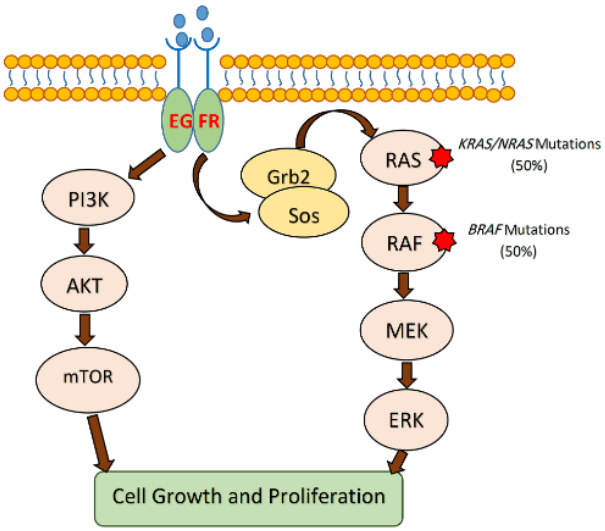


Figure 29: Schematic representation of the EGFR signaling pathway in colorectal cancer *(Zeinalian et al., 2021).*

II.3.2 MAPK Signaling Pathway

The MAPK signaling pathway, also known as the RAS-RAF-MEK-ERK pathway, is a highly conserved intracellular signaling cascade that plays a crucial role in regulating various cellular processes, including cell proliferation, differentiation, survival, and apoptosis. It is activated in response to a wide range of extracellular signals, such as growth factors, cytokines, and stress signals, allowing cells to respond appropriately to changes in their environment *(Guo et al.,2020)*.

The pathway is initiated when extracellular ligands, such as growth factors or cytokines, bind to specific cell surface receptors called receptor tyrosine kinases (RTKs). This binding leads to a conformational change in the RTKs, causing them to undergo autophosphorylation at specific tyrosine residues. This autophosphorylation activates the RTKs and enables them to serve as docking sites for downstream signaling molecules.

One of the key downstream effectors recruited by activated RTKs is the small GTPase protein RAS. The autophosphorylated RTKs facilitate the exchange of GDP for GTP on RAS, converting it into an active state (RAS-GTP). Activated RAS-GTP acts as a molecular switch, triggering a series of events that propagate the signal further downstream.

The next crucial step in the MAPK pathway is the activation of RAF, a serine/threonine kinase. Activated RAS-GTP recruits and activates RAF, which then phosphorylates and activates another serine/threonine kinase called MEK (Mitogen-Activated Protein Kinase Kinase).

MEK, in turn, phosphorylates and activates the terminal kinases of the pathway, ERK1 (Extracellular Signal-Regulated Kinase 1) and ERK2 (Extracellular Signal-Regulated Kinase 2), also known as MAPKs. Once activated, ERK1/2 undergo dually phosphorylation at specific threonine and tyrosine residues and translocate to the nucleus.

In the nucleus, ERK1/2 phosphorylate various transcription factors, modulating their activity and thus influencing gene expression. This leads to the upregulation or downregulation of specific genes involved in cell cycle progression, cell survival, differentiation, and other vital cellular processes. The phosphorylated transcription factors, in combination with other co-regulatory proteins, form a complex signaling network that orchestrates the cellular response to the initial extracellular stimuli (*Frémin and Meloche 2010)* **(Figure 30)**.

Beyond their role in gene expression regulation, ERKs can also phosphorylate other cytoplasmic and nuclear substrates, further influencing various cellular functions and processes. It's important to note that while the canonical MAPK pathway described above is well-established, there are also non-canonical MAPK pathways that respond to different extracellular signals and utilize distinct MAPKs. These non-canonical pathways may interact and converge with the canonical pathway at various points, forming a complex and tightly regulated signaling network that allows cells to respond precisely to a diverse array of environmental cues. This signaling network ensures that cells can adapt and coordinate their behaviors appropriately to maintain tissue homeostasis and respond to changing physiological conditions.

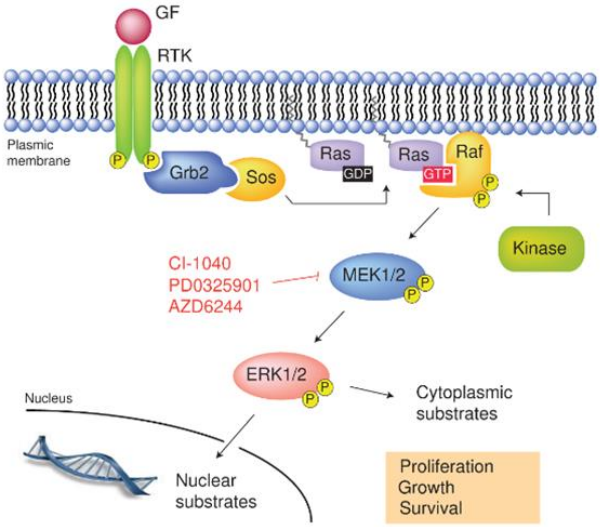


Figure 30: A simplified schematic representation of the RAS–RAF–MEK–ERK signaling pathway *(Frémin and Meloche 2010).*

II.3.2.1 Genetic Alterations in the *MAPK* Pathway

II.3.2.1.1 *RAS* Mutations

Genetic alterations in the MAPK pathway, particularly involving RAS mutations, play a pivotal and intricate role in the development and progression of CRC. RAS, a family of three small GTPases KRAS, NRAS, and HRAS, serves as a key downstream effector in the MAPK pathway, establishing a crucial link between the EGFR and intracellular signaling cascades *(Sforza et al., 2016)*.

Within the RAS gene family, KRAS takes center stage due to its prevalence in CRC. Approximately 40% of CRC cases harbor KRAS mutations, with the majority occurring at codons 12 (70%-80%) and 13 (15%-20%) in exon 2. A smaller subset of mutations affects codons 61 (5%) and 146 (5%). These specific point mutations engender significant functional consequences, impairing RAS's intrinsic ATPase activity and thereby disrupting its ability to hydrolyze GTP to GDP *(Sforza et al., 2016)*.

The result of these genetic alterations is the accumulation of mutant RAS proteins in an active conformation. Unlike their normal counterparts, these mutants remain persistently bound to GTP, failing to switch off downstream signaling by hydrolyzing GTP to GDP. This sustained activation of the MAPK pathway, even in the presence of EGFR inhibition, drives dysregulated mitogenic and antiapoptotic signaling, thus substantially contributing to uncontrolled tumor growth and enhanced survival of cancer cells *(Prior et al., 2012)*.

The clinical implications of RAS mutations are of paramount importance, particularly in the management and treatment of CRC. These mutations stand as a major mechanism behind resistance to anti-EGFR moAbs, which are frequently employed in CRC therapy. The continuous activation of the MAPK pathway, orchestrated by mutant RAS, significantly diminishes the effectiveness of anti-EGFR moAbs in impeding tumor growth. Consequently, the treatment landscape for CRC patients becomes more intricate, necessitating innovative approaches to address this challenge *(Zhao et al., 2016)*.

II.3.2.1.2 RAF Mutations

RAF mutations encompass genetic alterations affecting the RAF protein family, which consists of ARAF, BRAF, and CRAF. These proteins are integral to the MAPK signaling pathway, a critical regulator of cell growth, differentiation, and survival. When RAF genes undergo mutations, the MAPK pathway can become constitutively active, contributing to the initiation and progression of diverse cancer types *(Bahrami et al., 2017)*.

Effectively managing RAF mutations is pivotal in CRC treatment. Inhibitors targeting the MAPK pathway, including MEK inhibitors and BRAF inhibitors, have displayed promise in both preclinical investigations and clinical trials. Nevertheless, the activation of the MAPK pathway downstream of the RAF mutation can lead to resistance against these inhibitors. Consequently, researchers are concentrating on the development and assessment of combination therapies that target multiple nodes within the MAPK pathway or other pathways activated in RAF-mutated CRC. Ongoing clinical trials are shedding light on these approaches *(Li et al., 2020).*

In conclusion, RAF mutations represent a prevalent genetic anomaly within the MAPK signaling pathway, bearing significant consequences for CRC outcomes. While targeted therapies restraining the MAPK pathway exhibit potential, the hurdle of resistance looms large. The pursuit of combination therapies, targeting various points within the pathway or other activated pathways in RAF-mutated CRC, holds promise for treating this specific CRC subtype.

II.3.2.1.3 EGFR Mutations

EGFR mutations denote genetic modifications occurring within the EGFR gene, which encodes the epidermal growth factor receptor protein. EGFR is a transmembrane receptor critically involved in cellular growth and division, with its activation frequently associated with the initiation and progression of cancer *(Saletti et al., 2015).*

While EGFR mutations are more commonly recognized in non-small cell lung cancer (NSCLC), they are also detected in CRC, albeit at a lower frequency. Extensive investigations into the prevalence of EGFR mutations in CRC across diverse populations have estimated occurrence rates at approximately 3%. The presence of these mutations can significantly influence disease progression and holds potential implications for selecting appropriate treatment strategies *(Kim et al., 2020).*

Predominant EGFR mutations encountered in CRC include R165Q and S492R substitutions, each identified in five cases *(Kim et al., 2020)*. These specific alterations have undergone thorough examination and are recognized for rendering heightened sensitivity to specific medications known as EGFR tyrosine kinase inhibitors (TKIs). Examples of EGFR TKIs encompass gefitinib, erlotinib, and afatinib. These inhibitors effectively obstruct hyperactive EGFR signaling and display promise in treating cancers carrying EGFR mutations, including NSCLC and CRC *(Yang et al., 2022).*

The efficacious utilization of therapies targeting EGFR in CRC mandates meticulous patient selection, which hinges on various factors encompassing the mutational status of genes

and associated signaling pathways. Ongoing research is dedicated to unraveling novel biomarkers and refining targeted therapies tailored for CRC, with an emphasis on those aimed at EGFR and its downstream signaling cascades *(Janani et al., 2022)*.

II.3.3 PI3K Pathway

The PI3K (phosphatidylinositol 3-kinase) signaling pathway is a intricate and vital network of intracellular signaling pathways responsible for orchestrating various cellular processes such as cell growth, proliferation, survival, and metabolism *(He et al., 2021)*.

This pathway, often referred to as the PI3K/AKT/mTOR pathway, comprises three key components: phosphatidylinositol 3-kinase (PI3K), AKT (also known as protein kinase B), and mammalian target of rapamycin (mTOR) *(Fernandes et al., 2018)*. These components belong to a family of lipid kinases that are activated in response to external signals, often initiated by cell surface receptors such as receptor tyrosine kinases (RTKs) or G protein-coupled receptors (GPCRs) *(Zhang et al., 2011)*.

The PI3K pathway can be categorized into three classes of PI3Ks, which are lipid and protein kinases classified as class I, II, and III based on their structural characteristics and substrate specificity. Notably, within class I PI3Ks, there are subclasses IA and IB. It's worth noting that in CRC cell lines, there is a notable expression of class IA PI3K *(Yu and Grady, 2012)*.

In essence, the PI3K pathway serves as a crucial nexus where external signals converge to regulate fundamental cellular processes, making it a key player in various physiological and pathological contexts.

II.3.3.1 Activation and Function of PI3K/AKT/mTOR Pathway

The PI3K/AKT/mTOR pathway is activated by external signals, initiating a cascade of events that govern a range of essential cellular processes, such as protein synthesis, cell growth, proliferation, survival, and metabolism *(Zhang et al., 2011)* **(Figure 31)**.

1- **Initiation Signal:** The process is initiated when growth factors or insulin bind to their specific receptors on the cell surface. This interaction initiates the activation of receptor tyrosine kinases (RTKs) or insulin receptors, subsequently triggering the activation of class I PI3Ks. These class I PI3Ks comprise regulatory (p85) and catalytic (p110) subunits.

2- **Conversion of PIP2 to PIP3:** The primary consequence of PI3K activation is to catalyze the conversion of membrane-bound phosphatidylinositol-(4,5)-biphosphate

(PIP2) to phosphatidylinositol-(3,4,5)-biphosphate (PIP3). This conversion is mediated by the lipid kinase activity of the p110 subunit of PI3K. PIP3 is an important secondary messenger that recruits and activates downstream signaling molecules, including AKT and PDK1. PTEN (phosphatase and tensin homolog) is a tumor suppressor protein that functions as a negative regulator of the PI3K/AKT pathway by dephosphorylating PIP3 and converting it back to PIP2. This negative feedback loop helps to maintain the balance of PIP3 levels in the cell and prevent excessive activation of the PI3K/AKT pathway.

3- **Activation of AKT:** A pivotal downstream effector of the PI3K pathway is AKT, also known as protein kinase B (PKB). AKT is activated through phosphorylation at two distinct sites: threonine 308 (Thr308) in the activation loop and serine 473 (Ser473) in the hydrophobic motif. Phosphoinositide-dependent kinase 1 (PDK1) mediates the phosphorylation of Thr308, while mTOR complex 2 (mTORC2) facilitates the phosphorylation of Ser473. Once activated, AKT phosphorylates various downstream targets, including mTOR, glycogen synthase kinase 3 (GSK3), and forkhead box O (FOXO) transcription factors.

4- **Activation of mTORC1**: mTOR, a serine/threonine kinase, is present in two distinct complexes: mTORC1 and mTORC2. Activation of mTORC1 occurs downstream of AKT and plays a central role in governing protein synthesis, cell growth, and metabolism.

5- Regulation of protein synthesis: mTORC1 regulates protein synthesis by phosphorylating and activating the ribosomal protein S6 kinase (S6K) and the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). S6K phosphorylates and activates ribosomal protein S6, which is involved in ribosome biogenesis and protein synthesis. 4E-BP1 regulates the initiation of protein synthesis by binding to eIF4E, a key component of the translation initiation complex.

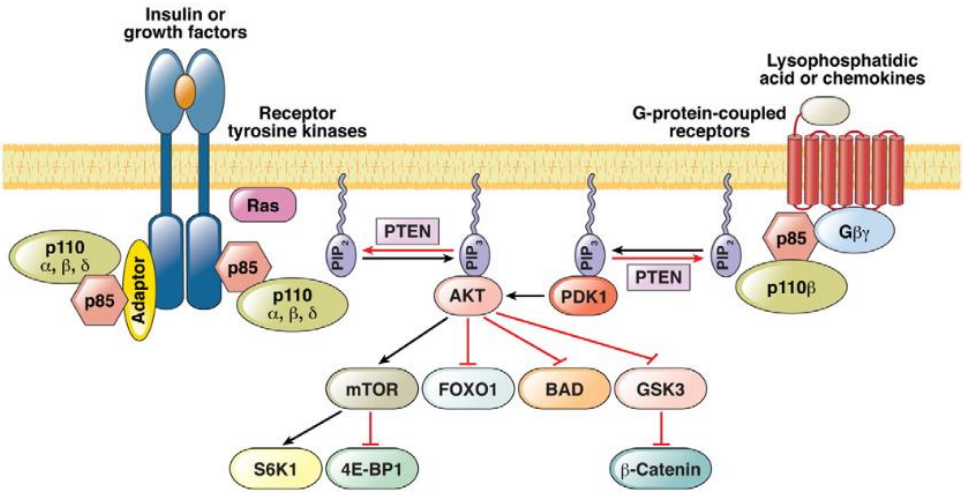


Figure 31: Schematic representation of PI3K Signaling Pathway *(ZHANG et al., 2011)*.

II.3.3.2 Genetic Alterations in the *PI3K-AKT-mTOR* Pathway

Mutations within the PI3K pathway have been intricately associated with the initiation and progression of diverse cancer types, including CRC. The pronounced hyperactivity of the PI3K-AKT-mTOR signaling pathway serves as a hallmark in the development of CRC, frequently coinciding with mutations in various pivotal genes, such as PIK3CA, PTEN, AKT, and mTOR. Among these genes, several show heightened mutation rates:

II.3.3.2.1 PIK3CA Mutations

PIK3CA mutations are frequently observed in CRC, with a prevalence of approximately 15% in cases of mCRC (Wang and Zhang 2014). These mutations can result in the overactivation of the PI3K-AKT-mTOR signaling pathway, which is associated with malignant behavior and a poor prognosis in CRC. The mutations in PIK3CA are concentrated in exons 9 and 20, which are critical regions of the PI3K protein that regulate its helical and kinase domains. When mutations occur in these regions, they can activate the PI3K pathway, leading to increased cell proliferation and survival *(Chen et al., 2014)*.

II.3.3.2.2 PTEN Mutations

PTEN emerges as a frequently mutated gene in CRC, with mutations emerging in approximately 5-10% of cases. Serving as a negative regulator of the PI3K-AKT-mTOR pathway, mutations in PTEN bear the potential to induce an excessive activation of the pathway, thereby contributing to the unfolding and progression of CRC. PTEN's functioning can be disrupted through a spectrum of mechanisms, including mutations associated with MSI (approximately 5% of CRC), allelic loss on chromosome 10q23 (approximately 23% of CRC), or hypermethylation of the PTEN gene's promoter region. These perturbations engender significant consequences for PTEN's regulatory capabilities *(De Roock et al., 2011)*.

II.3.4 Prognostic and Predictive Significance of KRAS Mutations

KRAS mutations have garnered substantial attention in the field of CRC research due to their clinical implications, playing a pivotal role in predicting patient outcomes and response to treatment. Their presence holds critical prognostic value, influencing both OS and DFS in CRC patients. Additionally, KRAS mutations bear significance in forecasting patient responses to therapies targeting the EGFR.

II.3.4.1 Prognostic Significance of *KRAS* Mutations

The prognostic implications of KRAS mutations in CRC have undergone extensive investigation. Multiple studies have consistently illustrated a connection between these mutations and a poorer prognosis, particularly in advanced stages of the disease. More specifically, mutations in KRAS exon 2 have been linked to inferior OS and DFS when compared to either KRAS wild-type cases or other forms of KRAS mutations *(Asako et al., 2023)*.

The influence of KRAS mutations on patient prognosis in CRC is evidenced by various studies that report decreased OS and DFS in individuals harboring these mutations in contrast to those without them. Furthermore, the prognostic implications of KRAS mutations are context-dependent and vary according to the disease stage. For instance, in stage IV CRC, KRAS mutations are associated with shorter OS, whereas NRAS mutations are connected to shorter OS in stage I-II CRC. Among KRAS mutations, those affecting codons 12 and 61 generally denote a worse prognosis, while codon 146 mutations correlate with a better prognosis. Nevertheless, the use of RAS genes as reliable prognostic indicators remains a topic of ongoing discussion *(Koulouridi et al., 2022)*.

Moreover, specific KRAS mutations, such as G12V and Q61, exhibit heightened aggressiveness and are linked to an unfavorable prognosis. In contrast, the A146 mutation is associated with a more favorable prognosis. Accurate identification of specific KRAS mutations in metastatic colorectal cancer patients is paramount for precise prognostication and tailoring treatment strategies accordingly *(Lavacchi et al., 2022)*.

II.3.4.2 Predictive Significance of *KRAS* Mutations

Beyond their prognostic value, KRAS mutations hold predictive relevance in the context of CRC treatment, notably in relation to therapies targeting the EGFR.

Clinical trials have consistently demonstrated that patients with KRAS wild-type tumors exhibit superior responses to anti-EGFR therapy in comparison to those with KRAS mutations. Consequently, KRAS mutation testing has become an integral component of the diagnostic

assessment for CRC patients, especially those grappling with metastatic disease. Typically, patients with KRAS wild-type tumors are considered candidates for anti-EGFR therapy, while those harboring KRAS mutations are not deemed suitable candidates *(Asako et al., 2023)*.

Other targeted treatment avenues, such as MEK inhibitors, have also emerged for the treatment of KRAS-mutant CRC; however, their efficacy remains limited *(Koulouridi et al., 2022)*. In conclusion, KRAS mutations wield both prognostic and predictive significance in CRC patients, underscoring the critical need for their assessment to facilitate personalized treatment and management strategies for CRC.

III. BRAF Signaling Pathway and its Implications in Colorectal Carcinogenesis

The intricate landscape of colorectal carcinogenesis is significantly influenced by the BRAF signaling pathway. Mutations within the BRAF gene trigger a complex cascade of events that drive uncontrolled cell proliferation and tumor formation. This exploration delves into the BRAF gene and its associated signaling pathway, shedding light on its multifaceted implications in the development of CRC.

III.1 The BRAF Gene and its Functions

The BRAF gene, also known as V-Raf murine sarcoma viral oncogene homolog B1, is situated on chromosome 7q34. This gene encodes a protein kinase that plays a pivotal role in the RAS/MAPK signaling pathway. This pathway is responsible for regulating vital cellular processes including growth, differentiation, and survival *(Croce et al.,2019)*. The activation of the BRAF protein is initiated by upstream signaling molecules like RAS. This, in turn, triggers the activation of downstream signaling molecules such as MEK and ERK. These molecules work in tandem to activate transcription factors and facilitate the expression of genes involved in cell proliferation and survival. Normally, the BRAF signaling pathway is meticulously regulated to ensure proper cell growth and differentiation. However, mutations within the BRAF gene can lead to pathway activation, resulting in uncontrolled cell proliferation and tumor growth *(Degirmenci et al., 2020)*. A particularly noteworthy mutation is the BRAF V600E mutation, identified in 8-10% of mCRCs, which leads to continuous BRAF protein activation. This mutation is also present in other cancer types including melanoma, lung, and thyroid cancers *(Mauri et al.,2021)*.

III.2 BRAF Mutations in Colorectal Cancer

BRAF mutations are observed in around 8-15% of CRC cases, and they are associated with distinct molecular and clinical characteristics. These mutations are more frequently found in tumors originating from the serrated pathway of colorectal carcinogenesis. This pathway is

marked by the accumulation of epigenetic and genetic changes in precursor lesions. Most BRAF mutations in CRC are missense mutations, resulting in the V600E substitution. This alteration leads to the constitutive activation of the BRAF protein kinase, instigating downstream signaling activation and promoting cell proliferation and survival. The most prevalent BRAF mutation involves a T1799A transversion in exon 15, causing the V600E substitution. This mutation is found in over 90% of cases and is recognized as the mutation V600E *(Fanelli et al., 2020)*.

The presence of BRAF mutations has substantial implications for diagnosis, prognosis, and treatment. It is associated with a poorer prognosis, particularly in microsatellite stable (MSS) tumors. Additionally, it serves as a diagnostic marker for Lynch syndrome, a hereditary cancer syndrome. Recent findings suggest that the position of BRAF downstream to EGFR and KRAS in the RAS/MAPK pathway makes it a predictive marker for resistance to anti-EGFR monoclonal antibodies, affecting treatment responses in mCRC *(Molina-Cerrillo et al., 2020)*.

III.3 Targeted Therapies for BRAF-Mutated CRC

Managing CRC patients with BRAF mutations presents unique challenges due to the hyperactive MAPK pathway. Similar to RAS-mutated CRC, patients with BRAF-mutated CRC often exhibit resistance to targeted therapies, necessitating innovative treatment approaches. Promising options include inhibitors that target the MAPK pathway, such as BRAF inhibitors and MEK inhibitors. However, the persistent pathway activation downstream of BRAF mutations can foster resistance against these inhibitors. In addition to MAPK pathway inhibitors, other targeted therapies have been explored for the treatment of BRAF-mutated CRC, including the combination of BRAF and EGFR inhibitors. Immune checkpoint inhibitors have also emerged as a promising treatment option for patients with BRAF-mutated CRC, particularly those with high MSI-H. Therefore, the management of BRAF-mutated CRC requires a personalized approach that considers the patient's molecular profile, disease stage, and treatment history *(Grassi et al., 2021)*.

III.4 The BRAF Signaling Pathway

The BRAF signaling pathway, like RAS, is an integral component of the MAPK pathway, which governs critical cellular processes including growth, differentiation, and survival. BRAF functions as a serine/threonine kinase, facilitating the translation of extracellular signals into intracellular responses via downstream effector activation *(Caputo et al., 2019)* **(Figure 32)**.

III.4.1 Initiation of Signaling

The pathway commences when specific receptors on the cell surface are bound by growth factors or extracellular ligands. This binding activates RTKs, initiating a series of events that culminate in BRAF activation.

III.4.2 Activation of BRAF

Activated RTKs set in motion a chain of intracellular events, leading to RAS activation. In the context of BRAF, active RAS molecules recruit RAF proteins, including BRAF, to the plasma membrane, thereby initiating activation.

III.4.3 Phosphorylation Cascade

BRAF activation triggers the phosphorylation and activation of MEK, a dual-specificity kinase. MEK, in turn, phosphorylates and activates ERK, also known as MAPK. Phosphorylated ERK translocates into the nucleus and phosphorylates transcription factors, influencing gene expression and diverse cellular responses.

III.4.4 Cellular Responses

The BRAF-MEK-ERK pathway activation regulates genes linked to cell proliferation, survival, differentiation, and apoptosis. These gene expression changes contribute to cellular responses encompassing growth, cell cycle progression, and evasion of apoptosis. Pathway dysregulation, frequently resulting from BRAF mutations, can lead to unchecked cell growth and facilitate cancer development and progression.

III.4.5 Feedback Mechanisms

Several feedback mechanisms maintain MAPK pathway activation equilibrium. Negative feedback loops regulate upstream components like RAF and RTKs. Furthermore, tumor suppressors such as PTEN inhibit PI3K signaling, which intersects with the MAPK pathway.

III.4.6 BRAF Mutations and Constitutive Activation

BRAF mutations, especially the V600E mutation, foster the continual activation of BRAF kinase activity. This activation circumvents normal regulatory mechanisms, resulting in continuous signaling via the MAPK pathway. The downstream consequences of this persistent activation contribute to oncogenesis and cancer progression.

In summary, the BRAF signaling pathway is an integral element of the MAPK pathway, governing diverse cellular processes. The activation of BRAF and the ensuing phosphorylation cascade are pivotal in transmitting extracellular signals to the nucleus, ultimately shaping gene expression and cellular responses. Dysregulation, often driven by BRAF mutations, can lead to uncontrolled cell growth and contribute to the development of cancer.

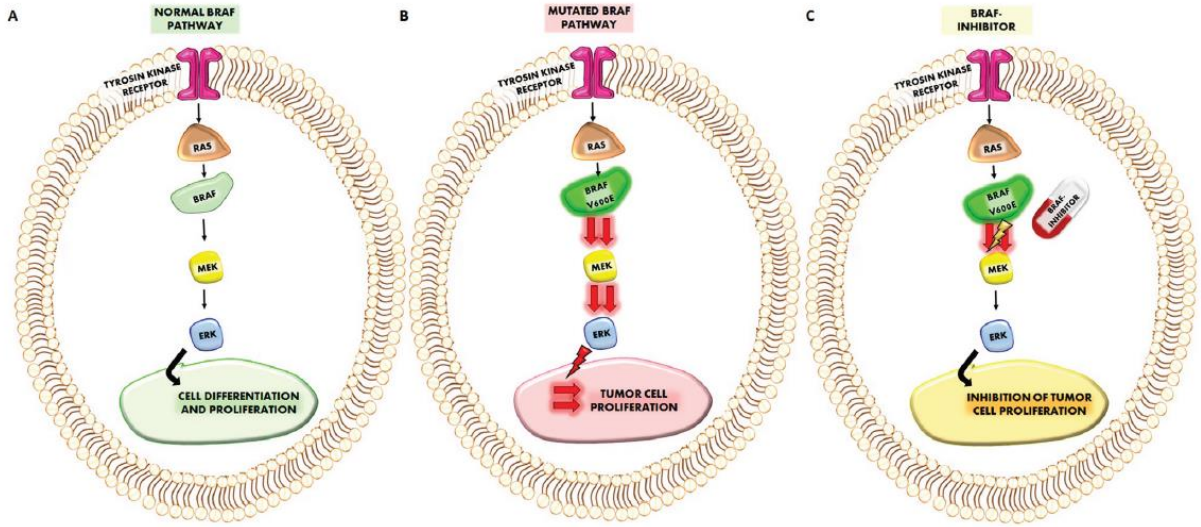


Figure 32: Pathway Alterations in BRAF Mutation and Treatment Response *(Croce et al., 2019).*

III.5 Prognostic and Predictive Significance of BRAF Mutations

The presence of BRAF mutations in CRC holds profound prognostic and predictive implications, significantly influencing disease progression, treatment response, and patient outcomes. Understanding the distinct roles that BRAF mutations play in prognosis and treatment response is crucial for tailoring effective therapeutic strategies.

III.5.1 Prognostic Significance:

BRAF mutations in CRC have been associated with a poorer prognosis, particularly in certain subsets of patients. Notably, the presence of BRAF mutations has been linked to more aggressive disease characteristics, including advanced tumor stage, lymph node metastasis, and distant metastasis. Patients harboring BRAF-mutated CRC tend to have shorter OS and DFS compared to those with wild-type BRAF tumors. This unfavorable prognosis is partly attributed to the intricate interactions between the BRAF signaling pathway and downstream effectors that regulate cell growth, proliferation, and survival *(Fanelli et al., 2020)*.

Moreover, the prognostic significance of BRAF mutations extends beyond CRC subtypes. In MSS CRC, BRAF mutations are indicative of a distinct molecular subtype that is characterized by an inflammatory microenvironment and immune evasion. This interaction

between BRAF mutations and immune responses shapes the tumor microenvironment and influences disease progression *(Nakayama et al., 2020)*.

III.5.2 Predictive Significance:

The predictive significance of BRAF mutations in CRC is evident in the context of treatment response to targeted therapies. In particular, the identification of BRAF mutations has influenced the efficacy of EGFR therapies. Patients with BRAF-mutated CRC have been shown to exhibit resistance to anti-EGFR monoclonal antibodies, such as cetuximab and panitumumab. This predictive marker has significant implications for treatment decision-making, as patients with BRAF-mutated tumors are less likely to benefit from anti-EGFR therapies *(Pietrantonio et al., 2015)*.

Additionally, the emergence of targeted therapies specifically designed to inhibit the hyperactive MAPK pathway offers potential avenues for personalized treatment of BRAF-mutated CRC. BRAF inhibitors and MEK inhibitors have shown promise in clinical trials, providing an alternative treatment strategy for patients with these mutations. Furthermore, ongoing research into combination therapies, immunotherapies, and strategies to overcome resistance holds promise in improving treatment responses in this subset of patients *(Garcia-Carbonero et al., 2020)*.

In conclusion, BRAF mutations in CRC have substantial prognostic and predictive significance. They are associated with a poorer prognosis, marked by aggressive disease characteristics and reduced survival. The predictive role of BRAF mutations is particularly evident in the context of treatment response to anti-EGFR therapies, guiding therapeutic decision-making. As targeted therapies continue to evolve, understanding the multifaceted impact of BRAF mutations on CRC prognosis and treatment response becomes increasingly pivotal in shaping individualized patient management strategies.

IV. Treatment of CRC

IV.1.1 Surgery

Surgery is the most common treatment for CRC. It is often called surgical resection (SR). It involves the removal of the tumor and some surrounding healthy tissue during an operation. Part of the healthy colon or rectum and nearby lymph nodes will also be removed. Surgical resection plays a crucial role in the treatment of CRC, especially in cases where the tumor is localized and has not spread to other organs or distant sites. The primary goal of surgical resection is to achieve curative intent by eliminating the cancerous tissue and preventing its further spread. However, in some cases where the cancer is more advanced, surgery may be employed to alleviate symptoms and improve the patient's quality of life. There

are different types of surgical resection procedures for CRC, each chosen based on the specific characteristics of the cancer and the patient's condition. Two common types of surgical resection for CRC are partial colectomy (segmental resection) and total colectomy, each with its own indications and outcomes.

IV.1.1.1 Partial Colectomy (Segmental Resection)

Partial colectomy, also known as segmental resection, involves removing a portion of the colon containing the tumor. The extent of the colon removed depends on the location and size of the tumor. After removing the affected segment, the healthy ends of the colon are then reconnected (anastomosis) to restore the continuity of the gastrointestinal tract. This procedure is often performed laparoscopically, which involves making several small incisions and using specialized surgical tools and a camera to guide the surgeon. Laparoscopic surgery generally results in faster recovery, reduced postoperative pain, and a shorter hospital stay compared to traditional open surgery.

A partial colectomy is commonly employed for early-stage CRC or when the tumor is limited to a specific region of the colon or rectum. In cases where the tumor is causing obstruction or bleeding, partial colectomy can provide significant relief and improve the patient's quality of life.

IV.1.1.2 Total Colectomy

Total colectomy involves the complete removal of the colon and is performed in cases of more extensive or aggressive CRC. Additionally, it may be considered for individuals with conditions such as FAP, a genetic disorder that predisposes patients to the development of multiple colon polyps that can progress to cancer.

After the removal of the colon, the surgeon may perform an ileorectal anastomosis, connecting the small intestine (ileum) to the rectum. Alternatively, if the rectum is also affected by cancer or if there is a risk of recurrent disease in this area, the surgeon may create an ileostomy. An ileostomy involves bringing the end of the small intestine (ileum) through an opening in the abdominal wall to create a stoma, through which waste products will be eliminated into a disposable bag. In some cases, a temporary ileostomy may be created to allow the anastomosis to heal before it is reversed in a subsequent procedure.

Total colectomy is more complex than partial colectomy and may require a longer recovery period. However, it is sometimes the most effective option to ensure the complete removal of cancerous tissue and prevent recurrence.

IV.1.1.3 Laparoscopic Surgery

Laparoscopic surgery, also known as minimally invasive surgery, is an approach used in both partial colectomy and total colectomy. During laparoscopic surgery, small incisions are made in the abdomen, and a laparoscope and specialized instruments are used to perform the surgery with the assistance of a camera and a video monitor. Laparoscopic surgery offers benefits such as smaller incisions, reduced pain, quicker recovery, and shorter hospital stays compared to traditional open surgery *(Chand et al., 2012)*.

IV.1.1.4 Colostomy

A colostomy is a surgical procedure that involves creating an opening (stoma) in the abdominal wall and bringing a portion of the colon to the surface. This procedure may be performed when the rectal cancer is very low in the rectum or when it is not possible to reconnect the colon and rectum after a partial colectomy. A colostomy allows stool to bypass the lower part of the rectum and is often temporary, allowing the surgical site to heal before a reversal in a subsequent procedure *(Banaszkiewicz et al., 2015)*.

IV.1.1.5 Radiofrequency Ablation (RFA)

RFA is a minimally invasive technique used to treat liver metastases from CRC. It involves using high-frequency electrical currents to heat and destroy cancerous tumors within the liver. RFA is not the same as colectomy or colostomy; it is specifically used for liver tumors and is performed percutaneously (through the skin) under image guidance *(Wang et al., 2020)*.

IV.1.2 Radiation Therapy

Radiation therapy, also known as radiotherapy, is a medical treatment that uses high-energy rays or particles to target and destroy cancer cells. It is one of the key treatment modalities for various types of cancer, including CRC. The primary goal of radiation therapy in CRC is to eliminate cancer cells, reduce the size of tumors, and prevent cancer from spreading or recurring.

Radiation therapy uses ionizing radiation, such as X-rays or gamma rays, to damage the DNA inside cancer cells. When DNA is damaged, the cancer cells lose their ability to divide and grow, leading to their death. Radiation therapy can be delivered externally or internally, depending on the location and stage of the cancer.

IV.1.2.1 Preoperative (Neoadjuvant) Radiation Therapy

Preoperative radiation therapy is given before surgery, especially for locally advanced rectal cancer. Its purpose is to shrink the tumor, making it easier to remove surgically and potentially increasing the chances of successful tumor removal without the need for extensive

surgery. This approach may also reduce the risk of cancer recurrence in the area after surgery *(Feeney et al., 2019).*

IV.1.2.2 Postoperative (Adjuvant) Radiation Therapy

Adjuvant radiation therapy is typically administered after surgery for patients with certain risk factors, such as positive margins or involvement of nearby lymph nodes. It aims to destroy any remaining cancer cells that might not have been completely removed during surgery, thereby reducing the risk of cancer recurrence. Adjuvant radiation therapy is often used in combination with other treatments, such as chemotherapy, to improve the effectiveness of the treatment and reduce the risk of cancer recurrence. However, the decision to use radiation therapy will depend on a number of factors, including the stage of cancer, the patient's overall health, and the risk of cancer recurrence *(Kosmider and Lipton 2007).*

IV.1.2.3 Palliative Radiation Therapy

Palliative radiation therapy is used to relieve symptoms and improve the quality of life for patients with advanced or metastatic CRC. It may be employed to shrink tumors causing blockages, control bleeding, or alleviate pain and other symptoms associated with the spread of cancer. This treatment can be delivered externally, using a machine called a linear accelerator, or internally, using radioactive implants. The type of radiation therapy used will depend on the location and size of the tumor, as well as the patient's overall health and treatment goals *(Ba et al., 2021).*

IV.1.3 Chemotherapy

Chemotherapy is a systemic treatment modality used in CRC management, which involves the administration of drugs to target and destroy cancer cells throughout the body. It plays a crucial role in both early and advanced stages of CRC, often used in combination with surgery or radiation therapy. The main objective of chemotherapy is to shrink tumors, prevent their growth, and reduce the risk of cancer recurrence.

IV.1.3.1 Adjuvant Chemotherapy

Adjuvant chemotherapy is a common treatment approach for early-stage CRC following surgical resection of the primary tumor. The goal of adjuvant chemotherapy is to eliminate any remaining cancer cells that may not be visible to the naked eye, reducing the likelihood of tumor recurrence and improving long-term survival rates. However, the use of adjuvant chemotherapy in early-stage CRC is not always necessary and depends on the individual patient's risk factors and tumor characteristics. For example, patients with stage II colon cancer may not benefit from adjuvant chemotherapy if their tumor has a low risk of recurrence. Additionally, the choice of

chemotherapy regimen may vary depending on the patient's overall health and other factors. Overall, adjuvant chemotherapy has proven particularly effective in preventing cancer relapse and improving disease-free survival (DFS) in patients with early-stage CRC *(Chan and Chee 2019)*.

IV.1.3.2 Neoadjuvant Chemotherapy:

Neoadjuvant chemotherapy is another application of chemotherapy in CRC management. It is administered before the primary treatment, which is usually surgery and is often used in combination with radiation therapy, which is called neoadjuvant chemoradiation therapy (CRT). The purpose of neoadjuvant chemotherapy is to shrink the tumor and make it more operable or reduce the extent of surgery required. By reducing the tumor size, neoadjuvant chemotherapy may improve the likelihood of successful surgical removal and potentially preserve organ function. The choice of chemotherapy regimen should be based on initial clinical staging, predicted circumferential resection margin (CRM) status, and pathological evaluation of the surgical specimen. Neoadjuvant chemotherapy is commonly used in locally advanced CRC to downsize the tumor and increase the chances of successful surgical resection *(Chan and Chee 2019)*.

IV.1.3.3 Palliative Chemotherapy:

Palliative chemotherapy is an essential component of care for patients with locally advanced or mCRC. The primary goal of palliative chemotherapy is to alleviate symptoms, improve quality of life, and prolong survival. While it does not aim for a complete cure, it can help shrink tumors, slow down cancer growth, and reduce symptoms such as pain, bleeding, and obstruction caused by the cancer. Palliative chemotherapy can also improve a patient's overall well-being and provide valuable time for patients to spend with their loved ones and engage in meaningful activities. By managing the disease and enhancing the patient's comfort, palliative chemotherapy can help patients live as fully and comfortably as possible *(Baek et al., 2019)*.

IV.1.4 Drug Classes Used in CRC Chemotherapy

IV.1.4.1 Fluoropyrimidines (see Vodenkova et al., 2019):

✓ **5-Fluorouracil (5-FU)**: 5-FU is one of the most commonly used drugs in CRC chemotherapy. It belongs to the class of fluoropyrimidines and acts by inhibiting the enzyme thymidylate synthase, essential for DNA synthesis. This interference leads to cell death in rapidly dividing cancer cells.

✓ **Capecitabine**: Capecitabine is an oral prodrug of 5-FU that gets converted to 5-FU within the body. It is convenient for patients as it can be taken orally and has demonstrated efficacy in CRC treatment.

✓ **Trifluridine/Tipiracil (TAS-102)**: Trifluridine is a nucleoside analog, and tipiracil is a thymidine phosphorylase inhibitor. The combination of trifluridine and tipiracil, known as TAS-102, is used in the treatment of mCRC that has become resistant to other chemotherapy drugs. Trifluridine incorporates into DNA during replication, leading to DNA breaks and cell death. Tipiracil inhibits the breakdown of trifluridine, enhancing its effectiveness and extending its activity in the body.

IV.1.4.2 Platinum Agents:

✓ **Oxaliplatin**: Oxaliplatin is a platinum-based drug used in combination with 5-FU or capecitabine. It exerts its anticancer effects by forming DNA crosslinks, which disrupt DNA replication and ultimately lead to cancer cell death. Oxaliplatin is a third-generation platinum drug and a cisplatin analogue, in which the two ammine ligands have been replaced by a 1,2-diaminocyclohexane (DACH) carrier ligand. Using various tumor cell lines, it was shown that oxaliplatin has a spectrum of activity which differs from that of cisplatin. Most importantly, pre-clinical investigations revealed that oxaliplatin has substantial activity in colon cancer cells, making it an approved chemotherapeutic drug for colorectal cancer treatment *(Köberle and Schoch 2021)*.

IV.1.4.3 Topoisomerase I Inhibitors:

✓ **Irinotecan**: Irinotecan is a topoisomerase I inhibitor, and it hinders the function of this enzyme, leading to the accumulation of DNA breaks in cancer cells. These DNA breaks result in cell death and the inhibition of cancer growth *(Walker et al 2014)*.

IV.1.4.4 Combination Therapies (see Aparicio et al., 2020):

✓ **FOLFOX**: FOLFOX is a widely used combination chemotherapy regimen that includes 5-FU, leucovorin, and oxaliplatin. It is effective in both adjuvant and metastatic CRC settings.

✓ **FOLFIRI**: FOLFIRI is another common combination therapy comprising 5-FU, leucovorin, and irinotecan. It is often used as a first-line treatment for metastatic CRC.

✓ **XELOX**: XELOX is a combination therapy consisting of capecitabine and oxaliplatin. It is an alternative to FOLFOX and can be administered orally, making it a more convenient option for some patients.

IV.1.5 Targeted Therapy

Targeted therapy has emerged as a promising approach in the management of CRC, offering a more personalized and precise treatment strategy. Unlike traditional chemotherapy, which affects both cancerous and healthy cells, targeted therapies focus on specific molecules or pathways critical for cancer cell growth and survival. In CRC, various targeted therapies have been developed to address specific genetic alterations and cellular signaling pathways associated with tumor progression. These targeted agents aim to selectively attack cancer cells while minimizing damage to normal tissues, resulting in improved treatment outcomes and reduced side effects. Let's delve into the different classes of targeted therapies used in colorectal cancer and their respective mechanisms of action *(Xie et al., 2020)*.

IV.1.6 Monoclonal Antibodies (moAbs) in CRC

Monoclonal antibodies (moAbs) represent a class of targeted therapeutic agents that intricately disrupt key signaling pathways in diseases like CRC. Specifically, in CRC, moAbs exert their effect by acting as antagonists to EGFRs. Through their competitive and selective binding to the extracellular domain of EGFR, moAbs curtail the activation of the receptor's kinase activity, thus hampering downstream signaling cascades responsible for cancer cell growth and survival. These antibodies are classified into distinct types mouse, chimeric, humanized, and fully human each contributing to their unique efficacy and compatibility *(Xu et al., 2017)*. Notable examples of EGFR-targeting moAbs include cetuximab, panitumumab, nimotuzumab, and necitumumab, which have attained clinical approval *(Cai et al., 2020)*. Structurally, moAbs are intricate Y-shaped molecules, characterized by four polypeptide chains two heavy (H) and two light (L) with constant (CH and CL) and variable (VH and VL) domains **(Figure 33)**. The vital antigen-binding fragments (Fabs) situated at the arms of the Y-shaped structure harbor the active binding site responsible for moAbs' inhibitory prowess. The precise efficacy of moAbs is further honed by three hyper-variable regions known as complementarity-determining regions (CDR1, CDR2, and CDR3), which flank the Fabs. The stem of the Y-shaped configuration, or the fragment crystallizable region, dictates the class of the antibody, intricately influencing its functional attributes *(Sadeghalvad & Rezaei, 2021)*. This intricate

interplay between moAb structure and EGFR antagonism underscores their pivotal role in reshaping therapeutic landscapes for CRC treatment.

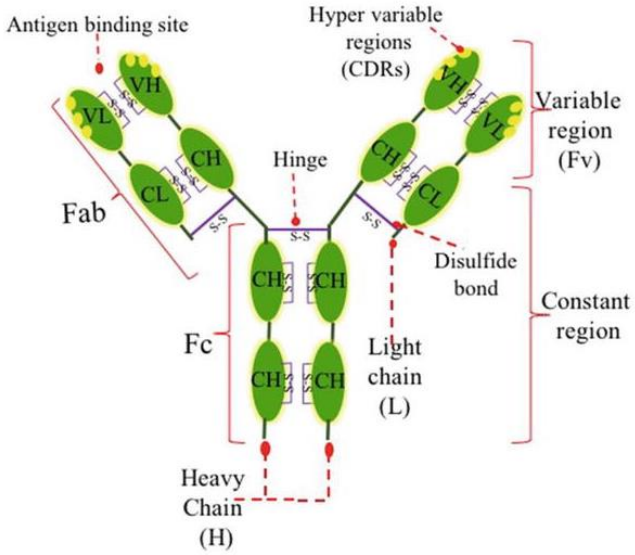


Figure 33*:* The schematic structure of an antibody *(Sadeghalvad & Rezaei, 2021).*

IV.1.6.1 EGFR Inhibition by Monoclonal Antibodies

moAbs designed to inhibit EGFR play a crucial role in CRC therapy. By competitively binding to the extracellular domain of the EGFR receptor, mAbs prevent the subsequent activation of EGFR's kinase activity. This interruption in EGFR signaling disrupts pathways that drive cancer cell proliferation and survival *(Yuan et al., 2022).*

IV.1.7 EGFR Inhibitors in CRC

EGFR inhibitors are a class of targeted therapies used in the treatment of CRC. EGFR is a cell surface receptor that plays a crucial role in cell growth and survival. In CRC, overexpression of EGFR is common and is associated with aggressive tumor behavior and poor prognosis. EGFR inhibitors specifically target the EGFR protein on the surface of cancer cells, disrupting the signaling pathways that drive cancer cell proliferation *(Janani et al., 2022).*

IV.1.7.1 Cetuximab:

Cetuximab, a moAb, effectively targets the EGFR and holds significant promise in the treatment of mCRC. This therapeutic agent, known as a human-mouse chimeric IgG1 monoclonal antibody, specifically homes in on EGFR by binding to its extracellular domain. Through this precise interaction, the activation of EGFR's kinase activity is effectively suppressed, resulting in a disruption of the EGFR signaling cascade. This disruption serves as a pivotal mechanism that curtails the proliferation and survival of cancerous cells, ultimately culminating in the inhibition of tumor growth *(Yuan et al., 2022).*

Incorporating cetuximab alongside chemotherapy has demonstrated substantial advancements in clinical trials. Notably, the combined treatment regimen has showcased enhanced OS and progression-free survival (PFS) rates in mCRC patients who express EGFR. The therapeutic approach zeroes in on the extracellular domain of the EGFR protein, where cetuximab's binding ability effectively impedes activation and downstream signaling. This interference in the EGFR pathway exerts a profound impact, leading to a tangible reduction in tumor growth and a promotion of cancer cell apoptosis *(Ohishi et al., 2023)*.

Cetuximab's utilization is particularly pertinent in mCRC cases, especially those featuring wild-type KRAS tumors. The term "wild-type KRAS" signifies the absence of mutations within the KRAS gene that could potentially hinder the efficacy of EGFR inhibitors. As part of an optimal treatment strategy, cetuximab is frequently administered in conjunction with chemotherapy agents such as irinotecan or FOLFIRI, which is a combination therapy involving 5-fluorouracil, leucovorin, and irinotecan *(Cann et al.,2023)*.

IV.1.7.2 Panitumumab:

Panitumumab, another notable moAb targeting the EGFR protein, functions similarly to its counterpart, cetuximab. It achieves its action by binding to EGFR, effectively impeding its activation and subsequent downstream signaling cascade. With extensive deployment in the field of metastatic mCRC treatment, panitumumab holds particular relevance for cases involving wild-type KRAS tumors. Its utility encompasses both collaborative use with chemotherapy and independent application as a single-agent therapy, adapting to the patient's distinct medical context and treatment goals. Distinguished as a fully humanized anti-EGFR monoclonal antibody, panitumumab excels in combating mCRC *(Battaglin et al., 2017)*. Its mechanism hinges on adept binding to the extracellular domain of EGFR, resulting in potent inhibition of EGFR's kinase activity. This disruption in EGFR signaling decisively suppresses cancer cell proliferation and survival, culminating in significant tumor growth inhibition.

Noteworthy clinical trials have underscored the potency of panitumumab, especially when teamed with chemotherapy. The collaboration has showcased tangible advancements in PFS among mCRC patients showcasing wild-type KRAS. Panitumumab's specific approval is reserved for the treatment of RAS wild-type mCRC patients.

Both cetuximab and panitumumab have substantiated their prowess in enhancing PFS and OS for individuals contending with mCRC. Yet, a pivotal precondition lies in determining the KRAS mutation status before embarking on EGFR inhibitor therapy. This meticulous assessment is paramount, as patients harboring KRAS mutations are unlikely to derive substantial benefits from these therapeutic agents *(Cann et al.,2023)*.

In sum, the tandem of cetuximab and panitumumab emerges as vital therapeutic avenues for mCRC patients marked by EGFR expression. Their concomitant integration with chemotherapy has, unequivocally, yielded favorable patient outcomes.

IV.1.8 VEGF Inhibitors in CRC

VEGF (Vascular Endothelial Growth Factor) inhibitors represent a pivotal class of targeted therapies designed to disrupt angiogenesis, a fundamental process that fuels tumor growth and metastasis. By blocking VEGF, these inhibitors effectively obstruct the formation of new blood vessels crucial for supplying nutrients to tumors. Consequently, the tumor's access to essential resources is curtailed, rendering it susceptible to regression. For more than 15 years, VEGF inhibitors have played a central role in the treatment of CRC by significantly augmenting PFS and OS when combined with chemotherapy. However, the development of resistance to VEGF inhibitors over time poses a substantial challenge, often leading to disease progression. As such, close and vigilant monitoring, along with strategic management strategies, are imperative for patients undergoing VEGF inhibitor therapies *(Hansen et al., 2021)*.

IV.1.8.1 Bevacizumab:

One prominent player in the realm of VEGF inhibitors is Bevacizumab. Operating as a monoclonal antibody directed against VEGF, it finds application in the treatment of mCRC. Frequently administered in tandem with chemotherapy regimens like FOLFOX or FOLFIRI, Bevacizumab has consistently demonstrated its efficacy in elevating both PFS and OS among patients grappling with mCRC. Its mechanism of action lies in the inhibition of VEGF, thereby hampering the formation of new blood vessels and inducing regression in the tumor *(Ohishi et al., 2023)*.

IV.1.8.2 Ramucirumab:

Ramucirumab is yet another noteworthy VEGF inhibitor, focusing on the VEGF receptor itself. This inhibitor stands as a versatile option, being employed either as a standalone therapeutic agent or in combination with chemotherapy. It caters to patients who have advanced or metastatic CRC and have previously undergone treatment with Bevacizumab. By targeting the VEGF receptor, Ramucirumab manages to exert its influence on angiogenesis, further curtailing the tumor's ability to access vital nutrients and promoting regression *(Ohishi et al., 2023)*.

IV.1.9 BRAF Inhibitors in CRC

BRAF is a gene that encodes a protein involved in cell signaling. Mutations in the BRAF gene are present in a subset of CRC tumors and are associated with a poor prognosis. BRAF

inhibitors specifically target cancer cells with BRAF mutations, inhibiting the aberrant signaling pathways and impeding tumor growth *(Caputo et al., 2019)*.

IV.1.9.1 Eucorafenib:

Encorafenib is a potent BRAF inhibitor designed to specifically target the mutated BRAF protein, particularly the V600E mutation. This mutation leads to a constitutively active BRAF protein, resulting in continuous activation of downstream signaling pathways that promote cell growth and survival. By inhibiting this mutated BRAF protein, Encorafenib helps normalize these signaling pathways, slowing down the excessive growth and division of cancer cells. Encorafenib belongs to a class of targeted therapies that aim to directly interfere with the molecular drivers of cancer while sparing normal cells, leading to potentially more effective treatment with fewer side effects *(Geel and Iersel, 2022)*.

IV.1.9.2 Binimetinib:

Binimetinib, on the other hand, is a MEK inhibitor. The MEK protein is downstream from BRAF in the RAS-RAF-MEK-ERK signaling pathway. When BRAF is mutated, it hyperactivates this pathway, contributing to uncontrolled cell growth and survival. By inhibiting MEK, Binimetinib disrupts this downstream signaling cascade, further reducing the aberrant activity driven by the mutated BRAF protein *(Subbiah et al., 2020)*.

IV.1.9.3 Other BRAF Inhibitors:

In addition to Encorafenib and Binimetinib, other BRAF inhibitors have also been studied in the context of CRC. Vemurafenib and Dabrafenib are among the notable inhibitors that have undergone investigation. These inhibitors, similar to Encorafenib, target the mutated BRAF protein to impede the abnormal signaling pathways that drive cancer growth. However, the combination of Encorafenib and Binimetinib stands out for its dual-targeting approach, addressing the BRAF mutation at two critical points in the signaling pathway, which can contribute to enhanced therapeutic efficacy and reduced risk of drug resistance *(Xu et al., 2022)*.

The combination of Encorafenib and Binimetinib is particularly effective because it addresses the BRAF mutation at two critical points in the signaling pathway. Encorafenib targets the mutated BRAF protein itself, while Binimetinib targets MEK downstream, amplifying the disruption of the signaling pathway. This dual inhibition strategy aims to maximize the therapeutic effect and reduce the likelihood of drug resistance that can occur when targeting a single point in the pathway.

IV.1.10 HER2 Inhibitors in CRC

HER2 (Human Epidermal Growth Factor Receptor 2) is a protein that plays a vital role in cell growth and division. In some cases, CRC tumors can exhibit an overexpression of the HER2 protein, which is associated with a more aggressive form of the disease. HER2 inhibitors are a class of targeted therapies designed to specifically target cancer cells that overexpress HER2, thereby interrupting the signaling pathways that drive tumor growth and progression *(Ivanova et al., 2022)*.

IV.1.10.1 Trastuzumab:

Trastuzumab, a monoclonal antibody, is a pioneer in HER2-targeted therapy. It binds to the HER2 protein on the surface of cancer cells, inhibiting its activity and interfering with the signaling that drives cell proliferation. Trastuzumab has proven effective in treating HER2-positive mCRC, especially when used in combination with chemotherapy. By blocking the HER2 pathway, trastuzumab helps to slow down tumor growth and improve clinical outcomes.

IV.1.10.2 Lapatinib:

Lapatinib takes a dual-targeting approach by inhibiting both HER2 and EGFR , another protein implicated in cell signaling. This small molecule inhibitor has shown promise in combination with chemotherapy for treating HER2-positive mCRC. By blocking these receptors, lapatinib disrupts the signaling cascades that promote cell growth, providing a potential therapeutic avenue for patients with HER2-positive mCRC.

IV.1.10.3 Pertuzumab:

Pertuzumab, another monoclonal antibody, complements trastuzumab's action by targeting a different region of the HER2 protein. When combined with trastuzumab and chemotherapy, pertuzumab creates a synergistic effect, further inhibiting HER2-driven pathways. This combination therapy has demonstrated improved outcomes for patients with HER2-positive mCRC, offering an enhanced therapeutic strategy.

IV.1.10.4 Tucatinib:

Tucatinib represents a newer addition to the HER2 inhibitor landscape. This promising drug has exhibited encouraging results in clinical trials for HER2-positive mCRC. Tucatinib can be used as a monotherapy or in combination with chemotherapy and/or other HER2 inhibitors. Its efficacy underscores the potential of multiple-target approaches in treating HER2-positive CRC.

IV.1.10.5 Neratinib:

Neratinib, a small molecule HER2 inhibitor, has been explored in combination with other targeted therapies, such as cetuximab, for HER2-positive mCRC. This combination strategy aims to comprehensively address the multiple signaling pathways involved in promoting cancer cell growth in HER2-positive tumors.

IV.1.11 Immunotherapy

Immunotherapy represents a groundbreaking paradigm in the realm of cancer treatment, capitalizing on the body's natural defense mechanism the immune system to recognize and combat malignant cells. In the context of CRC, immunotherapy has emerged as a beacon of hope, particularly for patients harboring distinct molecular signatures termed microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) tumors. These tumors, characterized by an elevated mutation burden, become conspicuous targets for the immune system's vigilant scrutiny, underpinning the success of immunotherapeutic interventions **(Figure 34)** *(Makaremi et al., 2021)*.

A pivotal strategy within immunotherapy is the employment of checkpoint inhibitors, a class of therapies adept at targeting specific proteins residing on immune cells, such as PD-1 (programmed cell death protein 1) or PD-L1 (programmed death ligand 1). These proteins ordinarily serve as gatekeepers of the immune response, shielding healthy cells from undue harm. However, cancer cells often exploit these checkpoints to evade immune recognition and attack. By deploying checkpoint inhibitors, this defense mechanism is dismantled, enabling immune cells to rekindle their assault on cancer cells. While the journey of checkpoint inhibitors in clinical trials has been promising, particularly for patients with MSI-H or dMMR tumors, not all CRC patients respond uniformly, and challenges including immune-related adverse events and the emergence of resistance persist *(Makaremi et al., 2021)*.

IV.1.11.1 Pembrolizumab:

Pembrolizumab, a trailblazing checkpoint inhibitor targeting PD-1, stands as a beacon of success in mCRC management. Approved for employment in cases of MSI-H or dMMR tumors that have progressed post-standard chemotherapy, this agent has etched its mark through clinical trials, boasting impressive response rates and enduring outcomes for this subgroup of patients *(Wookey and Grothey, 2021)*.

IV.1.11.2 Nivolumab:

Echoing the triumphs of pembrolizumab, nivolumab, another PD-1 inhibitor, steps onto the stage as an approved option for mCRC characterized by MSI-H or dMMR status and

progression post-standard treatment. Its prowess in unleashing immune responses within this specific patient cohort attests to the potency of checkpoint inhibitors in reshaping CRC treatment paradigms *(Motta et al.,2021).*

IV.1.11.3 Dostarlimab:

Dostarlimab emerges as a beacon of hope for recurrent or advanced MSI-H or dMMR solid tumors, including mCRC. A dedicated PD-1 immune checkpoint inhibitor, dostarlimab secured accelerated approval for its commendable achievements. A recent clinical trial, enrolling stage 2 or 3 CRC patients with dMMR, showcased remarkable efficacy, resulting in a remarkable 100% cure rate among treated CRC patients. The trial further revealed a lack of grade 3 or higher adverse events, unveiling the promising safety profile of dostarlimab. These findings underscore its potential as a highly effective and well-tolerated therapeutic avenue for this precise subset of CRC patients, catalyzing optimism within the medical community *(Hussain et al., 2022).*

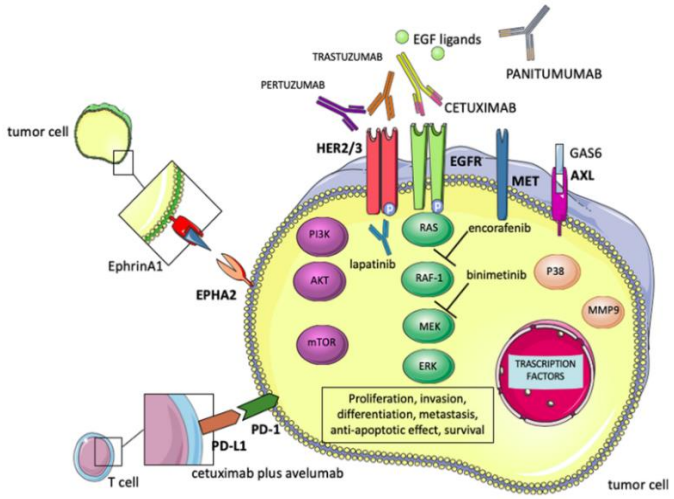


Figure 34: Unraveling Molecular Resistance Mechanisms to Anti-EGFR Therapies and Exploring Potential Therapeutic *Strategies (Martini et al., 2020).*

V. Materials and Methods

V.1 FFPE Blocks and Anatomopathological Analyses

The FFPE blocks utilized in our study were sourced from biopsies or surgical resections of the left or right colon and rectum. These samples were collected from histologically confirmed metastatic mCRC patients. Anatomopathological analyses were conducted within the anatomopathology department at the military hospital to ascertain the grade and TNM stage of each specimen. Grade determination involved assessing characteristics to assign appropriate grades, while TNM staging followed the evaluation of Tumor, Node, and Metastasis components. These analyses, performed through staining methods and microscopic examination, yielded valuable insights into the clinical implications of grade and TNM stage determinations for mCRC diagnosis, prognosis, and treatment planning. The contributions of these analyses to our study's objectives underscore their significance in advancing the understanding of mCRC.

V.2 DNA Extraction Methodology

DNA extraction was carried out using the QIAamp® DNA FFPE Tissue Kit (Qiagen GmbH, Germany), following the manufacturer's recommendations. This kit facilitates the extraction and purification of DNA from biological samples that have been formalin-fixed and paraffin-embedded, utilizing a silica membrane-based approach. The extraction process was meticulously conducted to ensure optimal results and maintain the integrity of the extracted DNA for subsequent analyses. The utilization of this well-established kit allowed us to effectively obtain high-quality DNA from our FFPE samples, which is crucial for accurate downstream molecular investigations and genetic analyses.

V.2.1 Composition of the QIAamp® DNA FFPE Tissue Kit (Qiagen GmbH, Germany)

The QIAamp® DNA FFPE Tissue Kit, a pivotal component of our methodology, encompasses a comprehensive set of elements meticulously designed for effective DNA extraction from formalin-fixed and paraffin-embedded (FFPE) samples. This kit, which adheres to the supplier's recommendations, provides an advanced and streamlined approach to DNA recovery, enabling subsequent genetic analyses. The key components of the kit, as illustrated in Figure 10, include:

- ATL Buffer (14 ml): A lysis buffer optimized for FFPE sample digestion and DNA release.

- ❖ AL Buffer (12 ml): A buffer essential for binding DNA to the silica membrane, facilitating its subsequent purification.
- ❖ AW1 Wash Buffer (19 ml) and AW2 Wash Buffer (13 ml): Wash buffers designed to remove impurities and contaminants, ensuring a clean DNA sample.
- ❖ Elution Buffer (20 ml): A buffer used for eluting the purified DNA from the silica membrane, enabling its collection for downstream analyses.
- ❖ Protéinase K (1.25 ml): An enzyme critical for digestion of proteins that might hinder DNA extraction.
- ❖ QIAamp MinElute® Columns: Specialized columns facilitating the binding, washing, and elution steps, contributing to the isolation of high-quality DNA.
- ❖ Collecting Tubes (2 ml): Tubes used for collecting and storing the processed samples at various stages of the extraction process.

By meticulously adhering to the manufacturer's instructions and utilizing each component judiciously, we ensured the successful extraction of high-quality DNA from our FFPE samples. This extracted DNA serves as a foundational resource for our subsequent molecular investigations, contributing to a deeper understanding of the mCRC under scrutiny.

V.2.2 Extraction Protocol

The DNA extraction protocol using the QIAamp® DNA FFPE Tissue Kit involved several key stages, each designed to ensure the successful isolation of high-quality DNA from FFPE samples. The protocol encompassed the following steps:

- ❖ **Section 1: Deparaffinization**

The initial step involved deparaffinization of FFPE tissue samples. Thin sections of FFPE samples were collected, and paraffin removal was achieved through the use of xylene, followed by ethanol washes This process effectively removed paraffin and prepared the samples for subsequent lysis.

- ❖ **Section 2: Cell and Tissue Lysis**

Lysis of cells and tissues was accomplished using ATL Buffer provided in the kit. The deparaffinized samples were incubated with ATL Buffer and Protéinase K, ensuring efficient

digestion of proteins and subsequent DNA release. The lysate obtained was then mixed with AL Buffer to facilitate DNA binding to the silica membrane.

❖ **Section 3: Protein Elimination**

To eliminate proteins and impurities, the lysate was loaded onto QIAamp MinElute® Columns. These columns selectively bound DNA while allowing removal of proteins, ensuring a pure DNA sample. Wash buffers (AW1 and AW2) were employed to further cleanse the DNA-bound column.

❖ **Section 4: DNA Precipitation**

Elution Buffer was added to the column, releasing the purified DNA from the silica membrane. The eluted DNA was collected and precipitated using ethanol, followed by centrifugation. This step concentrated the DNA, preparing it for downstream analyses.

V.3 DNA Assay and Quantification

The assessment and quantification of extracted DNA play a crucial role in ensuring the quality and suitability of DNA samples for downstream molecular analyses. In our study, we employed the Qubit 2.0 Fluorometer (Invitrogen Life Technologies, Washington, USA) in conjunction with the Qubit DNA HS Assay kit (Life Technologies, Washington, USA) for accurate and sensitive DNA quantification.

Methodology:

V.3.1 DNA Assay Setup:

Following the DNA extraction protocol, the eluted DNA samples were prepared for quantification using the Qubit DNA HS Assay kit. This kit employs a highly sensitive fluorescence-based method to accurately quantify low-concentration DNA samples, making it particularly well-suited for working with FFPE-extracted DNA.

V.3.2 Fluorometric Analysis:

The Qubit 2.0 Fluorometer, a state-of-the-art instrument designed for fluorometric quantification, was utilized. This device operates on the principle of measuring fluorescence emission resulting from the interaction between the DNA sample and the Qubit DNA-binding dye in the Qubit DNA HS Assay kit.

V.3.3 Standard Curve and Quantification:

To accurately quantify the DNA concentration, a standard curve was generated using known DNA standards provided with the Qubit DNA HS Assay kit. The fluorometer measured the fluorescence intensity of the DNA samples, and their concentrations were determined by comparison with the standard curve.

V.3.4 DNA Concentration Calculation:

The DNA quantification process involved adding 10 µl of extracted DNA to a volume of 190 µl of Qubit working solution provided by the assay kit. Two standard solutions (Standard 1 and Standard 2) were prepared, each containing 10 µl of Qubit DNA HS Standard 1 or Standard 2, respectively, along with 190 µl of Qubit working solution, resulting in a final volume of 200 µl (Table 1). The concentration of the sample was calculated using the formula: Sample Concentration = QF X 200 / x, where QF represents the value provided by the instrument.

Results and Importance:

Accurate quantification of DNA is essential for ensuring consistency and reliability in downstream molecular analyses, such as PCR, sequencing, and genotyping. The Qubit 2.0 Fluorometer, in combination with the Qubit DNA HS Assay kit, provided a precise and reproducible method for quantifying DNA extracted from FFPE samples. This quantitative data guided the subsequent dilution of DNA samples to standardized concentrations suitable for various experimental procedures **(Table 5)**.

Table 5: DNA Concentration Calculation Components and Volumes.

Solution	Added Reagent	Volume added
Qubit Working	Working Solution	190 µl
Standard 1	10 µl Qubit DNA HS Standard 1	190 µl
Standard 2	10 µl Qubit DNA HS Standard 2	190 µl
Sample	10 µl Extracted DNA	190 µl
Total Volume		200 µl

V.4 Study of the RAS Gene by Pyrosequencing

The study of genetic variations within the RAS gene is a fundamental aspect of our investigation into mCRC. To achieve this, we employed the pyrosequencing technique, a powerful method that provides insights into the presence of specific mutations and alterations within the RAS gene.

V.4.1 Principle of the Pyrosequencing Technique (PyroMark Q24):

The pyrosequencing technique enables real-time detection and quantification of specific mutations. This approach involves the sequential addition of nucleotides. The correct incorporation of a nucleotide by DNA polymerase leads to the release of inorganic pyrophosphate (PPi). ATP sulfurylase and adenosine 5'-phosphosulfate convert this PPi into ATP. The generated ATP molecule catalyzes the conversion of luciferin to oxyluciferin through luciferase, producing visible light in proportion to the liberated ATP quantity. Unincorporated nucleotides are degraded by an enzyme named apyrase. The light emitted by oxyluciferin is detected by a photomultiplier tube. This light is transformed into a pyrogram and analyzed by the bioinformatics system of the PyroMark Q24 pyrosequencing instrument **(Figure 35)**.

- **Template Preparation:**

Initially, DNA templates were prepared from the extracted DNA samples, focusing on the region of interest within the RAS gene. These templates served as the basis for subsequent pyrosequencing analysis.

- **PCR Amplification:**

Polymerase chain reaction (PCR) was employed to amplify the target region of the Ras gene. This generated a sufficient quantity of DNA for subsequent pyrosequencing reactions.

- **Pyrosequencing Reaction**:

The amplified DNA was subjected to pyrosequencing using the PyroMark Q24 platform. In this technique, each of the four nucleotides (adenine, cytosine, guanine, and thymine) is sequentially introduced to the reaction. As each nucleotide is added, a series of enzymatic reactions occur, resulting in the release of pyrophosphate (PPi). The released PPi triggers a chemiluminescent signal that is detected by the instrument, allowing the determination of the sequence **(Figure 35)**.

❖ **Data Analysis:**

The PyroMark Q24 software then interprets the chemiluminescent signals generated during the pyrosequencing reaction. This information is translated into a sequence readout, enabling the identification of specific mutations or variations within the Ras gene.

By employing pyrosequencing on the PyroMark Q24 platform, we aimed to uncover critical insights into the genetic landscape of the RAS gene in mCRC. This technique provides a high-resolution examination of sequence alterations, enabling us to better understand the role of RAS gene mutations in cancer progression and therapeutic responsiveness.

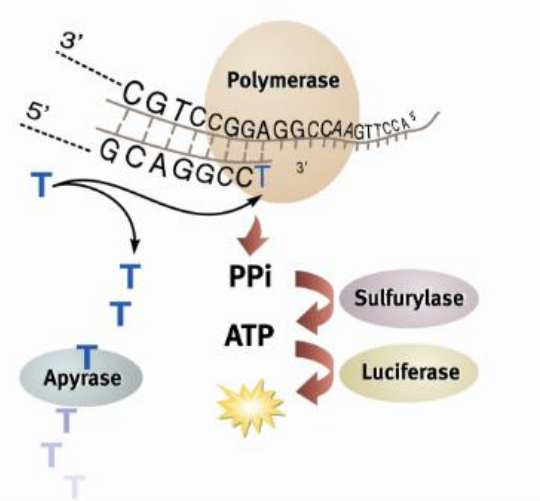


Figure 35:Principle of Pyrosequencing Process *(England and Pettersson 2005)*.

V.4.2 Therascreen Kit Composition

To facilitate this meticulous analysis, we enlisted the aid of the therascreen KRAS Pyro® kit (Qiagen, Germany) and the therascreen RAS extension pyro® kit. These carefully curated kits, intricately designed for the PyroMark Q24 platform, are indispensable components of our methodology, enabling the seamless execution of our comprehensive study.

❖ **Kit therascreen KRAS Pyro® Composition:**

The therascreen KRAS Pyro kit encompasses a meticulously formulated ensemble of elements tailored for the targeted amplification and pyrosequencing analysis of the KRAS gene. It facilitates the quantitative detection of specific mutations at codons 12, 13, and 61 of the KRAS oncogene.

❖ **Kit therascreen RAS Extension Pyro® Composition:**

Complementary to the KRAS Pyro kit, the therascreen RAS extension pyro kit extends our analysis to encompass a broader spectrum of RAS gene variants. This extension module

encompasses the analysis of specific mutations in exon 2 (codons 12/13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of the KRAS gene, as well as exon 2 (codons 12/13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of the NRAS gene **(Table 6)**. The detailed composition of the therascreen KRAS Pyro and therascreen RAS extension Pyro kits is as follows:

Table 6: Composition of therascreen KRAS Pyro Kit and therascreen RAS Extension Pyro Kit.

Component	Therascreen KRAS Pyro Kit	Therascreen RAS Extension Pyro Kit
PCR Primers	KRAS 12/13, KRAS 61	KRAS 59/61, KRAS 117, KRAS 146, NRAS 12/13, NRAS 59, NRAS 61, NRAS 117, NRAS 146
Sequencing Primers	KRAS 12/13, KRAS 61	KRAS 59/61, KRAS 117, KRAS 146, NRAS 12/13, NRAS 59, NRAS 61, NRAS 117, NRAS 146
PyroMark PCR Master Mix, 2x	Included	Included
CoralLoad®Concentrate, 10x	Included	Included
Non-methylated Control DNA	10 ng/µL	Included
PyroMark Binding Buffer	Included	Included
PyroMark Hybridization Buffer	Included	Included
PyroMark Denaturation Solution	Included	Included
PyroMark Wash Buffer, Concentrate 10x	Included	Included
Enzyme Mix	Included	Included
Substrate Mix	Included	Included
DATP, dCTP, dGTP, and dTTP	Included	Included
H2O	Included	Included

V.4.3 PyroMark Q24 System

The PyroMark Q24 system, an essential cornerstone of our study, furnishes the capabilities required for the intricate analysis of genetic variations and mutations. Designed by Qiagen, Germany, this cutting-edge platform empowers us to navigate the genetic landscape of the Ras gene with unparalleled precision.

V.4.4 Components of the PyroMark Q24 System:

The PyroMark Q24 system consists of several integrated components, each contributing to the seamless execution of our investigation:

V.4.4.1 PyroMark Q24 Instrument:

At the heart of the system lies the PyroMark Q24 instrument, a state-of-the-art device that orchestrates the pyrosequencing process. This instrument enables real-time sequencing analysis, providing insights into DNA sequences and mutations.

V.4.4.2 PyroMark Q24 Vacuum Workstation:

Complementing the instrument is the PyroMark Q24 vacuum workstation, an indispensable partner in our endeavor. This workstation creates the optimal environment for sample preparation, ensuring the accuracy and reproducibility of our results.

V.4.4.3 PyroMark Q24 Software (Version 2.0):

The PyroMark Q24 software, version 2.0, serves as the intellectual backbone of the system. This intuitive software facilitates experiment design, data collection, and analysis, streamlining the entire workflow.

V.5 Pyrosequencing Procedure

V.5.1 Configuration of Analysis on the PyroMark Q24 System:

The configuration of analysis on the PyroMark Q24 system is a pivotal initial phase, orchestrating the parameters and components essential for an insightful pyrosequencing exploration. This step encompasses several key tasks that harmonize to guide the subsequent journey of genetic investigation.

- **Designing the PyroMark Plate:**

Central to this phase is the meticulous design of the PyroMark plate within the PyroMark Q24 software. Each well on the plate is meticulously allocated, aligning with the number of samples and controls (extraction control, PCR control, and wild-type control) under scrutiny. The design defines the layout, ensuring precise placement for each element of analysis.

❖ **Transferring Analysis Data:**

Once the plate design is perfected, the analysis configuration is transferred to a USB drive. This step ensures the seamless transfer of the carefully curated design to the PyroMark Q24 system for subsequent processing and execution.

❖ **Automated Calculation of Reaction Components:**

The PyroMark Q24 software operates as a mastermind, intricately calculating the volumes of nucleotides, enzyme solutions, and substrates required for the pyrosequencing reaction. This automation streamlines the process, ensuring the accuracy and consistency of reagent volumes.

V.5.2 Pre-Pyrosequencing Polymerase Chain Reaction (PCR) Procedure

The pre-pyrosequencing PCR procedure is a fundamental phase that primes our investigation into the genetic landscape of the RAS gene. This meticulously orchestrated process sets the stage for subsequent pyrosequencing analysis, ensuring the preparation of DNA templates and the reliability of results. The procedure encompasses specific steps designed to amplify target DNA regions and establish a robust foundation for unraveling the intricate molecular variations associated with mCRC.

V.5.2.1 Conventional PCR and its Cycles:

The conventional PCR technique is employed, characterized by its cyclic nature. Each cycle consists of three pivotal stages:

1- **Denaturation:** DNA strands are denatured, separating the double-stranded DNA template into single strands.

2- **Primer Hybridization:** Primers, designed to match specific target sequences, anneal to the single-stranded DNA template.

3- **Elongation:** DNA polymerase extends the primers, synthesizing complementary DNA strands in the presence of deoxyribonucleotides (dNTPs).

V.5.2.2 Bi-directional Amplification with Biotinylated Primers:

In our pre-pyrosequencing approach, bi-directional amplification is employed, utilizing a primer pair with one primer biotinylated at its 5' end. This strategic design underpins subsequent pyrosequencing analysis, enabling precise detection of genetic variations.

V.5.2.3 Reaction Mixture Formulation:

The pre-pyrosequencing PCR begins with the formulation of a comprehensive reaction mixture. This mixture comprises key components:

- A master mix designed to provide optimal reaction conditions.
- CoralLoad concentrated 10X, facilitating efficient sample loading.
- Biotinylated PCR primers, instrumental in the targeted amplification of specific DNA segments.
- Molecular Biology Water (MBW) as a solvent for reagent dispersion.

V.5.2.4 Accurate Volume Calculation:

The calculation of precise reagent volumes is a critical aspect, ensuring consistent and reliable results. Factors such as the number of samples, controls (a positive control and no template control NTC), and a 10% margin of uncertainty are considered in this calculation.

V.5.2.5 Sample Distribution and Controls:

A volume of 20 µl of the reaction mixture is thoughtfully distributed into PCR tubes. Subsequently, 5 µl of DNA, possessing a concentration of 30 ng/µl, is added to each well. Controls include a non-methylated positive control (wild type: WT) and a negative control (no template control or NTC).

V.5.2.6 Thermocycling:

To initiate the PCR amplification, the PCR tubes are placed within the APOLLO ATC 201 thermocycler. The thermocycler meticulously executes cycles of denaturation, primer annealing, and DNA extension, fostering the targeted amplification of DNA segments **(Table 7)**.

Table 7:Program and Thermal Profile of the PCR.

Step	Temperature	Duration	No. of cycles
Initial Denaturation	95	15mn	1X
Denaturation	95	20 sec	
Annealing	53	30 sec	42X
Extension	72	20 sec	
Final Extension	72	5mn	1X

V.6 Electrophoresis on Agarose Gel

Electrophoresis on agarose gel is a pivotal technique employed to separate DNA fragments based on their size and charge. This process allows for the analysis and visualization of DNA samples, an indispensable step in genetic research. The electrophoresis was performed on a 1.6% agarose gel, executed through a meticulous sequence of steps designed to ensure accurate results and reliable data interpretation.

V.6.1 Gel Preparation:

The journey of electrophoresis commences with the preparation of the agarose gel. A solution comprising 1.6 g of agarose and 100 ml of 0.5X TBE (Tris, Borate, EDTA) buffer is meticulously concocted. This solution is brought to a boil using a microwave and then allowed to cool. Subsequently, 2 µl of ethidium bromide (EtBr) is added to the mixture. The resulting solution is then carefully poured into a gel tray and left to solidify at room temperature.

V.6.2 DNA Migration:

With the gel now set, it is placed within an electrophoresis chamber containing 0.5X TBE buffer. A volume of 2 µl of the PCR product is delicately loaded into each well of the gel. The electrophoresis is carried out at a constant voltage of 100V for a duration of 25 minutes . Once the electrophoresis is complete, the gel is visualized under ultraviolet (UV) light using a transilluminator, specifically the D4UN transilluminator. The gel's intricate patterns are then captured and analyzed using the sophisticated BIO-RAD apparatus.

V.7 Immobilization of PCR Products on Streptavidin-Coated Sepharose Beads (Streptavidin Sepharose High Performance)

The immobilization of PCR products onto streptavidin-coated Sepharose beads marks a pivotal step in our experimental protocol, capitalizing on the remarkable affinity between streptavidin and biotin. These Sepharose beads, consisting of cross-linked agarose, are adeptly chosen as a substrate due to their versatility and stability. The strategic coating of these beads with streptavidin further enhances their binding capacity, forming a robust biotin-streptavidin interaction that transcends varying environmental conditions such as pH, temperature, and denaturing agents *(Luecke and Gundry 2021)*.

V.7.1 Streptavidin-Coated Sepharose Bead Immobilization:

The journey into immobilization begins by preparing a master mix tailored for DNA immobilization, comprising a meticulously orchestrated ensemble of reagents, as outlined in **Table 8** below.

Table 8: Master Mix for DNA Immobilization

Component	**Mix Volume (ml)**
Streptavidin Sepharose High Performance	2
PyroMark Binding Buffer	40
Molecular Biology Grade Water (EBM)	28
Total Volume	70

With the master mix primed, a precisely measured 70 µl volume is aliquoted into individual tube wells. A controlled addition of 10 µl of biotinylated PCR products ensues, culminating in a final volume of 80 µl. Ensuring the uniform distribution of streptavidin-coated Sepharose beads is paramount, achieved through controlled agitation within an incubator set at room temperature. The plate shaker operates at 1400 revolutions per minute for a duration of 5 to 10 minutes, facilitating a harmonious intermingling of components.

This orchestrated agitation paves the way for optimal interaction between immobilized PCR products and the streptavidin-coated bead matrix. This delicate molecular dance sets the stage for subsequent analytical endeavors, laying a fundamental foundation for delving into the intricate genetic signatures concealed within the KRAS gene.

V.8 Sample Preparation Prior to Pyrosequencing Analysis

Before delving into the intricacies of pyrosequencing analysis, a meticulous preparation process readies the single-stranded DNA and orchestrates the priming of sequencing primers to the DNA matrix. This critical phase unfolds through a series of carefully orchestrated steps:

V.8.1 Preparation of Reaction Mix for Hybridization:

The journey begins with the meticulous crafting of a reaction mix, housing sequencing primers and hybridization buffer. Subsequently, a volume of 25 µl of this mix is distributed into the wells of the PyroMark Q24 plate.

V.8.2 Placing PCR and PyroMark Q24 Plates on the Vacuum Workstation:

The immobilized PCR products grace the PCR plate, while the PyroMark Q24 plate cradles the sequencing primers. These plates find their designated spots on the PyroMark Q24 MDx vacuum workstation, adhering to the order specified in the analysis file.

V.8.3 Vacuum Preparation Tool (VPT) Deployment:

With precision, the Vacuum Preparation Tool (VPT) is gently positioned within the wells of the PCR plate. The entire content is then meticulously aspirated through the filter probes under controlled command.

V.8.4 Bead Washing and Denaturation:

The aspirated beads embark on a journey of refinement. They undergo a thorough wash with 70% ethanol (50 ml), followed by immersion in a denaturation solution (50 ml), and finally suspension in a wash buffer (Wash Buffer, 50 ml).

V.8.5 Hybridization and Activation:

The sequencing primer-loaded probes are strategically placed within the PyroMark Q24 wells. The vacuum station is sealed to facilitate the release of the beads, securing the biotinylated strands to the sequencing primer-laden plate. A crucial step involves hybridizing the sequencing primers and immobilized PCR products at 80°C **(Table 9)**.

Table 9: Reaction Mix for Sequencing Primers

Components	**Mix Volumes (ml)**
Sequencing Primers	0.8
Hybridization Buffer	24.2
PyroMark Annealing Buffer	
Total Volume	25

V.9 Powering On the PyroMark Q24 System

The initiation of the PyroMark Q24 system encompasses a strategic loading process, orchestrated to infuse nucleotide mixes (A, C, G, and T), enzymes (DNA polymerase, ATP sulfurylase, luciferase, and apyrase), and substrates (adenosine 5’-phosphosulfate (APS) and luciferin) into the PyroMark Q24 cartridge (refer to Figure 42). Each reagent's precise volume, an instrumental directive during the analysis configuration on the PyroMark Q24 system, ensures a harmonious preparatory setup.

With this comprehensive assembly ready, the PyroMark Q24 cartridge and the dedicated PyroMark Q24 plate find their designated positions within the PyroMark Q24 instrument. The culmination of this installation is complemented by the introduction of a key participant – a USB drive housing the essential analysis file.

V.10 **Analysis of Results** using **PyroMark Q24 Software**

The PyroMark Q24 software transforms the raw data into a symphony of insights, guiding us through the intricate genetic landscape with meticulous precision. This crucial phase begins as the USB drive containing the test results is seamlessly integrated into a computer harboring the PyroMark Q24 software suite **(Figure 36)**.

Upon initiation, the software orchestrates a carefully choreographed data interpretation ballet. The outcomes elegantly unfold, first in the form of a comprehensive summary table that encapsulates the diverse range of samples scrutinized. Subsequently, a cascade of detailed pyrogram visualizations emerges, each pyrogram akin to a unique genetic fingerprint. These pyrograms intricately capture the delicate dance of nucleotide incorporation, with peak lengths elegantly reflecting the nucleotide count.

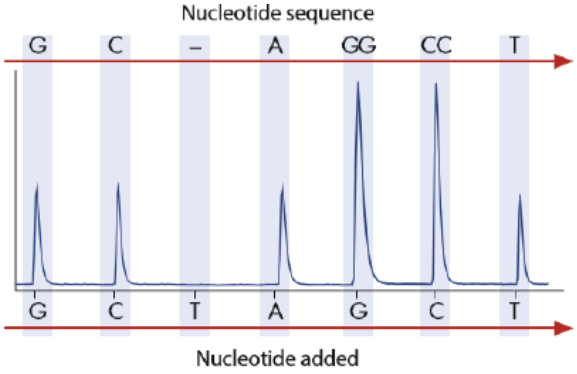
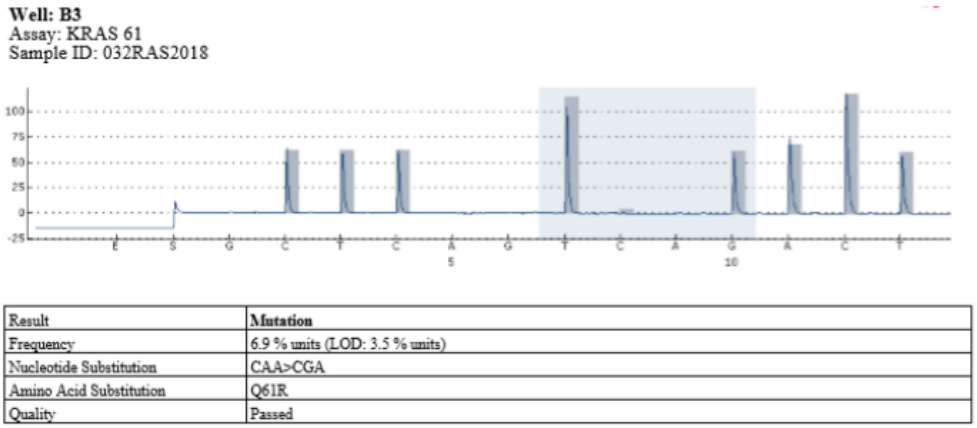


Figure 36: Illustrative Pyrogram following Results

The interpretation stage unfurls against the backdrop of the Limit of Detection (LOD). The revelations gracefully fall into distinct categories **(Figure 37)**:

- Mutation Frequency < LOD: Corresponding to the Wild Type.
- Mutation Frequency ≥ LOD and ≤ LOD + 3% units: Hinting at a potential low-level mutation.
- Mutation Frequency > LOD + 3% units: Confirming a significant mutation.

This structured analysis, masterfully facilitated by the PyroMark Q24 software, concludes with a crescendo of understanding. It unravels the genetic intricacies underlying the targeted sequence, casting a spotlight on the subtlest variations. Through this digital orchestration, our comprehension of the molecular canvas is heightened, shedding light on the enigmatic realm of mCRC.



Result	Mutation
Frequency	6.9 % units (LOD: 3.5 % units)
Nucleotide Substitution	CAA>CGA
Amino Acid Substitution	Q61R
Quality	Passed

Figure 37: Pyrogram Example Obtained after Analysis by PyroMark Q24 (Source: LRB-P3)

Objectives of the Thesis

VI. The aim of this Thesis

This thesis aims to comprehensively investigate the significance of the RAS gene in CRC. The research focuses on understanding the role of the RAS gene in CRC development, progression, and prognosis, as well as exploring its impact on the molecular characteristics of the disease. Additionally, the study assesses the clinical implications of RAS gene mutations in mCRC and their influence on treatment decisions and responses to anti-EGFR therapies.

Objectives:

1. Investigate the Role of the RAS Gene in CRC:
    - Examine the involvement of the RAS gene in CRC development, progression, and prognosis.
    - Analyze the impact of RAS gene mutations on the molecular characteristics of CRC.
2. Explore Genetic Heterogeneity and Molecular Characteristics:
    - Investigate the diversity of RAS gene mutations in CRC patients within the Moroccan population.
    - Understand the underlying molecular mechanisms associated with RAS gene mutations in colorectal carcinogenesis.
3. Assess the Clinical Significance of RAS Gene Mutations in mCRC:
    - Determine the clinical implications of RAS gene mutations in mCRC.
    - Evaluate the influence of RAS gene mutations on treatment decisions and response to anti-EGFR therapies.

Approach:

This thesis follows a two-fold approach to achieve its objectives. The initial phase we focus on investigating the occurrence and impact of KRAS, NRAS, and BRAF mutations in Moroccan CRC patients. Additionally, we explore the correlation between lifestyle factors and the risk of developing CRC. By identifying the genetic and lifestyle factors contributing to CRC in this specific population, our research aims to enhance the understanding of CRC in Morocco and provide insights into prevention and treatment strategies. The findings will form the basis for improving the diagnosis, prognosis, and targeted therapeutic approaches for CRC patients in Morocco, ultimately leading to a decrease in the incidence and mortality of this disease.

The second phase of this study entails a comprehensive literature review in the Middle East and North Africa. We analyze the prevalence and patterns of mutations in the RAS/RAF/MEK/ERK/MAPK signaling pathway among CRC patients. Additionally, this phase

investigates potential associations between dietary and lifestyle factors and CRC risk in North Africa.

Papers Presentation

Paper I: Clinical Significance of Somatic Mutations in *RAS/RAF/MAPK* Signaling Pathway in Moroccan and North African Colorectal Cancer Patients

Colorectal cancer is a significant global public health concern, characterized by high incidence and mortality rates. Targeted therapy utilizing anti-EGFR monoclonal antibodies has shown promising results in improving outcomes for patients with mCRC. However, the response rates to this treatment vary widely among individuals. Somatic mutations in the RAS/RAF/MAPK signaling pathway have been identified as key biomarkers that can predict the response to anti- EGFR therapy. This study aimed to investigate the frequency and distribution of RAS/RAF/MAPK pathway mutations in a cohort of Moroccan CRC patients.

To achieve this, we enrolled 80 patients with histologically confirmed CRC who had undergone surgical resection or endoscopic biopsy. All tissue samples were FFPE and histologically confirmed. We screened these CRC patients for clinically relevant mutations in RAS and BRAF genes at the Laboratory of Research and Biosafety P3, Mohammed V Military Teaching Hospital in Rabat, Morocco. DNA was extracted from FFPE tissue samples and analyzed for mutations in the KRAS, NRAS, and BRAF genes using polymerase chain reaction (PCR) and pyrosequencing. Additionally, we collected clinical and pathological data from medical records to correlate with the genetic findings.

Our study analyzed the clinico-pathological characteristics and genetic mutations in 80 CRC patients. The primary tumors were predominantly located in the colon (56.3%) or rectum (25%), with moderate-differentiated adenocarcinomas being the most prevalent (57.5%). Notably, RAS mutations were observed in 57.5% of patients, with KRAS mutations (56.2%) being more common than NRAS mutations (8.8%). Moreover, we found that KRAS codon 12 mutations were associated with higher tumor stages (III-IV) compared to wild-type carcinomas. However, no BRAF gene mutations were detected in CRC patients. Furthermore, NRAS mutations did not show significant associations with the examined molecular features.

The findings of this study provide valuable insights into the characteristics and genetic profile of CRC in the Moroccan population. Understanding the prevalence of RAS/RAF/MAPK pathway mutations is crucial as it can help in identifying potential candidates for targeted therapy with anti-EGFR monoclonal antibodies. Moreover, the knowledge gained from this research may contribute to the development of more personalized and effective treatment strategies for CRC patients in Morocco and beyond.

RESEARCH ARTICLE

Editorial Process: Submission:03/09/2022 Acceptance:11/02/2022

Clinical Significance of Somatic Mutations in *RAS/RAF/MAPK* Signaling Pathway in Moroccan and North African Colorectal Cancer Patients

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Abstract

Background: Mutations in *RAS* (*KRAS, NRAS*) and *BRAF* genes are the main biomarker predicting response to anti-EGFR monoclonal antibodies in targeted therapy in colorectal cancer (CRC). **Objective:** Our study aims to evaluate the frequencies of KRAS, NRAS and BRAF mutations and their possible associations with clinico-pathological features in CRC patients from Morocco. **Methods:** DNA was extracted from 80 FFPE samples using the QIAamp DNA FFPE-kit. *RAS* and BRAF mutations were assessed by pyrosequencing assays using Qiagen, KRAS Pyro®kit 24.V1, Ras-Extension Pyro®kit 24.V1 and BRAF Pyro®Kit 24.V1, respectively, and carried out in the PyroMark-Q24. **Results:** RAS mutations were identified in 57.5% (56.2% in KRAS, 8.8% in NRAS). In KRAS gene, exon 2 mutations accounted for 93.3% (68.9% in codon 12, 24.4% in codon 13). Within codon 12, G12D was the most prevalent mutation (37.7%), followed by G12C (13.4%), G12S (8.9%) and G12V (6.6%). Within codon 13, the most frequently observed mutation was G13D (22.3%). The mutation rates of exon 3 and 4 were 15.6% and 13.3%, respectively. In exon 3 codon 61, 2.3% patients were detected with two concurrent mutations (Q61R, Q61H), and 4.4% with three concurrent mutations (Q61R, Q61H, Q61L). In NRAS gene, the mutation rates of exon 2, 3 and 4 were 57.1%, 28.6%, and 14.3%, respectively. G13A and Q61H were the most common mutations, accounting for 42.9% and 28.5%, respectively. There were 13% patients with concurrent KRAS/NRAS mutation and 4.3% wt KRAS with NRAS mutations. No mutations were identified in BRAF gene. In both sexes, KRAS codon 12 mutations were associated with higher stage III/IV tumors. Moreover, Patients whose tumor is in the proximal colon (56.3%) are more likely to harbor KRAS mutations than those tumor located in rectum (25%). **Conclusion:** *RAS* mutations could be useful in future target anti-EGFR therapy and molecular CRC screening strategy in Morocco.

Keywords: *KRAS- NRAS- BRAF-* Colorectal cancer- Pyrosequencing

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Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer and is the fourth leading cause of death worldwide. There were 1.93 million estimated new diagnosed CRC cases and 0.94 million CRC caused deaths in 2020 worldwide (Xi et al., 2021). The CRC-global burden is expected to increase by 60% causing 2.2 million new cases and 1.1 million annual deaths by 2030 (Arnold et al., 2017). CRC incidence and mortality rates vary widely worldwide, with distinct gradients across human development levels and its trends point towards widening disparities and an increasing burden in countries in transition (Arnold et al., 2017). CRC is now recognized

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as one of the clearest markers of the disease transition in societies undergoing socioeco¬nomic development and transition to a lifestyle more typical of industrialized countries (Fidler et al., 2017).

In Morocco, CRC ranks first among gastro-intestinal cancers and it is the third most commonly diagnosed cancer (Fondation Lalla Salma Pévention et traitement des cancers, 2016). According to the data on GLOBOCAN 2020, there were 4324 new CRC patients and 2374 deaths, accounting for 7.3% and 2.8% of all cancers, respectively (Ferlay et al., 2020). There was a clear rising trend in CRC incidence with age-standardized increases in incidence rates of 3.8 to 8.4 and from 2.6 to 7.4 per 100,000 in men and women, respectively. CRC patients are a young population and are diagnosed between 55 and 59 years for both sexes (Fondation Lalla Salma Pévention et traitement des cancers, 2012). The detection rates of advanced adenomas and CRC were 4.0 in 1000 and 0.5 in 1000 screened individuals, respectively (Selmouni et al., 2017).

CRC is a complex and genetically heterogeneous disease involved in oncogene and tumor suppressor genes, as well as genes involved in DNA damage recognition and repair. CRC includes also different categories of tumors based on their specific spectrum of mutation and molecular phenotype, driving various oncogenic signaling pathways (Miele et al., 2020). The RAS-MAPK pathway is one of the most deregulated and extensively characterized pathways in CRC, with KRAS being the most frequently mutated gene. RAS oncogenes (KRAS and NRAS) encode small membrane-bound GTPase proteins that regulate cellular proliferation, differentiation, and survival. Under physiologic conditions, RAS proteins cycle between their GTP-bound active and GDP-bound inactive states to regulate the activation of downstream effectors proto-oncogene serine/threonine kinase (RAF), MAPK kinase (MEK), and extracellular-signal-regulated kinase (Kano et al. 2016). The BRAF oncogene encodes a protein belonging to the raf/mil serine/threonine kinases family that plays a role in intracellular signaling and cell growth, and is a downstream effector of KRAS in the MAPK signaling pathway. Mutations in BRAF gene will lead to cancer development and progression (Karimi et al., 2021; Bashir et al., 2022).

The frequency rates of KRAS, NRAS, and BRAF mutations in CRC differ among populations. Our study aims to evaluate the frequencies of KRAS, NRAS and BRAF mutations and their possible associations with clinico-pathological features in CRC patients from Morocco Materials and Methods

Materials and Methods

Subjects study

Between September 2020 and December 2021, a total of 80 FFPE specimens obtained from newly diagnosed CRC patients were screened for clinically relevant mutations in RAS and BRAF genes at the Laboratory of Research and Biosafety P3, Mohammed V Military Teaching Hospital at Rabat, Morocco. All tissue samples that were extracted from primary or metastatic tumor through surgical resection or endoscopic biopsy were FFPE, and histologically confirmed.

Clinicopathological data were recorded from the medical records, including: histologic subtype, histologic grade, tumor site, vascular invasion, and perineural invasion. Primary tumors located in the cecum and ascending colon were defined as proximal tumors, whereas tumors located in the splenic flexure, descending colon, and sigmoid colon were defined as distal tumors. Detailed information on demographic factors, anthropometric characteristics, and lifestyle habits were also collected. The exclusion criteria included the presence of any other cancers, and prior anti-cancer treatment. All participants were informed about the significance of molecular testing and signed an informed consent form prior to molecular genetic testing.

Genomic DNA extraction

DNA was extracted from five slices of 10 µm FFPE tissues using the QIAamp® DNA FFPE Tissue kit (QIAamp DNA FFPE tissue, Qiagen, GmbH, Hilden, Germany). DNA concentrations were assessed with the fluorometric method based on the binding of double-stranded DNA (dsDNA)-selective fluorescent dyes (dsDNA) (Qubit 4.0 Fluorometer/Life Technologies, Invitrogen). All steps were performed following providers' guidelines.

Genotyping of Full RAS and BRAF

KRAS, NRAS and BRAF gene mutations were assessed by pyrosequencing assays using Qiagen, K-Ras Pyro®kit 24.V1, Ras-Extension Pyro®kit 24.V1 and BRAF® Pyro® Kit 24.V1. The target sequence covering the polymorphic site was amplified with one of the specific biotinylated primers. Briefly, PCR was used for amplifications of a full RAS region containing codon 12, codon 13, and codon 61 in KRAS and NRAS, as well as codons 600 and 464-469 in BRAF gene. The PCR assay was performed in a reaction volume of 20 µl using 12.5 µl PyroMark PCR Master Mix, 2,5µl CoralLoad Concentrate, 10X, 1 µl PCR Primer and 4 µl Water (H_2O, supplied). The PCR conditions were as follows: Initial denaturation at 95°C for 15min, followed by 40 cycles of 95˚C for 20 sec, 53°C for 30s and extension at 72˚C for 20 sec and then final extension at 72°C for 5min). Unmethylated control DNA was incorporated in the product as a positive control for PCR and sequencing reactions. A negative control (without template DNA) was included. We subsequently immobilized, washed, and denatured the amplified products using the vacuum workstation and subjected those products to pyrosequencing using the PyroMark Q24 system (Qiagen, PyroMark Q24 MDx V2.0), Germany).

The pyrosequencing results were analyzed using the PyroMark-Q24 version 2.0.6 software (Qiagen, PyroMark Q24 MDx (version 2.0), Germany), which identifies the presence of a specific mutation and its percentage. Manufacturer-supplied Limits of detection (LOD) thresholds were used to call a mutation for LOD studies (≥ % LOD is positive). Real-time curves and programs were interpreted according to the kit instructions and PyroMark ID software (Qiagen) allowed determination of mutant allelic frequency according to

relative peak height.

Statistical Analysis

The data were analyzed by chi-square or Fisher's exact tests to study the correlation between the clinicopathological features and the occurrence of a particular mutation. The statistical analysis used the software IBM SPSS test version 23, with $p < 0.05$ taken as the threshold for a significant difference.

Results

Clinico-pathological characteristics of FFPE specimens

The clinicopathological features of the 80 CRC patients are summarized in Table 1. The primary tumor was localized in the colon and rectum in 45 (56.3%) and 20 (25%) of CCR patients, respectively. Histological analysis has demonstrated that 25 (31.3%) of the patients are high-differentiated adenocarcinomas, 46 (57.5%) are moderate-differentiated adenocarcinomas and 9 (11.3%) are low-differentiated adeno-carcinomas. At diagnosis, 6 (7.5%), 46 (57.5%), 28 (35%) patients had stage II, III, and IV carcinomas, respectively. The most common metastatic location is observed as the liver (11/80, 13.8%).

The baseline demographic characteristics of CCR patients

There were 52 (65%) males and 28 (35%) females; with a gender ratio of ~2:1. The mean age at tumor collection was 62 ± 10.6 years (range: 29-85 years) and the majority of patients were aged less than 60 years (35%, 28/80). The median age at CRC diagnosis is younger for rectal cancer (63 ± 16 years) than for colon cancer (69 ± 15 years). For colon cancer, the median age at the time of diagnosis for men was 62 ± 7.9 years and for women was 58.5 ± 14.5 years. For rectal cancer, it is 62 ± 7.3 years for both men and women. CRC peaked in the 29 - 85 years old age group. About 5% of patients were younger than 40 years, 20% were 70 years and older.

Status of full RAS and BRAF gene mutations

RAS (KRAS and NRAS) mutations were identified in 57.5% (46/80) with 56.2% (45/80) in KRAS and 8.8% (7/80) in NRAS. In KRAS gene, exon 2 mutations accounted for 93.3% patients including 68.9% (31/45) at codon 12 and 24.4% (11/46) at codon 13. Within codon 12, G12D was the most prevalent mutation (37.7%), followed by G12C (13.4%), G12S (8.9%) and G12V (6.6%). Within codon 13, the most frequently observed mutation was G13D (22.3%). Outside exon 2, the mutation rates of exon 3 and exon 4 were 15.6% and 13.3%, respectively. In exon 3 codon 61, 2.3% patients were detected with two concurrent mutations (Q61R, Q61H), and 4.4% with three concurrent mutations (Q61R, Q61H, Q61L). Outside exon 2, the mutation rates of exon 3 were 8.8% (4/45). In exon 4, the mutation rate was 4.4% (2/45) including 2.2% (1/45) in both codon 117 and codon 146. In KRAS codons, concurrent mutations were identified in 6.7% (3/45) including 2.3% (1/45) cases, with two distinct mutations (Q61R, Q61H) and 4.4% (2/45) with three distinct mutations (Q61R, Q61H, Q61L) in codon 61. In NRAS gene, the mutation rates of exon 2, exon 3 and exon 4 were 57.1% (4/7), 28.6% (2/7), and 14.3% (1/7), respectively. G13A and Q61H were the most common mutations and were found in 42.9% (3/7) and 28.5% (2/7) respectively. There were 13% (6/46) cases detected with both KRAS and NRAS mutations and 4.3% (2/46) wt KRAS was identified with mutations in the NRAS gene. No mutations in the BRAF gene were found in our patients

Table 1. Tumor Characteristics of 80 FFPE Specimens with Colorectal Cancer

Characteristics	No	%	KRAS			NRAS		
			Wild-type	Mutant	p-value	Wild-type	Mutant	p-value
Age (Years)								
Age<60	28	35	11	17	0.555	27	1	0.229
Age>60	52	65	24	28		46	6	
Gender								
Man	52	65	23	29	0.906	48	4	0.648
Women	28	35	12	16		25	3	
Primary tumor localization								
Rectum	20	25	14	6	0.022*	19	1	0.688
Colon	45	56.3	15	30		41	4	
Others	15	18.8	6	9		13	2	
Tumor histology (differentiated adenocarcinomas)								
High	25	31,3	11	14	0.793	22	3	0.712
Moderate	46	57.5	21	25		43	3	
Low	9	11.3	3	6		8	1	
Stages								
Stage II	6	7.5	3	3	0.162	6	0	0.701
Stage III	46	57.5	16	30		42	4	
Stage IV	28	35.0	16	12		25	3	

Table 2. Frequency and Distribution of KRAS and NRAS Mutations

Gene	Exon	Nucleotide Substitution	Codon Substitution	Amino Acid Substitution	Number	%
		c.35G>A	GGT>GAT	p.G12D	17	37.7
		c.34G>T	GGT>TGT	p.G12C	6	13.4
	2	c.34G>A	GGT>AGT	p.G12S	4	8.9
		c.38G>A	GGC>GAC	p.G13D	11	24.4
		c.35G>T	GGT>GTT	p.G12V	3	6.6
KRAS		c.35G>C	GGT>GCT	p.G12A	1	2.2
		c.182A>G	CAA>CGA	p.Q61R	1	2.2
	3	c.183A>C	CAA>CAC	p.Q61H	2	4.4
		c.182A>T	CAA>CTA	p.Q61L	1	2.2
	4	c.436G>A	GCA>ACA	p.A146T	1	2.2
		c.351A>C	AAA>AAC	p.K117N	1	2.2
	2	c.38G>C	GGT>GCT	p.G13A	3	42.9
		c.34G>C	GGT>CGT	p.G12R	1	14.2
NRAS	3	c.183A>T	CAA>CAT	p.Q61H	2	28.5
		c.175G>A	GCT>ACT	p.A59T	1	14.2
	4	c.351G>C	AAG>AAC	p.K117N	2	2.5
		c.436G>A	GCA>ACA	p.A146T	1	14.2

with CRC. The detailed distribution of the KRAS and NRAS identified mutations in 46 FFPE specimens have been given in Table 2.

Association between full RAS status and clinico-pathologic characteristics

Correlation of KRAS mutations with gender and age showed that KRAS mutations were slightly higher in women (57.1%) than men (55.7%), although the difference was not statistically significant. KRAS mutations were frequent in patients in the age range between 40 and 69 years and less frequent in patients younger than 40 years. The mean age of patients presenting KRAS mutations was 72 years for CCR patients with normal KRAS profiles. However, No significant differences were observed. KRAS codon 12 mutations, but not codon 13 mutations, were associated with higher tumor stages (III-IV) than those of wt carcinomas ($p < 0.05$). Patients whose tumor is in the proximal colon (56.3%) are more likely to harbor KRAS mutations than those tumor located in rectum (25%) in both sexes. NRAS mutations did not seem to be associated with any of the molecular features that were examined. Association between RAS status and clinicopathological features are summarized in Table 3.

Table 3. Association between RAS Status and Clinicopathological Features

Characteristics	RAS wild-type	RAS Mutants	p-Value
Age (Years)			
Age< 60	11 (32.3)	17 (36.9)	0.674
Age>60	23 (67.6)	29 (63.04)	
Gender			
Female	12 (35.2)	15 (32.6)	0.805
Male	22 (64.7)	31 (67.3)	
Primary tumor localization			
Colon	15 (44.1)	30 (65.2)	0.223
Rectum	13 (38.2)	8 (17.3)	
Others	6 (17.6)	8 (17.3)	
Tumor Histology			
High	11 (32.3)	13 (28.2)	
Moderate	14 (41.1)	26(56.5)	0.656
Low	9 (26.4)	7(15.2)	
Stages			
II	2 (5.8)	3(6.5)	
III	19 (55.8)	31(67.3)	0.318
IV	13 (38.2)	12(26.0)	

Discussion

CRC exhibits KRAS mutation rates of ≈33% in the Catalogue of Somatic Mutations in Cancer (COSMIC) database comprising ≈75,000 tested samples. The frequencies of 40%–45% suggested by the other datasets are based on small sample sizes of fewer than 500. Notably however, the private Foundation Medicine dataset comprising 13,336 colorectal samples reports a KRAS mutation frequency of ≈50% (Serebriiskii et al., 2019). According to the COSMIC database, mutations at codon 12 (G12A, G12V, G12S, G12R, G12C, G12D) are the most prominent (>90%), followed by those affecting codon 13 (G13D and G13C), codon 61 (Q61L, Q61R, Q61H), codon 146 (A146T, A146V, A146P), and codon 117 (K117N). NRAS mutations are found in 5%-10% of CRC. NRAS is mutated in the same codon of KRAS (therefore, these positions are true hot-spot mutations), particularly in exon 2 (found in approximately 3%-5% of CRC) and exon 3 (2%-6% of CRC). BRAF mutations are identified in about 8%-12% of CRC patients and 90%

of all identified mutations are a T1799A transversion in exon 15, which results in a valine amino acid substitution (V600E) (Cantwell-Dorris et al., 2011).

In our study, RAS mutations were identified in 57.5% (56.2% in KRAS and 8.8% in NRAS) of all 80 patients with CRC, respectively. No BRAF gene alterations were found. The prevalence of KRAS and NRAS mutations in our cohort is higher than that reported by previous Moroccan studies which is within the 23.9% to 51% range for KRAS mutations (El Agy et al., 2021; Houssaini et al., 2020; Dehbi et al., 2019; Jadda et al., 2014; Marchoudi et al., 2013; Bennani et al., 2010;) and 2% to 5.3% range for NRAS mutations (Houssaini et al., 2020; Bennani et al., 2010), respectively. There are some explanations for this difference. First, we analyzed the RAS mutations using the Pyrosequencing technology, which is considered to show higher sensitivity (5%) than that of other screening methods. A study demonstrated that pyrosequencing detected 17.9% of the KRAS mutations in patients with KRAS wt using direct sequencing alone (Tougeron et al., 2013). Second, the previous Moroccan studies have examined the distribution of KRAS mutations from archived FFPE tissues. The Length of formalin fixation and FFPE specimens archiving process often causes cross linking and degradation-fragmentation of the DNA molecules. These alterations have negative consequences on the quality and quantity of extracted DNA from FFPE, and inevitably adversely affect downstream analyses, which in turn would impact the accuracy of genetic tests to detect mutated genes (Gao et al., 2020; Gill Ferreira et al., 2014).

In our study, the majority of the KRAS gene mutations were at exon 2 within 93.3%. Within codon 12, G12D was the most common mutation (37.8%), followed by G12C (13.3%), G12S (8.9%) and G12V (4.4%). Within codon 13, G13D was the most common (22.3%). Previous Moroccan studies showed similar results (Marchoudi et al., 2013; Dehbi et al., 2019; Houssaini et al., 2020). However, El Agy et al. revealed a lower rate of G13D mutation in codon 13 (El Agy et al., 2021). Our findings showed that mutation rates in exon 3 and 4 were 15.6% and 13.3%, respectively, which consist with previous Moroccan studies (Dehbi et al., 2019; Houssaini et al., 2020) but higher than others (Dehbi et al., 2019; El Agy et al., 2021). In the NRAS gene, the majority of the mutations are at exon 2 within 57.1%. G13A and Q61H were the most common mutations and were found with 42.9% and 28.5% respectively. In previous Moroccan studies, the most common NRAS mutations were Q61K (2.6%) and Q61R (1.8%) (Dehbi et al., 2019). KRAS Concurrent mutations were identified in 6.7% including, 2.3% patients with two concurrent mutations (Q61R, Q61H), and 4.4% with three concurrent mutations (Q61R, Q61H, Q61L) in codon 61. NRAS mutations have been previously reported to have an association with KRAS wt and are generally found to be mutually exclusive with KRAS mutations. In our study, 4.3% (2/46) KRAS wt was identified with mutations in NRAS gene and 13% mutations concomitantly in KRAS and NRAS genes. Our findings suggest that multiple mutations can occur in the same codon or different codons.

KRAS, NRAS and BRAF mutations in North Africa

Studies of CRC in North Africa (including Morocco, Algeria, Tunisia, and Lybia) have shown striking differences in full RAS and patterns. In Algeria, KRAS gene mutations were identified in 31.3% and 51.2% of CRC patients (Boudida-Berkane et al., 2016; Mazouzi et al., 2017) with 94.5% of mutated cases in exon 2 (Mazouzi et al., 2017). Codon 12 was the most common KRAS mutation and the most frequently found mutation was G12D ((Boudida-Berkane et al., 2016; Mazouzi et al., 2017). KRAS exon 2 mutations were more frequent in the right colon 40.6% vs. 25% in the left colon (Boudida-Berkane et al., 2016). Outside exon 2, mutations in exon 3 have been identified in 3.5% of tumor cases (Boudida-Berkane et al., 2016). Additionally, 2.3% of CRC tumors harbor NRAS mutations (Mazouzi et al., 2017). BRAF mutations were identified in 4.9% of the tumors patients with CRC. V600E mutation was found in proximal colon in 60% (3/5) tumors in patients with older age >50 years (Boudida-Berkane et al., 2016). In Tunisia, the mutation frequency for the KRAS gene has been reported to be in the range of 23.1 % to 75.2%, dominated by those in exon 2 (Sammoud et al., 2012; Karim et al., 2011; Ines et al., 2014; Jouini et al., 2019; Aissi et al., 2013; Ouerhani et al., 2013). Most exon 2 mutations were detected in codons 12 and G12V and G12D were the most dominated KRAS mutations (Sammoud et al., 2012; Jouini et al., 2019; Aissi et al., 2013; Ouerhani et al., 2013). Mutations outside the KRAS exon2 presented 13.4% of mutated cases and almost a third (28.8%) of KRAS exon 2 wt mCRC (Jouini et al., 2019). Among those, 69.3% carried mutations in NRAS exons2, 3 and 4 and 30.7% in KRAS exons3 and 4 (Jouini et al., 2019). Mutations in BRAF have been found in ~8% (4/48) of mCRC (Bougatef et al., 2008). In Libya, KRAS codon 12/13 mutations were present in 38.2% (13/34) of the CCR patients. The most frequent mutation of codon12 was G12D (46.1%), followed by G12V (17/77, 22.1%) and by G12V (30.8%) and at codon13 were G13D (7.7%) (Aboudabous et al., 2021).

Our findings showed that the KRAS mutation rate was different with more studies that therefore the geographic location and the race/ethnicity backgrounds can influence the KRAS mutation occurrence in North Africa. However, information about ethnicity-based KRAS genotype differences in North Africa is not established. Worldwide, a persistent finding in the literature is the substantial variation in KRAS mutation frequency between race/ethnic groups. In Africans, the frequency of CRC with KRAS mutations was 21% (Abdulkareem et al., 2012). An analysis of the relationship between mutations and race revealed the predominance of the wt in the Asian group (51.4%). In Europeans, the KRAS gene mutation was more often detected (54.4%) CRC patients (Smagulova et al., 2020). Colon cancers in Asians had a lower rate of KRAS mutations than blacks or whites (Yoon et al., 2015). The finding of novel KRAS and BRAF gene mutations in cancerous tissues obtained from Saudi CRC patients were typical of those observed elsewhere (Rasool et al., 2021). In Turkey, the mutation frequency for the KRAS mutation gene has been reported to be in the range of

11–49.1% (Akkiprik et al., 2008; Demiralay et al., 2012; Selcukbiricik et al., 2013; Ozen et al., 2013; Baskin et al., 2014). Turkey is known from the geographical location between Europe, the Middle East and the Caucasus region. Thus, Turkey is comprised of many ethnic groups with European, Middle Eastern, Caucasian or Asian origins. The KRAS mutation prevalence may also vary in the same population. Studies from Japan with homogeneous ethnic groups found a wide range of KRAS mutation frequencies (9–71%) (Nishiyama et al., 2002; Mitomi et al., 2003). Studies with heterogeneous ethnic groups also reported various frequencies of KRAS mutation. In the US, CRC tumors from Non-Hispanic Black (48%) or Hispanic patients (44%) carried a greater KRAS mutation rate when compared with Non-Hispanic White (39%) or Asian or Pacific Islander patients (37%) (Leah 2021). The assessment of the impact of KRAS mutation within each race/ethnic group, comparing patients with KRAS mutation vs. wt KRAS on cause-specific survival risk show a 7% risk increase for Non-Hispanic White and a 15% risk increase for Non-Hispanic Black (Bien et al., 2021).

The aspects that seem to play an important role in the incidence of the KRAS gene mutations in CRC in North Africa are lifestyle and dietary patterns. In Morocco, An inverse association between physical activity and CRC risk and a positive association between sedentary behavior and rectal cancer risk has been established recently (Hatime et al., 2022). Furthermore, a marked shift was documented in dietary habits and nutritional intake as part of the nutritional transition. This was associated with an increase in overweight and obesity. According to the body mass index, 29.6% of women were overweight and 15.4% were obese (Barich et al., 2018). These prevalences were considered higher than those reported in the 2003-2004 survey.

The major pattern of dietary habits and nutritional intake includes a large increase in the consumption of high calorie diets and fatty foods in the Moroccan population. Consumption of high amounts of these foods was associated with increased body weight, BMI, and risk of overweight and obesity. High weight-to-height ratio and BMI and obesity were related to increased odds of the KRAS gene mutations (Dolatkhah et al., 2018). Furthermore, significant associations were found between the highest intakes of red meats, cold meats, sausages and the risk of CRC in a case-control study on dietary risk factors for CRC in Moroccan population (Imad et al., 2020. Another study found a significant relationship between the consumption of red meat and colon cancer (OR = 1.23, 95% CI = 1.05–1.44) and CRC risk (OR = 1.14, 95% CI = 1.02–1.27) in patients have been established (Douala et al., 2020). High levels of animal protein, acrylamide foods, and low levels of vitamin A consumption have been shown to be associated with increased risk of CRC tumors with KRAS mutations in patients from Morocco (El Asri et al., 2020). El Asri et al., showed that an increase in the consumption of Polyunsaturated Fatty Acids (PUFA) above 16.9 g/day was associated with an increase in the presence of KRAS mutations (OR = 2.48, 95% CI = 1.22–4.96) as compared to the reference group whose consumption was less than 16.9 g in CRC Moroccan patients (El Asri et al., 2022). A high intake of PUFA, in particular linoleic acid, may be an important dietary risk factor for KRAS mutated colon tumors, possibly by generating G>A transitions or G>T or G>C transversions in the KRAS oncogene (Brink et al., 2003).

In Algeria, occupational exposures showed a significant link to an increased risk of CRC, as did obesity, alcohol consumption, and passive smoking. Yogurt, cereals, sugar, butter, and margarine consumption were significant protective factors, while cheese, dried fruits, red meat, juice, and fizzy drink consumption was associated with increased CRC risk (Negrichi et al., 2021).

In Tunisia, the CRC incidence has increased markedly from 1994 to 2009, and it is suggested that if no interventions are implemented it is going to double by 2024. It is relevant to highlight that an upward trend in obesity largely explains the increasing incidence of CRC in the Tunisian population. The trend of the incidence of obesity had increased from 10.9% in 1998 to 26.9% in 2016 (El Ati et al., 2007) and 80% of Tunisians aged over 15 years did not consume enough fruits and vegetables per day (Saidi et al., 2019). High total day meat consumption (> 100 g) was significantly associated with a high risk of CRC compared to low consumption (< 50 g) in the Tunisian population (Gharbi et al., 2020).

Limitations

Our study had some limitations. It was a retrospective study and the number of patients was relatively small. It is recommended that future studies be undertaken with larger samples to better understand the frequency and the contribution of the KRAS, NRAS, and BRAF genotype in Morocco CRC patients. In addition, other oncogenic mutations, such as KRAS exon 3 (codons 59, 60, 61), KRAS exon 4 (codons 119, 146, 147) were not assessed. Despite these limitations, our findings may help in implementing effective strategies for RAS (KRAS and NRAS) testing in Moroccan CRC patients.

In conclusion, RAS proteins and their pathway play a causal role in different human cancer, as oncogenic drivers, prognostic and predictive markers, and possible targets. The high prevalence of RAS (KRAS and NRAS) mutations across cancer types makes it an interesting therapeutic target (Cefalì et al, 2021). In CRC, screening for KRAS and NRAS mutations is extremely important predictive and prognostic markers for anti-EGFR monoclonal antibody therapy. In our study RAS (KRAS and NRAS) mutations were identified in 57.5% (56.2% in KRAS and 8.8% in NRAS), indicating that RAS mutation testing is crucial for targeted therapy management in CRC in Morocco. Furthermore, G12C mutation was detected in 13.4% of all KRAS mutated patients. This finding provides an indication of the size of the population that could benefit from treatment with mutant-specific inhibitors. The Code Break 100 trial revealed the potent antitumor effects of AMG510, a novel KRAS G12C inhibitor, against KRAS G12C-mutant solid tumors, including mCRC (Hong et al., 2020).

Abbreviations

BRAF: B-Raf proto-oncogene
COSMIC: Catalogue of Somatic Mutations in Cancer
CRC: Colorectal Cancer;
DNA: Deoxyribonucleic Acid
EGFR: Epidermal Growth Factor Receptor
FFPE: Formalin-Fixed Paraffin-Embedded
GDP-bound: Guanosine Bi-Phosphate-bound
GTPase: Guanosine Tri-Phosphate ase
GTP-bound: Guanosine Tri-Phosphate-bound
HCAs : Heterocyclic Amines
KRAS : Kirsten Rat Sarcoma
LOD: Limits of detection
MAPK pathway: Mitogen-Activated Protein kinase pathway
NRAS : Neuroblastoma Rat Sarcoma
PAHs: Polycyclic Aromatic Hydrocarbons
PCR: Polymerase Chain Reaction
PUFA: Polyunsaturated Fatty Acids
RAS: Rat Sarcoma; wt: Wild-type.

Author Contribution Statement

SB, FB and AL have conceived the study, exploited data, coordinated and drafted the paper. FH, TB, CH, TM, and RT participated in the designed. SB, AL, MJ, SE, WB, and BEM generated data and involved in data analyses. FE, IAL, MO, MI, KE, ND, and YS have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript

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Ethics approval and consent to participate

Not applicable, the results obtained are part of the normal diagnostic procedure within the hospital for the clinico-pathological evaluation in order to decide on the treatment protocol and the prognosis of the patients.

Availability of data and material

Data involving participants or patients cannot be publicly shared. Individual requests for further information on the study can be sent to the corresponding author.

Conflicts of interest

The authors declare no conflict of interest.

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Paper II: *RAS/RAF/MAPK* Pathway Mutations as Predictive Biomarkers in Middle Eastern Colorectal Cancer: A Systematic Review

This study is an extension of a previous review conducted in paper I, which focused on CRC in North Africa. The current investigation aims to broaden the scope by including the Middle East region in order to comprehensively understand the prevalence and patterns of RAS and BRAF mutations in both North Africa and the Middle East. Through a systematic review of available literature and conducting meta-analyses, the study seeks to shed light on the clinicopathological characteristics and mutation prevalence in CRC patients in this particular region.

A rigorous search strategy was employed, encompassing multiple databases and relevant sources. Inclusion criteria were set to ensure the quality and relevance of the studies included in the review. After a thorough screening process, 19 studies were ultimately included in the meta-analysis, covering various Middle Eastern countries, including Bahrain, Iran, Iraq, Israel, Jordan, Lebanon, and Saudi Arabia.

This study reviewed CRC patients in the Middle East, with 58.6% being male. RAS mutations were found in 38.1% of cases, mainly KRAS mutations (37.1%) in different exons. G12D was the most frequent mutation (23.2%). NRAS mutations occurred in 3.3% of samples, commonly in codon 61. BRAF mutations were at 2.6%, with V600E as the prevalent mutation. These findings reveal distinct mutation patterns in the Middle East compared to other ethnic groups.

This comprehensive review provides valuable insights into the prevalence and distribution of RAS and BRAF mutations in CRC patients in the Middle East. The observed regional variability holds important implications for treatment strategies and the potential for targeted therapies. Ultimately, these findings advance personalized medicine in the region and underscore the significance of understanding the genetic landscape of CRC to improve patient care and outcomes.

RAS/RAF/MAPK Pathway Mutations as Predictive Biomarkers in Middle Eastern Colorectal Cancer: A Systematic Review

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ABSTRACT

BACKGROUND: This review article aims to investigate the prevalence and spectrum of rat sarcoma (RAS) and V-Raf Murine Sarcoma Viral Oncogene Homolog B (BRAF) mutations, and their connection with geographical location, clinicopathological features, and other relevant factors in colorectal cancer (CRC) patients in the Middle East.

METHODS: A systematic literature review, employing the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) framework, was conducted to investigate the association between the frequency of relevant mutations and the descriptive clinicopathological characteristics of CRC patients. Multiple electronic databases, including PubMed, Science Direct, Web of Science, Scopus, and Google Scholar, were searched to analyze the relevant literature.

RESULTS: A total of 19 eligible studies comprising 2960 patients with CRC were included in this review. A comprehensive analysis of the collected literature data as well as descriptive and methodological insights is provided. Men were predominant in reviewed studies for the region, accounting for 58.6%. Overall, RAS mutation prevalence was 38.1%. Kirsten RAS Viral Oncogene Homolog (KRAS) mutations were the most common, accounting for 37.1% of cases and distributed among different exons, with the G12D mutation being the most frequent in exon 2 (23.2%) followed by G12V (13.7%), G13D (10.1%), G12C (5.1%), G12A (5.04%), and G12S (3.6%). Neuroblastoma RAS Viral Oncogene Homolog (NRAS) mutations were identified in 3.3% of tumor samples, with the most common mutation site located in exons 2, 3, and 4, and codon 61 being the most common location for the region. The total mutation frequency in the BRAF gene was 2.6%, with the V600E mutation being the most common.

CONCLUSION: The distribution patterns of RAS and BRAF mutations among CRC patients exhibit notable variations across diverse ethnic groups. Our study sheds light on this phenomenon by demonstrating a higher prevalence of KRAS mutations in CRC patients from the Middle East, as compared with those from other regions. The identification of these mutations and geographical differences is important for personalized treatment planning and could potentially aid in the development of novel targeted therapies. The distinct distribution patterns of RAS and BRAF mutations among CRC patients across different ethnic groups, as well as the regional variability in mutation prevalence, highlight the need for further research in this area.

KEYWORDS: Colorectal cancer, RAS, BRAF, mutation, Middle East

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Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers, ranking as the third most commonly diagnosed cancer worldwide and the fourth leading cause of cancer-related deaths.[1] Globocan 2020 data reported 1.9 million new cases of CRC globally, resulting in 935 000 CRC-related deaths.[2] By

2040, these numbers are expected to increase significantly, with annual global CRC cases predicted to reach 3.2 million.[3] Colorectal cancer incidence rates are rising worldwide in the Middle East and other regions, affecting individuals of all ages and sexes.[4,5] It is important to note that most countries with high or very high human development index (HDI) have higher CRC incidence rates than countries with lower HDI,[6] showing the effect of lifestyle factors.

RAS genes are pivotal in CRC due to their frequent mutation in this malignancy. These genes, proto-oncogenes, encode small GTPase proteins that regulate cell division and proliferation. Mutations in RAS lead to constitutively active forms, driving unchecked cell growth. KRAS and NRAS are RAS gene variants, with KRAS located on chromosome 12 and NRAS on chromosome at position 13.1.[7] KRAS mutations are more prevalent than NRAS mutations, with approximately 40% of colorectal tumors bearing KRAS mutations.[8] The most common KRAS mutations occur at codon 12 (>90%), followed by codons 13, 61, 146, and 117. NRAS mutations, sharing similar codon mutations with KRAS, are found in 5% to 10% of CRC cases.[9] BRAF, a downstream effector of RAS located on chromosome 7 (7q34), is a serine/threonine kinase. BRAF mutations, identified in approximately 8% to 12% of CRC patients,[10] activate the Mitogen-activated protein kinase (MAPK) pathway, governing cell proliferation and differentiation.[11] The most common BRAF mutation, observed in 90% of cases, is a T1799A transversion in exon 15, resulting in a valine amino acid substitution (V600E).[10] RAS and BRAF mutations correlate with aggressive tumor behavior and resistance to targeted therapy in CRC, contributing to a poorer prognosis. Recent studies[12,13] suggest that advanced-stage CRC and tumors on the right side of the colon are more likely to have RAS and BRAF mutations. Hence, detecting these mutations is crucial for assessing treatment response and tailoring treatment strategies for CRC patients.

Colorectal cancer is a highly heterogeneous disease with various tumor phenotypes that are distinguished by specific molecular and morphological features. Colorectal cancer is a highly heterogeneous disease with various tumor phenotypes that are distinguished by specific molecular and morphological features. Colorectal cancer is caused by various genetic alterations that affect tumor suppressor genes, oncogenes, and genes involved in DNA repair mechanisms. Three major pathways have been identified in CRC: chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylation phenotype (CIMP). These 3 groups have involved pathological, genetic, and clinical characteristics.[14]

Chromosomal instability is the most common genetic mechanism in CRC (85% of all CRCs). Furthermore, CIN tumors have been linked to the accumulation of mutations in several oncogenes and tumor suppressor genes including *KRAS*, *BRAF*, adenomatous polyposis coli (APC), and TP53.[15] Colorectal cancer development is primarily driven by these genetic mutations and defective cell regulation.[16] The accumulation of these mutations activates multiple signaling pathways, including the *RAS-RAF-MAPK* pathway, which plays critical roles in essential cellular processes such as angiogenesis, cell proliferation, and motility. Mutations in specific genes, such as *KRAS*, *NRAS*, and *BRAF*, contribute to the dysregulation of this pathway and are frequently observed in CRC.[17-19]

Microsatellite instability is another significant pathway found in approximately 15% of all CRC cases; however, in metastatic CRC (mCRC), the prevalence of MSI decreases to approximately 4% to 5%.[20] This highlights the crucial role of BRAF mutation status and microsatellite stability (MSS) in CRC. The interaction between these 2 factors has significant implications for disease aggressiveness and clinical management. BRAF mutation is associated with MSI through its relationship with high-level CpG island methylator phenotype (CIMP) and MLH1 promoter methylation.[21] This interaction serves as a biomarker in CRC.[22] Importantly, the combined MSI/BRAF status can serve as a prognostic molecular biomarker. For instance, compared with most subtype of microsatellite stable (MSS)/BRAF-wild-type, MSS/BRAF-mutant, microsatellite instability-high (MSI-H)/BRAF-mutant, and MSI-H/BRAF-wild-type subtypes showed different CRC-specific mortality hazard ratios.[21] Furthermore, it is noteworthy that BRAF mutant MSS CRCs are particularly aggressive, and in some cases, the MSS status seems to override the BRAF status. Therefore, understanding the interaction between BRAF mutation status and MS, as well as the broader role of MSI status in CRC, can help to stratify prognostic risk, guide clinical management in CRC, and determine eligibility for certain treatments like immunotherapy.

The importance of detecting CRC early cannot be overstated. Early-stage CRC carries a good prognosis, boasting a 5-year survival rate of 91% for colon cancer and 90% for rectal cancer.[23] However, these rates drop to 13% and 18%,[23] respectively, indicating a poor prognosis, when the cancer metastasizes to distant organs such as the liver, lungs, lymph nodes, and peritoneum.[24,25] The tumor stage at diagnosis emerges as a crucial survival determinant, with rates dropping to 14% for cases with distant metastasis.[26] Despite medical advancements, the prevalence of advanced-stage CRC diagnoses underscores the urgent need for enhanced screening strategies and early detection methods.

In mCRC with an unmutated RAS gene, anti-epidermal growth factor receptor (EGFR) antibodies such as cetuximab and panitumumab are commonly employed. These antibodies prolong progression-free survival (PFS) and overall survival (OS) while also enhancing the overall response rate (ORR) by inhibiting the EGFR pathway.[27] Encorafenib, a BRAF inhibitor, has demonstrated efficacy in treating mCRC with the BRAF V600E mutation, particularly when combined with cetuximab. This underscores the significance of assessing the RAS/BRAF status in mCRC patients undergoing EGFR inhibition.[28] Despite the promising outcomes associated with these treatments, it is essential to consider potential side effects or limitations.

Ethnic disparities in cancer biology, including CRC, are a significant area of research. These disparities, observed among different population groups globally, are influenced by various factors such as socioeconomic status, culture, diet, stress, environment, and biology.[29] For instance, Black/African American individuals often face higher death rates for many cancer types, including CRC, despite similar rates of breast cancer.[30] Focusing on the Middle Eastern and North African context, unique genetic profiles and environmental exposures also contribute to differential patterns of CRC incidence and treatment responses, with distinct susceptibilities to CRC influenced by genetic predispositions, lifestyle factors, and socio-cultural determinants.[31] These disparities highlight the need for health care policies and practices that ensure equitable access to CRC prevention, detection, and treatment services across different ethnic groups. Therefore, this study builds on a prior review conducted by Jafari et al[32] in 2022 based on CRC in North Africa to expand the scope of the examination, by investigating the Middle East region. By incorporating this additional geographical area, our objective is to comprehensively understand the prevalence and patterns of RAS and BRAF mutations in the Middle East. This extension contributes to advancing knowledge in the field and facilitates the development of targeted strategies for CRC prevention and management across neighborhood regions. The aim of this study is to systematically review the available literature and determine the prevalence of RAS and BRAF mutations among CRC patients in the Middle East, while also examining their clinicopathological characteristics through descriptive outputs of the studies. The organization of the study is as follows. The next section provides the applied method in this study whereas the "Review Results" section provides a comprehensive analysis of the collected literature data and provides descriptive and methodological insights. "Discussion" section discusses the outputs more in-depth whereas the last section shows a fundamental gain of this study.

Methods

In this systematic review, we conducted a comprehensive examination of the literature to assess the prevalence of RAS mutations and their correlation with geographical location, clinicopathological features, and other relevant factors in CRC patients in the Middle East. To achieve this goal, we employed a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) mechanism relying on a rigorous search strategy that encompassed multiple databases, including PubMed, Science Direct, Web of Science, Scopus, and Google Scholar. Only case-control studies published between 2002 and 2022 are considered in this review study. Figure 1 summarizes the flowchart of PRISMA process. The search terms used in this study were carefully selected to capture a wide range of relevant keywords related to CRC and the Middle East region.

Our search terms specifically included variations of "colorectal cancer" or "CRC," combined with terms related to RAS mutations such as "RAS mutation," "KRAS mutation," "NRAS mutation," "BRAF mutation," "Chromosomal Instability" (or CIN), and "Microsatellite Instability" (or MSI)." In addition, we included terms specifying the Middle East region such as "Middle East," "Middle Eastern," and specific country names within the area. This comprehensive approach ensured that we captured relevant studies exploring the prevalence and characteristics of RAS mutations, BRAF mutations, and the status of CRC patients across the Middle Eastern region.

To ensure the quality and relevance of the studies included in this review, we established specific inclusion criteria. Specifically, studies must have focused on the role of the RAS gene and/or BRAF gene in CRC, analyzed also mutations in exons 2, 3, and 4 of the RAS gene, as well as exon 15 of the BRAF gene, provided sufficient information on the clinicopathological characteristics of included CRC patients, and included at least 100 CRC patients analyzed for RAS mutations. Studies that reported on MSI status were also included, even although they were limited.

After conducting an initial literature search in multiple databases, a total of 80 publications were identified. Following the removal of unrelated and duplicated records, 51 records remained, which were then screened using the title and abstract. Of these, 25 records did not meet the inclusion criteria and were subsequently excluded. The full text of the remaining 26 records was thoroughly reviewed, and 7 records were excluded due to the study status (such as unpublished study or report) provided in Figure 1. Ultimately, 19 studies met the desired criteria and were included in the review analysis. The studies were all case-control studies published between 2002 and 2022. Of the 19 included studies, 12 focused on KRAS mutations,[33-44] 3 on KRAS and NRAS mutations,[45-47] 1 on KRAS and BRAF mutations,[48] and 3 on KRAS, NRAS, and BRAF mutations.[49-51] Notably, 3 of these studies provided insight into MSI status, contributing to a more comprehensive understanding of the genetic landscape of CRC.[33,37,49] The original articles included in the present review were identified from various Middle Eastern countries, including Bahrain,[49] Iran,[33-38,45,48] Iraq,[39] Israel,[40] Jordan,[41,46] Lebanon,[50] and Saudi Arabia.[42-44,47,51] After reviewing the relevant literature, we present the descriptive patterns between RAS mutation status and the descriptive clinicopathological characteristics of CRC patients in the collected studies which are explained below.

Review Results

This section presents a comprehensive examination of the collected literature data and provides descriptive and methodological insights into the findings of the studies.

Description of sample sizes and included regions

The characteristics of *RAS* and *BRAF* mutation studies have been summarized in Tables 1 and 2, respectively. The sample

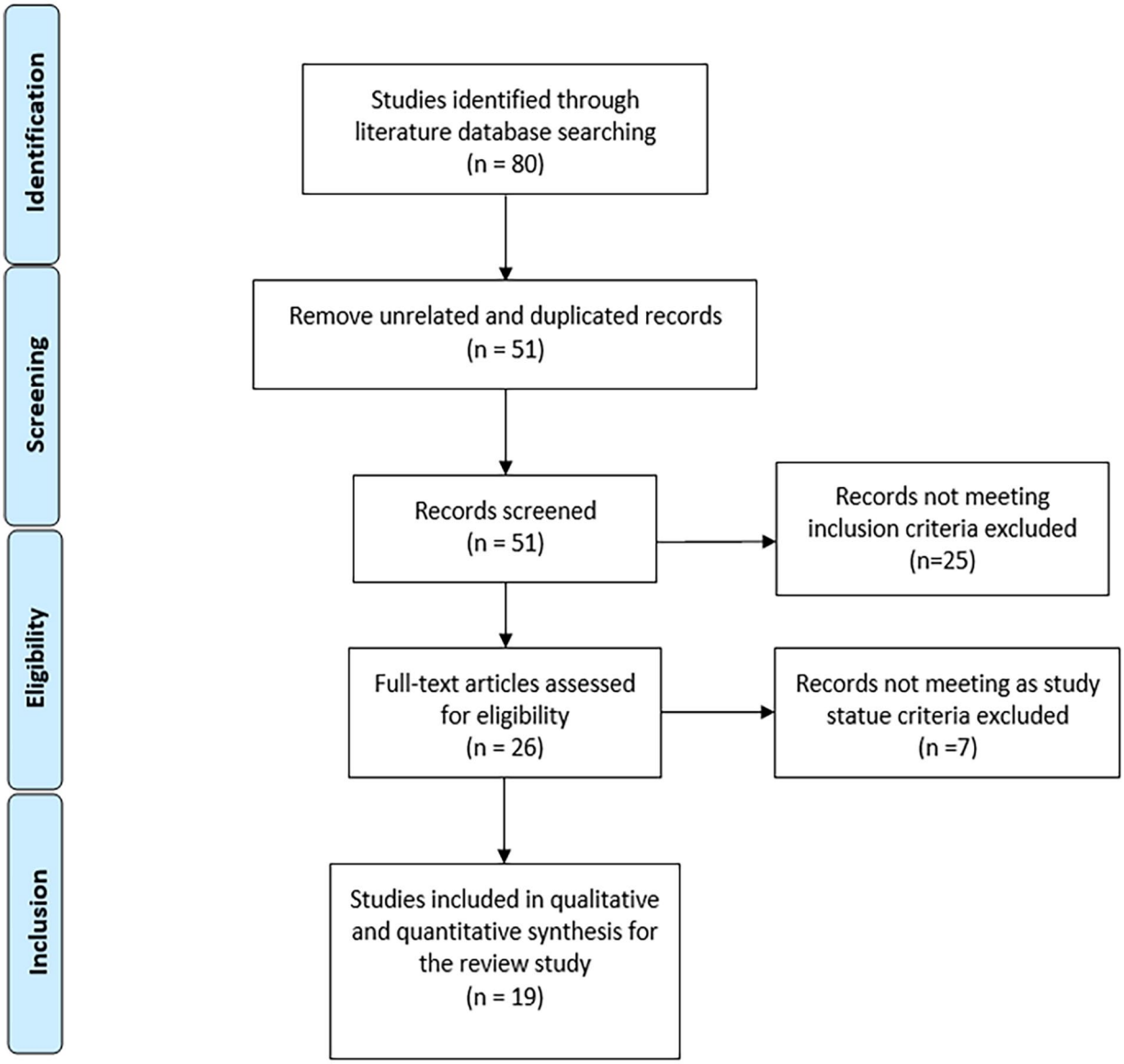

Figure 1. Flowchart of PRISMA process.

sizes of the studies analyzing *RAS* and *BRAF* mutations ranged from 33 to 1000 patients. Most analyzed patients, 92.6% (2742 of 2960), were derived from studies conducted in the Middle Eastern region, including Iran (8 studies, 1647 patients),[33-38,45,48] Iraq (1 study, 50 patients),[39] Israel (1 study, 105 patients),[40] Jordan (2 studies, 290 patients),[41,46] Lebanon (1 study, 273 patients),[50] and Saudi Arabia (5 studies, 423 patients).[42-44,47,51] Especially, 2 studies, 1 from Bahrain (172 patients)[49] and 1 from Saudi Arabia (46 patients),[43] did not provide information on the distribution of *RAS* mutations.

Specimens and methods used in the RAS and BRAF mutation identification

In most studies investigating mutations in *KRAS*, *NRAS*, and *BRAF* genes within Middle Eastern populations, formalin-fixed paraffin-embedded (FFPE) tissues were used as specimens. These specimens included both biopsies and resection materials. DNA extraction was performed on tissue samples utilizing paraffin block DNA extraction kits. Among Middle Eastern series, exon 2, 3, and 4 mutations of *KRAS* and *NRAS* genes were analyzed in 15.7% (3 of 19).[45-47] In 12 studies (15.7%, 3 of 19),[33-44] exon 2, 3, and 4 mutations of the *KRAS* gene were evaluated and whereas *NRAS* exons 2 and/or 3 were evaluated in 26.3% of studies.[45-47,50,51] Most studies (94.7%, 18 of 19)[33-48,50,51] assessed *KRAS* mutations in exon 2 codons 12 and 13. *BRAF* gene mutations were analyzed in 21.1% (4 of 19) studies.[48-51] Microsatellite instability was examined in a subset of these studies (15.7%, 3 of 19), whereas the remaining studies did not report on MSI.[33,37,49] One study from Bahrain (172 patients) did not mention specific exons genotyped.[49] Various molecular methods were employed for mutation screening, with sequencing assays being the most widely used method (21.1% of studies).[33,37,40,48] Other methodologies described in the considered studies included Pyrosequencing,[35,36,45,49] Sanger sequencing,[34,38,41] array-based techniques such as ARRAY[43]

Table 1. Details of studies investigating *KRAS*, *NRAS*, and *BRAF* genes in the Middle East (+, including genetic analysis;–, not including genetic analysis).

COUNTRY / AUTHOR (REFERENCE)	MATERIALS	KRAS			*NRAS*			*BRAF*	DETECTION METHOD
		EXON 2	EXON 3	EXON 4	EXON 2	EXON 3	EXON 4	EXON 15	
Bahrain									
Al Shaikh and Shubbar[49]	FFPE	–	–	–	–	–	–	+	PCR/ Pyrosequencing
Iran									
Shemirani et al[33]	Fresh tissue	+	–	–	–	–	–	–	PCR/Sequencing
Omidifar et al[34]	FFPE	+	–	–	–	–	–	–	PCR/Sanger sequencing
Yari et al[48]	FFPE	+	+	–	–	–	–	+	PCR/Sequencing
Amirifard et al[35]	FFPE	+	–	–	–	–	–	–	PCR/ Pyrosequencing
Naseri et al[45]	FFPE	+	–	–	–	–	+	–	PCR/ Pyrosequencing
Niya et al[36]	FFPE	+	–	–	–	–	–	–	HRM/ Pyrosequencing
Bishehsari et al[37]	FFPE	+	+	–	–	–	–	–	PCR/Sequencing
Hamzehzadeh et al[38]	Fresh tissue	+	–	–	–	–	–	–	HRM/Sanger sequencing
Iraq									
Al-Allawi et al[39]	FFPE	+	–	–	–	–	–	–	PCR/Hybridization StripAssay
Israel									
Kislitsin et al[40]	Frozen tissue	+	–	–	–	–	–	–	PCR/Sequencing
Jordan									
Elbjeirami et al[41]	FFPE	+	–	–	–	–	–	–	PCR/Sanger sequencing
Awidi et al[46]	FFPE	+	+	+	+	+	–	–	PCR/Hybridization StripAssay
Lebanon									
Baba et al[50]	FFPE	+	+	+	+	+	–	+	PCR/Hybridization StripAssay
Saudi Arabia									
Bader et al[42]	FFPE	+	–	–	–	–	–	–	PCR/LCD Array
Zekri et al[43]	FFPE	+	–	–	–	–	–	–	PCR/Array
Zahrani et al[44]	FFPE	+	–	–	–	–	–	–	PCR/LCD Array
Mulla et al[47]	FFPE	+	–	–	+	+	–	–	PCR/Next-generation sequencing
Saharti[51]	FFPE	+	+	+	–	+	–	+	PCR/Next-generation sequencing

Abbreviation: HRM, high-resolution melting.

Table 2. Characteristics and clinicopathologic features of studies included in this review.

COUNTRY AUTHOR (REFERENCE)	SAMPLE SIZE	MEAN YEARS (±SD)	WOMEN/ MEN, NO (%)	TUMOR SITE, NO (%)			STAGES, NO (%)	TUMOR GRADE
				COLON		RECTUM		
				RIGHT	LEFT			
Bahrain								
Al Shaikh and Shubbar[49]	172	60	79 (46%)/93 (54%)	NA	NA	38 (22%)	I: 6 (4%) II: 17 (10%) III: 47 (27%) IV: 21 (12%)	Well: 5 (3%) Moderate: 145 (83%) Poor: 10 (6%)
Iran								
Shemirani et al[33]	95	49	22 (23%)/73 (76%)	NA	NA	NA	NA	NA
Omidifar et al[34]	100	59.08 (±15.55)	45 (45%)/55 (55%)	NA	NA	NA	NA	NA
Yari et al[48]	100	59.60 (±15.24)	36 (36%)/64 (64%)	29 (29%)	30 (30%)	41 (41%)	I: 11 (11%) II: 17 (17%) III: 59 (59%) IV: 13 (13%)	W: 8 (8%) M: 78 (78%) P: 14 (14%)
Amirifard et al[35]	33	51.48 (±12.6)	7 (21%)/26 (79%)	NA	NA	15 (45%)	NA	W: 22 (66.7%) M: 7 (21.2%) P: 3 (9.1%)
Naseri et al[45]	50	61.3 (±13)	15 (30%)/35 (70%)	26 (52%)	NA	13 (26%)	II: 2 (4%) III: 11 (22%)	W: 15 (30%) M: 18 (36%)
Niya et al[36]	1000	NA	427 (42.7%)/573 (57.3%)	NA	NA	NA	NA	W: 439 (43.9%) M: 384 (38.4%) P: 164 (16.4%)
Bishehsari et al[37]	182	NA	79 (43.3%)/103 (56.6%)	58 (32%)	65 (35.7%)	53 (29%)	NA	NA
Hamzehzadeh et al[38]	87	NA	36 (41.3%)/51 (58.6)	76 (87.3%)	NA	11 (12.6%)	NA	W: 16 (18.3%) M: 67 (77%) P: 4 (4.6%)
Iraq								
Al-Allawi et al[39]	50	59 ± 15.25	23 (46%)/27 (54%)	10 (20%)	12 (24%)	28 (56%)	I: 12 (24%) II: 13 (26%) III: 23 (46%) IV: 2 (4%)	W: 10 (20%) M: 31 (62%) P: 9 (18%)

(Continued)

Table 2. (Continued)

COUNTRY AUTHOR (REFERENCE)	SAMPLE SIZE	MEAN YEARS (±SD)	WOMEN/ MEN, NO (%)	TUMOR SITE, NO (%)			STAGES, NO (%)	TUMOR GRADE
				COLON		RECTUM		
				RIGHT	LEFT			
Israel								
Kislitsin et al[40]	105	NA	NA	NA	NA	33 (31%)	NA	W: 7 (7%) M: 37 (35%) P: 2 (2%)
Jordan								
Elbjeirami et al[41]	100	55	45 (45%)/55 (55%)	58 (58%)	NA	22 (22%)	I: 0 (0%) II: 5 (5%) III: 8 (8%) IV: 87 (87%)	NA
Awidi et al[46]	190	58	76 (40%)/114 (60%)	62 (32.63%)	107 (56.32%)	5 (2.63%)	NA	NA
Lebanon								
Baba et al[50]	273	58	112 (41%)/160 (59%)	41 (15%)	163 (60%)	NA	NA	NA
S. Arabia								
Bader et al[42]	83	55	35 (42.2%)/48 (57.8%)	63 (76%)	NA	20 (24%)	I: 3 (3.6%) II: 8 (9.63%) III: 15 (18.07%) IV: 57 (68.67%)	W: 7 (8.43%) M: 68 (81.92%) P: 8 (9.63%)
Zekri et al[43]	46	61	16 (34%)/30 (65%)	17 (37%)	4 (9%)	8 (17%)	II: 14 (30%) III: 15 (33%) IV: 17 (37%)	W: 1 (2%) M: 38 (83%) P: 7 (15%)
Zahrani et al[44]	150	56.7	NA	21 (18%)	123 (82%)	32 (21.3%)	I: 2 (1%) II: 22 (14.6%) III: 26 (17.3%) IV: 100 (66.6)	NA
Mulla et al[47]	51	60.2	25 (49%)/26 (51%)	14 (27.5%)	37 (72.5%)	NA	I: 5 (9.8%) II: 13 (25.5%) III: 16 (31.4%) IV: 17 (33.33%)	W: 6 (11.8%) M: 43 (84.3%) P: 2 (3.9%)
Saharti[51]	93	57.3	NA	NA	NA	NA	NA	NA

Abbreviations: M, moderately differentiated; No (%), percentage of number of individuals (women/men); NA, not available; P, poorly differentiated; SD, standard deviation; W, well differentiated.

and LCD Array,[42,44] next-generation sequencing,[47,51] and hybridization StripAssay.[39,46,50] A summary of the details of these studies is presented in Table 1.

Statistical analysis in most studies performed using the chi-squared test and SPSS software, and a *P* value less than .05 was considered to indicate statistical significance. The chi-squared

test is a widely employed statistical analysis in research that aims to evaluate the independence or association between categorical variables. It proves especially valuable when dealing with data that involves frequencies or counts distributed across distinct categories. Through a comparison of observed and expected frequencies, the chi-square test calculates a test statistic that adheres to the chi-square distribution. This test statistic quantifies the degree of deviation between the observed and expected frequencies, providing insights into whether a significant association exists between the variables being examined.

Patients' clinicopathological characteristics

A total of 2960 patients diagnosed with CRC were included in this analysis. The median age of patients was 57 years with a range of 49 to 74 years. Among the studies considered, men constituted the predominant proportion, accounting for 1533 (58.6%) of 2612 patients. However, the men-to-women ratio was not available in 3 studies, 1 from Israel[40] and 2 from Saudi Arabia.[44,51]

Tumor site information was available for a subset of the specimens. It was found that 23.6% (319 of 1348) of the tumors were located in the rectum,[35,37-46,48,49] 34.8% (475 of 1362) were located in the right colon,[37-39,41-48,50] and 51.9% (541 of 1042) were located in the left colon.[37,39,43,44,46-48,50] Furthermore, the percentages of patients with well-differentiated, moderately differentiated, and poorly differentiated histology were 30.1%,[35,36,38-40,42,43,45,47-49] 51.5%,[35,36,38-40,42,43,45,47-49] and 12.5%,[35,36,38-40,42,43,47-49] respectively. Of the tumors, 41.7% (314 of 752) were reported to be in stage IV.[39,41-44,47-49] Formalin-fixed paraffin-embedded blocks were the primary source of specimens in 16 studies,[34-37,39,41-51] whereas fresh tissue was used in 2 studies[33,38] and frozen tissue in 1 study.[40] Further details regarding the baseline characteristics and clinicopathologic features of the enrolled studies can be found in Table 2.

RAS and BRAF mutation prevalence

The prevalence of *RAS* mutations among CRC patients in the Middle East region was found to be 1128 (38.1%) of 2960 based on analysis of available studies. Iran had the highest *RAS* mutation prevalence at 33.6% (336 of 1000),[36] whereas the lowest prevalence also was observed in another study from Iran at 6.3% (6 of 95).[33] The prevalence of *BRAF* mutations in the region was 2.6% (17 of 638),[48-51] with the highest prevalence observed in Iran at 7%[48] and the lowest in Saudi Arabia at 2.2%.[51] A summary of *RAS* and *BRAF* mutation prevalence in the Middle East is presented in Table 3.

RAS and BRAF mutation spectrum

The prevalence of *KRAS*, *NRAS*, and *BRAF* mutations has been reported in 19,[33-51] 6,[45-47,49-51] and 4[48-51] of the 19 included studies, respectively. In total, *KRAS* mutations were most frequently detected among Middle East patients with CRC, accounting for 37.1% (1098 of 2960).[33-51] Iran highlights a wide range of *KRAS* mutation rates, ranging from 6.3% (6 of 95)[33] to 33.6% (336 of 1000),[36] as compared with other countries from the region. Overall, *KRAS* mutations were distributed among the different exons as follows: 97.9% (1030 of 1052) exon 2,[33-51] 1.9% (7 of 361) exon 3,[37,46,48,50,51] and 5.6% (15 of 264) exon 4[46,50,51] (Table 3). As seen in Table 4 (see Appendix), the G12D was the most frequently identified exon 2 mutation (23.2%, 239 of 1030),[33-42,44-48,50,51] followed by G12V (13.7%, 142 of 1030),[35-42,44-48,50,51] G13D (10.1%, 105 of 1030),[34-42,44-46,48,50,51] G12C (5.1%, 53 of 1030),[34-42,44,46-48,50,51] G12A (5.0%, 52 of 1030),[34,36,38,39,41,44,46-48,50,51] G12S (3.6%, 38 of 1030).[34-36,38-42,44,46,47,50,51] G12R, G13C, and G13R are less than 5%.[33,34,36,41,44,50,51] However, there are important differences among Middle East countries. In Lebanon, there is a higher prevalence of G12D mutation 49.[50] The most frequent mutation type in exon 3 was Q61H (85%, 6 of 7).[46,48,50,51] In exon 4, the most common mutation was K117N (33.3%, 9 of 15),[46,48,50,51] followed by A146T (33%, 5 of 15)[46,50,51] and A146V (6.6%, 1 of 15).[50] One study from Saudi Arabia found a G138E mutation.[51]

NRAS total mutations were identified in 30 (3.3%) of 892[45-47,49-51] tumor samples. A higher prevalence of *NRAS* mutations has been reported in Lebanon (5.8%, 16 of 273).[50] Overall, the common mutation site of the *NRAS* gene was located in exons 2, 3, and 4 with 40% (12 of 30),[46,47,50] 40% (12 of 30),[46,47,50,51] and 3% (1 of 30)[45] of CRC patient (Table 3). *NRAS* mutations were more common in codon 61, accounting for 40% (12 of 30)[46,47,50,51] (Table 3). The most common mutations were G12D in exon 2 and Q61L in exon 3 and accounted for 58% (7 of 12)[47,50] and 41% (5 of 12)[47,50,51] of patients with CRC, respectively (Table 4). The total mutation frequency in the *BRAF* gene was 2.6% (17 of 638) as reported in the literature.[48-51] The highest frequency of *BRAF* mutations was found in Iran, where it was reported in 7% (7 of 100) of patients with CRC.[48] Overall, the *BRAF* V600E mutation frequency was 2.6% (17 of 638),[48-51] as shown in Tables 3 and 4.

In addition to the mutation analysis, some studies also investigated MSI and microsatellite stability (MSS). In Bahrain, MSI was observed in 11% (19 of 172) of the cases, whereas 58% (100 of 172) were found to be MSS.[49] In Iran, 2 studies reported on MSI and MSS. One study reported MSI in 17% of the cases, with the remaining 83% being MSS.[33] The other study found a slightly higher rate of MSI at 24.5%, with MSS accounting for 80.6% of the cases.[37] These findings on MSI and MSS add another layer of complexity to the genetic landscape of CRC in the Middle East, complementing the mutation data on KRAS, NRAS, and BRAF genes.

Discussion

This systematic review conducted on the prevalence of RAS and BRAF mutations in CRC patients in the Middle East

Table 3. KRAS, NRAS, and BRAF mutation rates in Middle East populations in this review.

COUNTRY AUTHOR	GENE FREQUENCY, NO (%)		*KRAS* EXON 2		*KRAS* EXON 3		*KRAS* EXON 4		*NRAS* EXON 2		*NRAS* EXON 3		*NRAS* EXON 4		*BRAF* EXON 15
	KRAS	*NRAS*	C12	C13	C59	C61	C117	C146	C12	C13	C59	C61	C117	146	V600E
Bahrain															
Al Shaikh and Shubbar[49]	46 (27%)	3 (2%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	6 (3.5%)
Iran															
Shemirani et al[33]	6 (12.5%)	NA	5 (83%)	1 (17%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Omidifar et al[34a]	32 (32%)	NA	23 (71.8%)	8 (25%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Yari et al[48]	29 (29%)	NA	21 (77.8%)	6 (22.2%)	NA	2 (2%)	NA	NA	NA	NA	NA	NA	NA	NA	7 (7%)
Amirifard et al[35]	12 (36.3%)	NA	11 (91.7%)	1 (8.3%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Naseri et al[45]	14 (28%)	1 (2%)	10 (71.4%)	4 (28.5%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	1 (100%)	NA
Niya et al[36]	336 (33.6%)	NA	286 (85.1%)	50 (14.9%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Bishehsari et al[37]	68 (37.4%)	NA	45 (66%)	22 (32.5%)	NA	1 (1.5%)	NA	NA	NA	NA	NA	NA	NA	NA	NA
Hamzehzadeh et al[38]	25 (28.7%)	NA	18 (72%)	7 (28%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Iraq															
Al-Allawi et al[39b]	24 (48%)	NA	21 (89.7%)	3 (10.3%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Israel															
Kislitsin et al[40]	44 (42%)	NA	32 (73%)	12 (27%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Jordan															
Elbjeirami et al[41]	44 (44%)	NA	39 (89%)	5 (11%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Awidi et al[46]	92 (48%)	6 (6%)	75 (82%)	13 (17%)	NA	1 (1.08%)	2 (2%)	6 (6%)	1 (1.08%)	2 (2.17%)	NA	3 (3.26%)	NA	NA	NA
Lebanon															
Baba et al[50c]	130 (47.6%)	16 (7.8%)	107 (82.30%)	17 (13.07%)	NA	1 (0.8%)	1 (0.8%)	2 (1.53%)	6 (37.5%)	2 (12.5%)	NA	6 (37.5%)	NA	NA	2 (1.2%)
Saudi Arabia															
Bader et al[42]	35 (42.2%)	NA	31 (88%)	4 (11%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Zekri et al[43]	15 (32%)	NA	13 (87%)	2 (13%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Zahrani et al[44]	84 (56%)	NA	73 (48.7%)	11 (7.3%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Mulla et al[47]	20 (91%)	2 (9%)	15 (75%)	4 (20%)	NA	NA	NA	NA	1 (50%)	NA	NA	1 (50%)	NA	NA	NA
Saharti[51d]	42 (45.2%)	2 (2.2%)	32 (76.19%)	3 (7.14%)	NA	2 (4.76%)	2 (4.76%)	2 (4.76%)	NA	NA	NA	2 (2.2%)	NA	NA	2 (2.2%)

Abbreviations: C, codon (eg, C12: codon 12); NA, not available.
[a]One case (3.1% of mutant cases) had a mutation in both codons 12 and 13 of KRAS.
[b]With 24 mutations in KRAS exon 2 (3 patients had double mutations in codon 12, whereas 1 patient had triple mutations with 2 in codon 12 and 1 in codon 13).
[c]The study revealed the presence of concomitant mutations in 2 out of 130 positive CRC cases with positive KRAS mutations; notably, 2 patients also exhibited concomitant mutations in NRAS.
[d]The presence of the KRAS G138E mutation in the analyzed sample.

sheds light on significant disparities compared with other regions stated below, underscoring the complexity of genetic and environmental factors influencing RAS (KRAS and NRAS) and BRAF mutation occurrence. Understanding these differences is crucial for tailoring effective prevention and treatment strategies in the region.

The prevalence of KRAS gene mutation in CRC patients varies globally, ranging from 11% to 66.1% (mostly 30%-45%).[52] This diversity in findings may be attributed to factors such as ethnicity, geographical area, environmental differences, and lifestyle.[53-56] KRAS mutation correlates with various clinical and clinicopathological features in CRC populations, including sex,[57-61] age at diagnosis (>50 years),[58] tumor location,[56,57,61] tumor differentiation,[56,61-63] and Tumor, Lymph-node, Metastasis (TNM) stage.[60] However, some studies did not find significant associations[64,65] in this context. In our study, men constituted most (58.6%) of the cohort, with tumor sites predominantly in the left colon (51.9%). Tumor differentiation varied, with 30.1%, 51.5%, and 12.5% classified as well-differentiated, moderately differentiated, and poorly differentiated, respectively. Stage IV was reported for 41.7% of tumors. Although some studies suggest a higher likelihood of mutant KRAS in women,[66,67] others indicate the opposite trend.[43,68]

This review shows a higher prevalence of KRAS mutations among Middle Eastern CRC patients compared with some other regions, suggesting unique genetic profiles within the region. The results showed that the frequency of KRAS mutations in the Middle Eastern population was 37.1%, which is consistent with reported data from other Asian countries such as China (32%),[69] Japan (33.5%),[70] and Taiwan (33.5%),[71] as well as from Western countries including the United States (35.7%, 35%, and 31%),[72-74] France (33.8%),[75] and the United Kingdom (36.9%).[76] However, there were variations when compared to some other regions such as Germany (41%),[77] Italy (62.2%, 43%, 43%, and 52.2%),[78-81] Turkey (44%),[82] India (20.5% and 23%),[52,83] Pakistan (13%),[84] Morocco (24%),[85] Egypt (11% and 18.4%),[86,87] Thailand (23%),[88] and Korea (20.7%).[89] Our study findings also align with prior research, indicating that most KRAS mutations in CRC patients occur in codons 12 and 13.[90,91] For instance, Dobre et al[92] found that 79.3% of KRAS mutations were in codon 12 and 19.7% in codon 13. Similarly, in India, KRAS mutations were detected in 87% and 13% of cases in codons 12 and 13, respectively.[93] Consistent results were reported in a large-scale study in Brazil, with KRAS mutations in codons 12 and 13 found in 87% and 13% of patients, respectively.[68] However, a study on the Greek population reported a lower frequency of KRAS mutations in codon 12, at 29.3%.[94] Various genetic and environmental factors contribute to the frequency and distribution of KRAS mutations, leading to variations across different ethnic populations. Notably, KRAS G12D and G12V mutations were the most common, followed by G12S, G12A, and G12C. In our study, the most prevalent KRAS mutations observed in CRC patients were G12D (23.2%) and G12V (13.8%), followed by G12C (5.2%), G12A (5.0%), and G12S (3.6%). In addition, we identified a p.G13D mutation in 10.1% of cases. Comparing our findings to North African populations, previous studies[85,95-109] reported variations in the frequency and distribution of KRAS mutations in CRC patients from this region. For example, in Morocco, the frequency ranged from 23.9% to 51%,[85,95-99] while in Tunisia, it ranged from 23.1% to 68.2%.[100-106] Algerian studies reported a KRAS mutation frequency of 31.4% to 50%,[107,108] whereas in Libya, it was 38.2%.[109]

Colorectal cancer in Arab patients also exhibits a distinct histological pattern, with a higher incidence of mucinous adenocarcinoma (10.1%) compared with Western populations (6.0%).[110] This suggests potential differences in tumor biology or genetic factors specific to the Middle East. However, comprehensive data on rarer subtypes such as signet ring cell carcinoma or medullary carcinoma are limited in the Middle East, making direct comparisons challenging. The geographical variations in CRC subtype distribution could be attributed to factors such as genetic diversity, lifestyle habits, and health care accessibility. These findings highlight the importance of region-specific epidemiological studies for developing tailored prevention and management strategies.

In the context of the NRAS gene, mutations in NRAS codons 12, 13, 61, and 146 exhibit similar effects to KRAS activation. In our review, NRAS mutations were observed in 3.3% of CRC tumor samples, predominantly in exons 2, 3, and 4, representing 4%, 40%, and 3% of cases, respectively. Particularly, codon 61 was the most frequently mutated site, accounting for 40% of cases. The most prevalent mutations were G12D in exon 2 and Q61L in exon 3, found in 40% and 41% of CRC patients, respectively. Other studies have also reported NRAS mutations in CRC patients, with mutation rates varying across different populations. For instance, Irahara et al[111] reported NRAS mutations in 2.2% of their patients, consistent with our findings. Similarly, Chinese and Greek/Romanian patients had mutation rates of 4.2% and 9.6%, respectively.[62,112] In Moroccan studies, Q61K and Q61R were the most common NRAS mutations, accounting for 2.6% and 1.8% of cases, respectively.[97] Focusing on BRAF gene, the most common mutation, V600E, results from a substitution at c.1799T > A. Our review identified a prevalence of 2.6% for BRAF V600E, which is lower than the reported global rates of 5% to 15%.[113-117] The incidence of BRAF mutations varies significantly across populations, with Taiwan recording the lowest at 1% and the Netherlands and the United States reporting the highest at 19.8% and 21.8%, respectively.[52-56,62,68,71-109,111-116] Interestingly, Asian populations generally exhibit lower incidences, with rates ranging from 3.8% to 7% in China and 4.7% to 6.7% in Japan.[118]

Microsatellite instability is a crucial factor in CRC, impacting prognosis, treatment response, and disease management.[119]

Our systematic review assessed MSI status in a limited subset of studies, underlining its clinical significance. Microsatellite instability-high tumors, often linked with BRAF mutations, display different outcomes compared with microsatellite stable (MSS) tumors. The combined MSI/BRAF status serves as a valuable prognostic and predictive marker in CRC.[21] One recent study[120] indicates that both mismatch repair (MMR)-deficient and MMR-proficient tumors, subsets of MSI-H and MSS tumors, can respond to neoadjuvant immunotherapy with ipilimumab, nivolumab, and celecoxib for mismatch repair proficient (pMMR). This treatment, which was well-tolerated by patients, resulted in pathological responses in all mismatch repair deficiency (dMMR) tumors and in 27% of pMMR tumors. These findings suggest the potential for this approach to become a standard of care for specific colon cancer patients. However, the presence of inconsistencies in MSI reporting underscores the need for standardized methodologies. Our findings enhance our understanding of CRC's molecular landscape, promoting personalized treatment approaches.

These findings underscore the heterogeneity of CRC biology and the importance of comprehensive molecular profiling for guiding treatment decisions. Such insights emphasize the significance of regional variations in understanding CRC pathogenesis and tailoring personalized treatment approaches that are touched on below.

Cancer development, including CRC, is influenced by a myriad of factors, ranging from genetic predisposition to environmental exposures whereas multi-gene mutation signatures are used for providing diagnosis, pathological classification, staging, and prognosis.[121] Environmental factors (eg, lifestyle related factors such as improper diets and alcohol consumption, and exposure to pathogenic bacteria) are recognized as contributors to CRC development.[122] Although environmental factors generally play a predominant role in most common cancers, it is important to note that a significant proportion of cancer-related mutations stem from random DNA replication errors, underscoring the combined influence of inherited and environmental factors.[123] Some studies indicate that both genetic and environmental factors contribute to approximately 92% of cancer risk variation across various tissues.[124] Encouraging a high-fiber diet, weight management, smoking cessation, and physical activity are lifestyle interventions aimed at addressing modifiable risk factors linked to CRC. Screening methods, such as colonoscopy and stool-based tests like fecal immunochemical tests (FITs), are pivotal for early detection, particularly in resource-limited settings where FIT is more preferred. National screening initiatives, like organized FIT programs in countries such as Israel and Qatar, target specific age groups.[125] In addition, endoscopic procedures offer a direct visualization of the colon and rectum, enabling the detection and removal of precancerous lesions and early stage tumors before they progress. It is noteworthy that the incidence rates of CRC have seen a reduction of up to 50% in older age groups in the United States, coinciding with the widespread adoption of screening colonoscopy.[126] This is despite the presence of adverse CRC risk factors and a rise in CRC incidence in younger age groups. Integrating both stool-based tests and endoscopic procedures offers a comprehensive approach to CRC management, enhancing the ability to identify high-risk individuals and facilitating personalized treatment strategies. Although lifestyle interventions and screening programs are crucial for CRC prevention, their effectiveness may vary across different cultural and resource contexts in the Middle East; therefore, tailored region-specific guidelines are essential to optimize participation and efficacy in prevention efforts.[125]

The current standard of treatment for CRC in Middle Eastern countries generally follows international guidelines and recommendations. Treatment approaches for CRC typically involve a multidisciplinary team of health care professionals and may include a combination of surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy, depending on the stage and characteristics of the cancer. Surgical resection of the tumor is often the primary treatment for localized disease, whereas adjuvant chemotherapy may be recommended for certain stages. For advanced or mCRC, systemic therapies such as chemotherapy, targeted therapy (eg, anti-EGFR or anti-Vascular Endothelial Growth Factor (anti-VEGF) agents), and immunotherapy (eg, checkpoint inhibitors) are commonly used methods.[127]

Personalized medicine is transforming CRC treatment by tailoring strategies to each patient's genetic profile, potentially enhancing therapy effectiveness and reducing side effects. This includes targeted therapies that precisely attack cancer cells with specific mutations. Studying ethnic differences in CRC tumor biology is crucial, as it can reveal significant variations in disease progression and response to treatment. For instance, research has shown that Black, White, and Asian/Pacific Islander patients with early onset CRC have different patterns of non-silent mutations.[128] However, most large phase 2 to 3 clinical trials predominantly recruit participants from Western countries, potentially leading to a lack of representation of Middle Eastern populations. This is a significant issue because drugs are approved based on the results of these trials, which may not fully account for the unique genetic and environmental factors influencing CRC tumor biology in the Middle East.[129] Therefore, it is important to suggest for more inclusive clinical trials that adequately represent diverse populations, including those in the Middle East. This could lead to a better understanding of ethnic differences in CRC tumor biology and more effective, personalized treatment strategies. In the context of these personalized strategies, immunotherapy, which uses the body's immune system to combat cancer, has shown promise, especially for CRC types with high microsatellite instability (MSI-H) or dMMR.[130] Liquid biopsy techniques further advance personalized medicine by enabling non-invasive monitoring of disease progression and treatment response.[131] In addition, the integration of artificial intelligence and machine learning in oncology can help predict outcomes, guide

treatment decisions, and identify new therapeutic targets. However, these approaches are still under investigation, and their clinical implementation requires further validation. The availability and accessibility of these treatments may also vary across regions due to health care resource disparities.[132]

The clinical implications of RAS and BRAF mutations in CRC patients in the Middle East are significant. These mutations, including KRAS, NRAS, and BRAF, can influence disease progression and treatment effectiveness. Patients with these mutations face challenges in treatment selection due to their resistance to anti-EGFR therapies such as cetuximab and panitumumab. For instance, KRAS and NRAS mutations necessitate a focus on chemotherapy-based treatments like FOLFOX or FOLFIRI. BRAF V600E mutations, linked to poor prognosis and anti-EGFR resistance, may benefit from combination therapies such as FOLFOXIRI or targeted therapies such as BRAF and mitogene-activated protein kinase kinase (MEK) inhibitors.[133] Knowledge of a patient's mutation status can guide personalized treatment plans, combining chemotherapy and targeted therapies to enhance treatment efficacy. Moreover, understanding the genetic landscape of CRC can inform targeted screening strategies for earlier detection and guide research efforts toward studying prevalent mutations. Participation in clinical trials exploring novel therapies is also crucial for advancing treatment options for these patients. Although genetic testing is crucial for individual patients, understanding the broader genetic landscape of CRC in different populations can inform treatment, screening, and research strategies, ultimately contributing to improved patient outcomes.[134]

In the rapidly evolving field of cancer genomics, it is crucial to consider the potential impact of a broader spectrum of genetic alterations in CRC. For instance, mutations in homologous recombination repair (HRR) genes such as breast cancer gene (BRCA) and ataxia-telangiectasia mutated (ATM) are gaining attention in the oncology community. These genes play a critical role in DNA repair, and their mutations can lead to genomic instability, a hallmark of cancer.[135] Moreover, the prevalence of HRR mutations can vary across different geographical regions as stated along with the review. This geographical variation could be due to differences in genetic backgrounds, environmental factors, or a combination of both. Understanding these geographical differences in mutation prevalence could provide valuable insights into the regional variations in CRC pathogenesis and response to treatment. Importantly, these mutations can confer sensitivity to poly (adenosine diphosphate (ADP)-ribose) polymerase (PARP) inhibitors, a class of targeted cancer drugs. Poly (ADP-ribose) polymerase inhibitors work by trapping PARP proteins on damaged DNA, which leads to the formation of cytotoxic DNA lesions and ultimately results in cell death.[136] Although PARP inhibitors have shown efficacy in treating cancers with BRCA1/2 mutations, they are also being investigated for other types of cancers with HRR gene mutations or deficiencies, as well as in tumors with high levels of replicative stress. It is important to note that PARP inhibitors are not effective in all patients with HRR gene mutations, and further research is needed to identify predictive biomarkers of response. Nonetheless, PARP inhibitors represent a promising avenue for targeted cancer therapy and have the potential to improve outcomes for patients with HRR-deficient tumors.[136]

Genetic testing plays a pivotal role in the early detection and intervention of diseases, particularly in the field of cancer disease. One of the key advantages of genetic testing in the context of CRC is its potential for early identification. Certain genetic mutations are known to significantly increase the risk of CRC. By identifying these mutations in individuals, we can classify them as high risk. Then, individuals can be monitored more closely for the early signs of CRC, allowing for earlier intervention and potentially better outcomes.[137] The detection of mutations such as RAS and BRAF genes can significantly contribute to the early detection and management of CRC, demonstrating the transformative potential of the advanced diagnostic tools. This proactive approach to early identification is essential as it enables the implementation of preventive measures and early treatments, potentially altering the disease's course.[137]

One recent study[138] has shed light on a significant disruption in DNA repair processes in digestive system cancers, marked by the simultaneous loss of MLH1, PMS2, and MSH6 immuno-expression. This disruption could have far-reaching effects on how we understand and treat a variety of cancers, including CRC. This study found that several MMR/HRR-related genes were affected, including ATM, BARD1, BRCA1, CDK12, CHEK1, CHEK2, FANCA, MLH1, MSH6, PALB2, and TP53. These findings could have a direct impact on the efficacy of PARP inhibitors in the treatment of CRC.

This systematic review possesses a notable strength in its comprehensive examination of studies conducted across various countries in the Middle East, encompassing diverse populations. By including a substantial number of studies while upholding statistical power, a thorough comprehension of the prevalence and range of RAS and BRAF mutations within the region were achieved. However, it is essential to acknowledge the limitations of the review, including data heterogeneity due to variations in specimen types and genotyping methods, as well as potential biases arising from limited accessibility to molecular testing. For example, some studies employed sequencing to confirm mutations, whereas others did not follow this approach. Moreover, demographic diversity within the region may contribute to variability in study results, warranting cautious interpretation.

Conclusions

In essence, this systematic review provides valuable insights into the molecular landscape of CRC in the Middle East, highlighting the importance of region-specific considerations in both research and clinical practice. The distribution patterns

of RAS and BRAF mutations among CRC patients exhibit notable variations across diverse ethnic groups. Our study sheds light on this phenomenon by demonstrating a higher prevalence of KRAS mutations in CRC patients from the Middle East, as compared with those from several regions. The identification of these mutations and geographical differences is important for personalized treatment planning and could potentially aid in the development of novel targeted therapies. Further studies are needed to address the identified gaps and enhance our understanding of CRC pathogenesis and treatment outcomes in the region. Also, more studies are needed to investigate histopathology, in addition to genetic alterations, as hematoxylin and eosin (H&E) histopathology remains the cornerstone of establishing cancer diagnosis.

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SB and AL conceived the study, exploited review data, and coordinated and drafted the paper. SB, FH, TB, CH, TM, and RT participated in the study design. SB, AL, MJ, SE, and WB involved in review analyses. SB, HE, IAL, MO, MI, KE, ND, and YS reviewed the manuscript. All authors have read and agreed to the version of the manuscript.

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Appendix

Table 4. Frequency and distribution of KRAS, NRAS, and BRAF mutations.

COUNTRY AUTHOR (REFERENCE)	GENE	EXON	CODON	AMINO ACID	NUCLEOTIDE	PROTEIN	FREQUENCY, NO (%)
Bahrain							
Al Shaikh and Shubbar[49]	KRAS	NA	NA	NA	NA	NA	NA
	NRAS	NA	NA	NA	NA	NA	NA
	BRAF	15	600	V600E	1799T > A	p.Val600Glu	6 (3.5%)
Iran							
Shemirani et al.[33]	KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	5 (83%)
			13	G13C	c.37G > T	p.Gly13Cys	1 (17%)
Omidifar et al.[34]	KRAS	2	12	G12A	c.35G > C	p.Gly12Ala	12 (12%)
				G12D	c.35G > A	p.Gly12Asp	9 (9%)
				G12S	c.34G > A	p.Gly12Ser	1 (1%)
				G12C	c.34G > T	p.Gly12Cys	1 (1%)
			13	G13D	c.38G > A	p.Gly13Asp	6 (6%)
				G13S	c.38G > C	p.Gly13Ser	1 (1%)
				G13R	c.37G > C	p.Gly13Arg	1 (1%)
Yari et al.[48]	KRAS	2	12	G12C	c.34G > T	p.Gly12Cys	1 (3.7%)
				G12D	c.35G > A	p.Gly12Asp	13 (48.1%)
				G12A	c.35G > C	p.Gly12Ala	1 (3.7%)
				G12V	c.35G > T	p.Gly12Val	6 (22.2%)
			13	G13D	c.38G > A	p.Gly13Asp	6 (22.2%)
		3	61	Q61H	c.183A > C	p.Gln61His	2 (100%)
	BRAF	15	600	V600E	1799T > A	p.Val600Glu	7 (7%)

(Continued)

Table 4. (Continued)

COUNTRY AUTHOR (REFERENCE)	GENE	EXON	CODON	AMINO ACID	NUCLEOTIDE	PROTEIN	FREQUENCY, NO (%)
Amirifard et al.[35]	KRAS	2	12	G12V	c.35G > T	p.Gly12Val	3 (25%)
				G12D	c.35G > A	p.Gly12Asp	5 (41%)
				G12S	c.34G > A	p.Gly12Ser	1 (8.3%)
				G12C	c.34G > T	p.Gly12Cys	1 (8.3%)
			13	G13D	c.38G > A	p.Gly13Asp	1 (8.3%)
Naseri et al.[45]	KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	5 (35%)
				G12V	c.35G > T	p.Gly12Val	5 (35%)
			13	G13D	c.38G > A	p.Gly13Asp	4 (28%)
	NRAS	4	146	A146T	c.436G > A	p.A146T	1 (100%)
Niya et al.[36]	KRAS	2	12	G12A	c.35G > C	p.Gly12Ala	NA
				G12C	c.34G > T	p.Gly12Cys	NA
				G12D	c.35G > A	p.Gly12Asp	NA
				G12F	c.34_35GG > TT	p.Gly12Phe	NA
				G12R	c.34G > C	p.Gly12Arg	NA
				G12S	c.34G > A	p.Gly12Ser	NA
				G12V	c.35G > T	p.Gly12Val	NA
			13	G13D	c.38G > A	p.Gly13Asp	NA
Bishehsari et al.[37]	KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	16 (8.8%)
				G12C	c.34G > T	p.Gly12Cys	9 (4.9%)
				G12V	c.35G > T	p.Gly12Val	18 (9.9%)
			13	G13D	c.38G > A	p.Gly13Asp	21 (11.5%)
Hamzehzadeh et al.[38]	KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	7 (28%)
				G12V	c.35G > T	p.Gly12Val	4 (16%)
				G12A	c.35G > C	p.Gly12Ala	3 (12%)
				G12S	c.34G > A	p.Gly12Ser	3 (12%)
				G12C	c.34G > T	p.Gly12Cys	1 (4%)
			13	G13D	c.38G > A	p.Gly13Asp	7 (28%)
Iraq							
Al-Allawi et al.[39]	KRAS	2	12	G12V	c.35G > T	p.Gly12Val	9 (31%)
				G12D	c.35G > A	p.Gly12Asp	7 (24.1%)
				G12A	c.35G > C	p.Gly12Ala	5 (17.2%)
				G12C	c.34G > T	p.Gly12Cys	3 (10.3%)
				G12S	c.34G > A	p.Gly12Ser	2 (6.9%)
			13	G13D	c.38G > A	p.Gly13Asp	3 (10.3%)

(Continued)

Table 4. (Continued)

COUNTRY AUTHOR (REFERENCE)	GENE	EXON	CODON	AMINO ACID	NUCLEOTIDE	PROTEIN	FREQUENCY, NO (%)
Israel							
Kislitsin et al.[40]	KRAS	2	12	G12C	c.34G > T	p.Gly12Cys	4 (14%)
				G12S	c.34G > A	p.Gly12Ser	4 (14%)
				G12V	c.35G > T	p.Gly12Val	7 (23%)
				G12D	c.35G > A	p.Gly12Asp	14 (43%)
			13	G13D	c.38G > A	p.Gly13Asp	12 (27%)
Jordan							
Elbjeirami et al.[41]	KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	24 (54.5%)
				G12V	c.35G > T	p.Gly12Val	6 (13.6%)
				G12C	c.34G > T	p.Gly12Cys	5 (11.4%)
				G12A	c.35G > C	p.Gly12Ala	2 (4.5%)
				G12R	c.34G > C	p.Gly12Arg	2 (4.5%)
				G12S	c.34G > A	p.Gly12Ser	1 (2.3%)
			13	G13D	c.38G > A	p.Gly13Asp	5 (11.4%)
Awidi et al.[46]	KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	18 (19.56%)
				G12A	c.35G > C	p.Gly12Ala	16 (17.39%)
				G12T	c.(34G > A; 35G > C)	p.Gly12Thr	13 (14.13%)
				G12V	c.35G > T	p.Gly12Val	10 (10.87%)
				G12S	c.34G > A	p.Gly12Ser	3 (3.26%)
				G12C	c.34G > T	p.Gly12Cys	2 (2.17%)
			13	G13D	c.38G > A	p.Gly13Asp	7 (7.60%)
				G13A	c.38G > C	p.Gly13Ala	6 (6.52%)
		3	61	Q61H	c.183A > C	p.Gln61His	1 (1.08%)
		4	117	K117N	c.351A > C	p.Lys117Asn	6 (6.52%)
			146	A146T	c.436G > A	p.Ala146Thr	2 (2.17%)
	NRAS	2	12	G12V	c.35G > T	p.Gly12Val	1 (1.08%)
			13	G12C	c.37G > T	p.Gly13Cys	1 (1.08%)
				G13A	c.38G > C	p.Gly13Ala	1 (1.08%)
		3	61	Q61R	c.182A > G	p.Gln61Arg	1 (1.08%)
				Q61P	c.182A > C	p.Gln61Pro	1 (1.08%)
				A61T	NA	p.Ala61Thr	1 (1.08%)

(Continued)

Table 4. (Continued)

COUNTRY AUTHOR (REFERENCE)	GENE	EXON	CODON	AMINO ACID	NUCLEOTIDE	PROTEIN	FREQUENCY, NO (%)
Lebanon							
Baba et al.[50]	KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	49 (37.4%)
				G12V	c.35G > T	p.Gly12Val	28 (21.4%)
				G12C	c.34G > T	p.Gly12Cys	15 (11.5%)
				G12A	c.35G > C	p.Gly12Ala	7 (5.3%)
				G12S	c.34G > A	p.Gly12Ser	6 (4.6%)
				G12R	c.34G > C	p.Gly12Arg	2 (1.5%)
			13	G13C	c.37G > T	p.Gly13Cys	1 (0.8%)
				G13D	c.38G > A	p.Gly13Asp	16 (12.9%)
		3	61	Q61H	c.183A > T	p.Gln61His	1 (0.8%)
		4	117	K117N	c.351A > T	p.Lys117Asn	1 (0.8%)
			146	A146T	c.436G > A	p.Ala146Thr	1 (0.8%)
				A146V	c.437C > T	p.Ala146Val	1 (0.8%)
	NRAS	2	12	G12D	c.35G > A	p.Gly12Asp	6 (37.5%)
			13	G13R	c.37G > C	p.Gly13Arg	2 (12.5%)
		3	61	Q61R	c.182A > G	p.Gln61Arg	3 (18.75%)
				Q61L	c.182A > T	p.Gln61Leu	3 (18.75%)
	BRAF	15	600	V600E	1799T > A	p.Val600Glu	2 (1.2%)
Saudi Arabia							
Bader et al.[42]	KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	16 (45.7%)
				G12V	c.35G > T	p.Gly12Val	11 (31.4%)
				G12C	c.34G > T	p.Gly12Cys	3 (8.5%)
				G12S	c.34G > A	p.Gly12Ser	1 (2.8%)
			13	G13D	c.38G > A	p.Gly13Asp	4 (11.4%)
Zekri et al.[43]	KRAS	2	12	NA	NA	NA	NA
			13	NA	NA	NA	NA
Zahrani et al.[44]	KRAS	2	12	G12V	c.35G > T	p.Gly12Val	26 (35.6%)
				G12D	c.35G > A	p.Gly12Asp	26 (35.6%)
				G12S	c.34G > A	p.Gly12Ser	10 (13.7%)
				G12C	c.34G > T	p.Gly12Cys	6 (8.2%)
				G12R	c.34G > C	p.Gly12Arg	3 (4.2%)
				G12A	c.35G > C	p.Gly12Ala	2 (2.7%)
			13	G13D	c.38G > A	p.Gly13Asp	10 (90.9%)
				G13R	c.37G > C	p.Gly13Arg	1 (9.1%)

(Continued)

Table 4. (Continued)

COUNTRY AUTHOR (REFERENCE)	GENE	EXON	CODON	AMINO ACID	NUCLEOTIDE	PROTEIN	FREQUENCY, NO (%)
Mulla et al.[47]	KRAS	2	12	G12A	c.35G>C	p.Gly12Ala	1 (6.7%)
				G12C	c.34G>T	p.Gly12Cys	1 (6.7%)
				G12D	c.35G>A	p.Gly12Asp	8 (53.3%)
				G12S	c.34G>A	p.Gly12Ser	2 (13.3%)
				G12V	c.35G>T	p.Gly12Val	3 (20%)
	NRAS	2	12	G12D	c.35G>A	p.Gly12Asp	1 (50%)
		3	61	Q61L	c.182A>T	p.Gln61Leu	1 (50%)
Saharti[51]	KRAS	2	12	G12D	c.35G>A	p.Gly12Asp	17 (18.3%)
				G12V	c.35G>T	p.Gly12Val	6 (6.5%)
				G12S	c.34G>A	p.Gly12Ser	4 (4.3%)
				G12A	c.35G>C	p.Gly12Ala	3 (3.2%)
				G12R	c.34G>C	p.Gly12Arg	1 (1.1%)
				G12C	c.34G>T	p.Gly12Cys	1 (1.1%)
			13	G13D	c.38G>A	p.Gly13Asp	3 (3.2%)
		3	61	Q61H	c.183A>T	p.Gln61His	2 (2.2%)
		4	117	K117N	c.351A>T	p.Lys117Asn	2 (2.2%)
			146	A146T	c.436G>A	p.Ala146Thr	2 (2.2%)
	NRAS	3	61	Q61K	c.181C>A	p.Gln61Lys	1 (1.1%)
				Q61L	c.182A>T	p.Gln61Leu	1 (1.1%)
	BRAF	15	600	V600E	1799T>A	p.Val600Glu	2 (2.2%)

Abbreviation: NA, not available.

Paper III: Prevalence and patterns of mutations in *RAS/RAF/MEK/ERK/MAPK* Signaling pathway in colorectal cancer in North Africa

Colorectal cancer is a significant health concern globally, with increasing research focusing on understanding its molecular characteristics and risk factors. In this review, we present the findings from analyzed data that examined the prevalence of KRAS, NRAS, and BRAF mutations in CRC patients in North Africa. Additionally, we explore the potential role of dietary and lifestyle factors in CRC risk. Understanding the genetic mutations and risk factors associated with CRC in this region can aid in improving treatment strategies and implementing preventive measures.

To gather relevant information, we conducted a systematic literature search using electronic databases to study KRAS, NRAS, and BRAF mutations in CRC patients from North Africa (Morocco, Tunisia, Algeria, and Libya). Seventeen relevant studies were analyzed, involving 1843 CRC patients, with an average age of 52.7 years and a male predominance of 56.6%. Among the CRC patients, KRAS mutations were most prevalent (46.4%), followed by NRAS (3.2%) and BRAF (3.5%) mutations. The frequency of KRAS mutations varied significantly: 23.9% to 51% in Morocco, 23.1% to 68.2% in Tunisia, 31.4% to 50% in Algeria, and 38.2% in Libya. Specific KRAS mutations were also identified, with G12D being the most common (31.6%). Morocco and Tunisia had a higher prevalence of G12D (approximately 50%). The study highlights the importance of understanding CRC genetics in North Africa and the potential role of dietary and lifestyle factors in CRC risk.

Our findings suggest that CRC patients with KRAS mutations in North Africa exhibit an incidence rate similar to that observed in European populations. This emphasizes the importance of understanding the genetic characteristics of CRC in the region to improve treatment outcomes. Furthermore, alongside established anti-CRC treatments, a better understanding of the causality of CRC can be established by exploring the potential role of dietary and lifestyle factors. These insights can contribute to the development of more targeted therapeutic approaches and effective preventive strategies for CRC in North Africa.

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Prevalence and patterns of mutations in *RAS/RAF/MEK/ERK/MAPK* signaling pathway in colorectal cancer in North Africa

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Abstract

Background: Our review discuss (i) the findings from analyzed data that have examined *KRAS*, *NRAS* and *BRAF* mutations in patients with colorectal cancer (CRC) in North Africa and to compare its prevalence with that shown in other populations and (ii) the possible role of dietary and lifestyle factors with CRC risk.

Methods: Using electronic databases, a systematic literature search was performed for the *KRAS*, *NRAS*, and *BRAF* mutations in CRC patients from Morocco, Tunisia, Algeria and Lybia.

Results: Seventeen studies were identified through electronic searches with six studies conducted in Morocco, eight in Tunisia, two in Algeria, and one in Libya. A total of 1843 CRC patients were included 576 (31.3%) in Morocco, 641 (34.8%) in Tunisia, 592 (32.1%) in Algeria, and 34 (1.8%) in Libya. Overall, the average age of patients was 52.7 years old. Patients were predominantly male (56.6%). The mutation rates of *KRAS*, *NRAS* and *BRAF* were 46.4%, 3.2% and 3.5% of all patients, respectively. A broad range of reported *KRAS* mutation frequencies have been reported in North Africa countries. The *KRAS* mutation frequency was 23.9% to 51% in Morocco, 23.1% to 68.2% in Tunisia, 31.4% to 50% in Algeria, and 38.2% in Libya. The G12D was the most frequently identified *KRAS* exon 2 mutations (31.6%), followed by G12V (25.4%), G13D (15.5%), G12C (10.2%), G12A (6.9%), and G12S (6.4%). G12R, G13V, G13C and G13R are less than 5%. There are important differences among North Africa countries. In Morocco and Tunisia, there is a higher prevalence of G12D mutation in *KRAS* exon 2 (≈50%). The most frequently mutation type in *KRAS* exon 3 was Q61L (40%). A59T and Q61E mutations were also found. In *KRAS* exon 4, the most common mutation was A146T (50%), followed by K117N (33.3%), A146P (8.3%) and A146V (8.3%).

Conclusion: *KRAS* mutated CRC patients in North Africa have been identified with incidence closer to the European figures. Beside established anti-CRC treatment, better understanding of the causality of CRC can be established

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by combining epidemiology and genetic/epigenetic on CRC etiology. This approach may be able to significantly reduce the burden of CRC in North Africa.

Keywords: *KRAS*, *NRAS*, And *BRAF* mutations, Colorectal cancer, Lifestyle factors, North Africa

Introduction

Colorectal cancer (CRC) is the third most commonly occurring cancer in men and the second most commonly occurring cancer in women. According to GLOBOCAN 2020 data, there are 1.15 million new cases of colon cancer, 0.7 million new cases of rectal cancers, and 50,000 new cases of anal cancer in 2020 globally [1]. With continuous progress, these numbers are predicted to increase to 1.92 million, 1.16 million, and 78,000 in 2040, respectively [2]. Globally it is one of the cancers whose incidence is increasing comprising 11% of all cancer diagnoses [3]. Over the world, the risk of CRC is highest and 3–4 times more common in countries with a very high human development index (HDI) than in countries with a low HDI [4]. CRC is considered one of the clearest markers of epidemiological and nutritional transition in societies undergoing socioeconomic development and transition to a lifestyle more typical of industrialized countries. Moreover, the data suggest the existence of a threshold at which CRC incidence stabilizes or declines according to the HDI [5].

Studies of CRC in the North of Africa (including Morocco, Algeria, Tunisia, and Libya) have shown rising trend in incidence rate. It was 7.1/100,000 [6]. When compared with sub-Saharan Africa (SSA), North Africa had the highest age-standardized incidence rates (ASR) of 8.66, while SSA had an ASR of 5.91 [7]. Libya reports the highest ASR for CRC with an incidence closer to the European figures. The ASR per 100,000 for CRC was 17.5 and 17.2 for males and females, respectively [8]. CRC ASR in Northern Tunisia was of 12.4 in 2009 (13.6 against 11.1, respectively, in men and women). This incidence increased during the period 1994–2009, from 6.4/100,000 in 1994 to 12.4/100,000 in 2009 [9]. CRC in young patients is more common in North Africa countries with a proportion of 11% in Morocco and much higher in the Middle East with 46% in UAE [10] and 25% in Egypt [11].

CRC is a complex and genetically heterogeneous disease with involving in oncogene and tumor suppressor genes, as well as genes involved in DNA damage recognition and repair. CRC includes also different categories of tumors based on their specific spectrum of mutations and molecular phenotype, driving various oncogenic signaling pathways [12]. The *RAS/RAF/MEK/ERK/MAPK* is the most well-known pathway in the pathogenesis of CRC. Mutations in genes of this pathway have been reported in about 50% of patients with CRC [13]. *RAS* and *BRAF* are members of the *RAS/RAF/MEK/ERK/MAPK* pathway which mediates cellular response to growth signals. Both *RAS* and *RAF* are members of multi-gene families and there are three *RAS* members (*KRAS*, *NRAS* and *HRAS*) and three *RAF* members (*BRAF*, *RAF1* (a.k.a c-Raf) and *ARAF*). The *KRAS* proto-oncogene (locus 1p13.2) is the most commonly mutated RAS family member (75% of *RAS* mutations). The *KRAS* proto-oncogene encodes a protein (p21-ras) belonging to the family of GTP/GDP-binding proteins with GTPase activity and is involved in the transduction of mutagenic signals. *KRAS* gene mutations occur commonly in colon [14, 15]. The *NRAS* proto-oncogene (locus 1p13.2) is also a member of the *RAS* gene family which encodes proteins involved in signal transmission in cells and participates in the regulation of cell growth. *NRAS* gene mutations is associated with of CRC tumors [14, 15]. The *BRAF* gene on chromosome 7 (7q34) encodes for a serine/threonine protein kinase) that sends signals from outside of the cell to the nucleus that in turn drives the growth of a cell. *BRAF* is a downstream target of *RAS*, playing a pivotal role in the MAPK/ERK signaling pathway. Mutations of *BRAF* are found in CRCs [16] and are associated with a poor prognosis in stage II, III, and IV [17].

This systematic review aims to discuss (i) the findings from analyzed data that have examined *KRAS*, *NRAS* and *BRAF* mutations in patients with CRC in North Africa and to compare its prevalence with that shown in other populations and (ii) the possible role of dietary and lifestyle factors with CRC risk.

Materials and methods

We included articles that described the prevalence of *KRAS*, *NRAS*, or *BRAF* gene mutations in CRC patients in North Africa. Pub Med, Science Direct and Google Scholar were searched up to December 2021 for eligible studies using the following keywords: "colorectal cancer", or "colon cancer", or "rectum cancer", or "*RAS*", or "*KRAS*", or "*NRAS*", or "*BRAF*", or "mutation", or "oncogenic mutation", or "oncogenic driver mutation", or "activating mutation", or "prevalence", or "rate", or "incidence", or "frequency".

An additional literature search was also conducted using North Africa and specific country names belonging to the considered region and any other variant names for any of North Africa countries (ex: Mediterranean

countries, Maghreb, Arab population). We manually checked reference lists of the included studies and relevant reviews to identify additional studies. We also searched relevant abstracts reported in the most important multi-disciplinary societies of medical oncology such as the American Society of Clinical Oncology (ASCO) meetings to identify unpublished studies.

Eligible analytical study designs were prospective or retrospective cohort studies, cross-sectional, and case–control studies and were conducted in CRC patients. The study must give information on the prevalence of *KRAS*, *NRAS*, and *BRAF* mutations in a specific or selected coding region of *KRAS*, *NRAS* genes (exon 2: codon 12 and 13; exon 3: codon 59 and 61; exon 4: codon 117 and 146) and *BRAF* (exon 15). Studies published as original articles on human subjects, evaluating *KRAS, NRAS,* and *BRAF* mutation status and recording clinico-pathological features in CRC patients, and written in English, were included for analysis.

The following information data were abstracted from each of the eligible studies: 1) characteristics of the study (including publication year, country where the study was conducted, study type, sample size, number of subjects with mutation results); 2) characteristics of study participants (including gender, age); 3) characteristics of study outcomes (including mutation detection assay, type of mutation, mutation exon/codon numbers, mutation prevalence, whether mutation data were from primary or metastatic tumor, and whether mutation data were obtained from tissue or liquid biopsy using plasma); 4) clinicopathological characteristics (including disease stage, T stage, metastasis status, tumor site, tumor differentiation and tumor histology).

Letters, comments, and review articles or meta-analysis without original data, were excluded from analysis.

Results

The search of the databases yielded 17 relevant references which are closely related to defining the inclusion criteria and was included in this review. The retrieved articles describe studies conducted in Morocco ($n=6$) [18–23], Tunisia ($n=8$) [24–31], Algeria ($n=2$) [32, 33], and Libya ($n=1$) [34]. A total of 1843 patients with CRC were included for our analysis, including 576 (31.3%) in Morocco [18–23], 641 (34.8%) in Tunisia [24–31], 592 (32.1%) in Algeria [32, 33], and 34 (1.8%) in Libya [34]. The average age of patients was 52.7 years old [18–20, 22–28, 31–33], with 45.1% (260/577) [18, 20, 26, 29, 34] of patients diagnosed above the age of 50 years. Male patients were predominant in all of the considered studies, accounting for 56.6% (960/1697) [18–20, 22–26, 28, 29, 31–34].

From the specimens where tumor site information was available, 31.4% (344/1095) of them were in the rectum [22, 23, 26, 28, 29, 31–34], 30.1% (188/625) were in the right colon [18, 22, 24, 25, 28, 31], and 56.5% (353/625) in the left colon [18, 22, 24, 25, 28, 31]. The predominant histopathological type of CRC is adenocarcinoma (82.2%, 374/455) [18, 20, 25]. Almost half (58%, 302/521) of the tumors were reported to be in stage III and IV [18, 23, 24, 27, 33]. Baseline characteristics and clinico-pathologic of enrolled studies are summarized in Table 1.

Among the 17 studies, 13 (76.5%) [18–25, 27, 30–33] used FFPE tumor samples for *KRAS*, *NRAS* and *BRAF* mutation analysis. Both exon 2, 3, and 4 mutations of *KRAS* and *NRAS* genes were analyzed in 23.5% (4/17) [18, 19, 25, 32] of North Africa series, exon 2, 3 and 4 mutations of *KRAS* gene were evaluated in one study (5.9%, 1/17) [33], specific *KRAS* exons (exon 2 and/or exon3) were assessed in 58.8% (10/17) [20–24, 26–30] and *NRAS* exons (exon 2 and 3) in 11.8% (2/17) investigations [20, 24]. *KRAS* mutations in exon 2 codon 12 and 13 have been assessed in the most of the studies (94.1%, 16/17) [18–30, 32–34]. *BRAF* gene mutation was analyzed in 41.2% (7/17) studies [18, 19, 21–23, 31, 33].

Different molecular methods were used for *KRAS*, *NRAS* and *BRAF* mutations screening. Sequencing assay was broadly used, as it was used in 64.70% (11/17) studies [21, 23, 26–34]. Other methodologies described in the considered studies include Pyrosequencing [18, 20, 25], array based techniques Mass ARRAY [19, 22], amplification-refractory mutation system-PCR (ARMS-PCR) [24], Allele-specific PCR [32], End-point genotyping [22], High Resolution Melting [30] and Denaturating High Performance Liquid Chromatography [30]. The details of studies examining *KRAS*, *NRAS* and *BRAF* genes in North Africa are presented in Table 2.

The prevalence of *KRAS*, *NRAS* and *BRAF* mutations has been reported in 16 [18–30, 32–34], 6 [18–20, 24, 25, 32], and 7 [18, 19, 21–23, 31, 33] of the 17 included studies, respectively. In total, *KRAS* mutations were most frequently detected among North Africa patients with CRC, accounting for 41.7% (749/1795) [18–30, 32–34]. Tunisia highlights a wide range of *KRAS* mutations rates, ranging from 23.1% (12/52) [29] to 68.2% (88/129) [25], as compared with other countries from the region. Overall, *KRAS* Mutations were distributed among the different exons as follows: 95.9% (718/749) exon 2 [18–27, 29, 30, 32–34], 2.7% (13/481) exon 3 [18, 20, 25, 32], and 2.9% (14/481) exon 4 [18–20, 25] (Table3). Among mutations in exon 2, 79.7% (94/118) had single mutations in codon 12 [18, 22, 27, 34] and 20.3% (24/118) had point mutations in codon 13 [18, 22, 27, 34] (Table 4).

As seen in Table 5, the G12D was the most frequently identified exon 2 mutations (31.6%, 137/433) [18,

Table 1 Baseline characteristics and clinico-pathologic of enrolled studies from North Africa countries

Country Author [reference]	Sample size	Mean age Years (± SD)	Women/Men N (%)	Tumor site N (%)			Disease stages Stage: N (%)
				Colon		Rectum	
				Right	Left		
Morocco							
El Agy et al. [18]	210	55.6 (± 14.3)	97 (46.2%)/113 (53.8%)	67 (31.9%)	143 (68.1%)	na	I-II: 126 (60%), III-IV: 84 (40%)
Houssaini et al. [19]	51	59,8	24 (48.1%)/27 (52.9%)	na	na	na	na
Dehbi et al. [20]	114	55.4	50 (43.9%)/64 (56.1%)	na	na	na	na
Jadda et al. [21]	47	na	na	na	na	na	na
Marchoudi et al. [22]	92	62.3	38 (41.3%)/54 (58.7%)	15 (16.3%)	34 (37%)	12 (13.0%)	na
Bennani et al. [23]	62	53	31 (50%)/31 (50%)	na	na	28 (44.4%)	II: 22 (35.5%), III: 26 (41.9%), IV: 14 (22.6%)
Tunisia							
Ounissi et al. [24]	96	62.4	36 (37.5%)/60 (62.5%)	25 (26.1%)	71 (73.9%)	na	I-II: 32 (33.3%), III-IV: 64 (66.6%)
Jouini et al. [25]	131	56.1 (± 12.6)	56 (42.7%)/75 (57.3%)	37 (78.7%)	75 (92.6%)		na
Chaar et al. [26]	167	57	83 (49.7%)/84(50.3%)	93 (55.7%)		74 (44.3%)	I-II: 30 (17.9%), III-IV: 137 (82%)
Aissi et al. [27]	51	48.5	na	na	na	na	II: 13 (48.1%), III: 12 (44.4%), IV: 2 (7.4%)
Ouerhani et al. [28]	48	61 (± 13)	25 (52%)/23 (47.1%)	21 (43.7%)	18 (37.5%)	9 (18.8%)	na
Sammoud et al. [29]	52	na	23 (44.2%)/29 (55.8%)	25 (48.1%)		27 (51.9%)	na
Bougatef et al. [30]	48	na	na	na	na	na	na
Bougatef et al. [31]	48	61 (± 13)	25 (52%)/23 (47.1%)	22 (45.8%)	15 (31.3%)	9 (18.8%)	na
Algeria							
Mazouzi et al. [32]	490	52.6	190 (38.8%)/300 (61.2%)	331 (67.6%)		141 (28.8%)	na
Boudida-Berkane K et al. [33]	102	48(47.1%) < 50 54(52.9%) > 50	44 (43.2%)/58 (56.8%)	66 (64.7%)		36 (35.3%)	III: 46 (45%), IV: 56 (55%)
Lybia							
Abudabous et al. [34]	34	na	15 (44.1%)/19 (55.9%)	11 (32.35%)	11 (32.35%)	8 (23.5%)	na

na not available

20–27, 29, 33, 34], followed by G12V (25.4%, 110/433) [18, 20–26, 29, 33, 34], G13D (15.5%, 67/433) [18, 20–27, 29, 33, 34], G12C (10.2%, 44/433) [18, 20–27, 29, 33, 34], G12A (6.9%, 30/433) [18, 20–22, 24, 27, 29, 33], G12S (6.4%, 28/433) [20, 23–25, 27, 33]. G12R, G13V, G13C and G13R are less than 5% [18, 20, 21, 24–26]. However, there are important differences among North Africa countries. In Morocco and Tunisia there is a higher prevalence of G12D mutation (50%) [22, 27]. The most frequently mutation type in exon 3 was Q61L (40%, 2/5) [18, 20]. A59T [20] and Q61E [25] mutations were also found in our review. In exon 4, the most common mutation was A146T (50%, 6/12) [18, 20, 25], followed by K117N (33.3%, 4/12) [18, 25], A146P (8.3%, 1/12) [18] and A146V (8.3%, 1/12) [18].

NRAS total mutations were identified in 3.2% (35/1090) [18–20, 24, 25, 32] tumor samples. Morocco highlights a wide range of *NRAS* mutations rates, ranging from 2% (1/51) to 5.3% (6/114) [19, 20]. A higher prevalence of *NRAS* mutations has been reported in Tunisia (7.3%, 7/96) [24]. Overall, the common mutation site of *NRAS* gene was located in exons 2 and 3, with 28.6% (10/35) [18, 24, 25] and 48.6% (17/35) [18, 20, 24, 25] of CRC patient (Table 3). *NRAS* mutations were more common in codon 61, accounting for 48.6% (17/35)[18, 20, 24, 25] (Table 4). The most common mutations were G12D in exons 2 and Q61K in exon 3 and accounted for 40% (4/10) [18, 25] and 35.3% (6/17) [18, 20, 25] of patients with CRC, respectively (Table 5).

The total mutation frequency in the *BRAF* gene was 2.8% (17/602) [18, 19, 21–23, 31, 33]. The highest frequency of *BRAF* mutations was found in Tunisia and reported in 8.3% (4/48) of patients with CRC [31]. The overall frequency of the *BRAF* V600E mutation was 2.8% (17/602) [18, 19, 21–23, 31, 33] (Tables 3 and 5).

Table 2 Details of studies examining *KRAS*, *NRAS* and *BRAF* genes in North Africa

Country Author [reference]	Materials	KRAS			NRAS			BRAF	Molecular analysis
		Exon 2	Exon 3	Exon 4	Exon 2	Exon 3	Exon 4	Exon 15	
Morocco									
El Agy et al. [18]	FFPE	+	+	+	+	+	+	-	Pyrosequencing
Houssaini et al. [19]	FFPE	+	+	+	+	+	+	-	MassARRAY
Dehbi et al. [20]	FFPE	+	+	-	+	+	-	-	Pyrosequencing
Jadda et al. [21]	FFPE	+	-	-	-	-	-	+	Sequencing
Marchoudi et al. [22]	FFPE	+	-	-	-	-	-	+	KRAS array, End-point genotyping
Bennani et al. [23]	FFPE	+	-	-	-	-	-	+	HRM and sequencing
Tunisia									
Ounissi et al. [24]	FFPE	+	-	-	+	+	-	-	PCR
Jouini et al. [25]	FFPE	+	+	+	+	+	+	-	Pyrosequencing
Chaar et al. [26]	Tumoral mucosa	+	+	-	-	-	-	-	PCR- SSCP, Sequencing
Aissi et al. [27]	FFPE	+	-	-	-	-	-	-	Sequencing
Ouerhani et al. [28]	Fresh tumour or FFPE	+	-	-	-	-	-	-	Sequencing
Sammoud et al. [29]	Normal mucosa and tumoral mucosa of epithelial tissue	+	-	-	-	-	-	-	Sequencing
Bougatef et al. [30]	FFPE	+	-	-	-	-	-	-	Sequencing, HRM, DHPLC
Bougatef et al. [31]	FFPE	-	-	-	-	-	-	+	Sequencing
Algeria									
Mazouzi et al. [32]	FFPE	+	+	+	+	+	+	-	ASPCR-Sequencing
Boudida-Berkane K et al. [33]	FFPE	+	+	+	-	-	-	+	Sequencing
Lybia									
Abudabous et al. [34]	Frozen tissue	+	-	-	-	-	-	-	Sequencing

+ genetic analysis realized, - genetic analysis not realized, *HRM* High Resolution Melting, *DHPLC* Denaturating High Performance Liquid Chromatography, *ASPCR* Allele-specific PCR

Discussion

CRC is a pathologically and clinically heterogeneous malignancy. Phenotypic and molecular characteristics of CRC represents a significant key step in defining diagnosis, prognosis and treatment predictive value both in localized and in the setting of CRC [35]. Mutation detection in any of the genes involved in the *RAS*/*RAF*/*MEK*/*ERK*/*MAPK* pathway has been used to predict the outcomes of *EGFR*-targeted therapy for CRC. *KRAS* mutations were found in ≈33% of in the COSMIC dataset comprising 75,000 tested specimens samples. In other datasets based on small sample sizes (< 500), *KRAS* was found mutated in ≈40%-45% CRCs. Notably however, the private Foundation Medicine dataset comprising 13,336 colorectal samples reports a *KRAS* mutation frequency of ≈50% [36]. According to the COSMIC database (Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cosmic), mutations at codon 12 (G12A, G12V, G12S, G12R, G12C, G12D) are the most prominent (> 90%), followed by those affecting codon 13 (G13D, G13C), codon 61 (Q61L, Q61R, Q61H), codon 146 (A146T, A146V, A146P), and codon 117 (K117N). *NRAS* mutations are found in 5%-10% of CRC. *NRAS* is mutated in the same codon of *KRAS* particularly in exon 2 (3%-5%) and exon 3 (2%-6%). *BRAF* mutations are identified in about 8%-12% of CRC patients and 90% of all identified mutations are a T1799A transversion in exon 15, which results in a valine amino acid substitution (V600E) [37].

In our review, *KRAS* mutation was detected in the CRC tumor of 41.7% of the patients among whom 95.9% had a single mutation at exon 2, 2.7% at exon 3, and 2.9% at exon 4. Among mutations in *KRAS* exon 2, 79.7% of cases had single mutations in codon 12 and 20.3% of cases in codon 13. The G12D was the most frequently identified exon 2 mutations (31.6%), followed by G12V, G13D, G12C, G12A, and G12S. The most frequently mutation type in exon 3 was Q61L (40%). In exon 4, the most common mutation was A146T, K117N, A146P and A146V. *NRAS* total mutations were identified in 3.2% tumor samples. The common mutation site of *NRAS* gene was located in exons 2 and 3, with 28.6% and 48.6% of CRC

Table 3 Prevalence of *KRAS, NRAS* and *BRAF* mutations in North Africa

Country Author [reference]	Gene frequency n/N (%)			Exon frequency n/N (%)						
	KRAS	NRAS	BRAF	KRAS Exon2	KRAS Exon3	KRAS Exon4	NRAS Exon2	NRAS Exon3	NRAS Exon4	BRAF Exon15
Morocco										
El Agy et al. [18]	77/210 (36.7%)	6/210 (2.9%)	0/210 (0%)	68 (83.3%)	1 (1.3%)	8 (10.4%)	2 (33.3%)	4 (66.7%)	na	na
Houssaini et al. [19]	22/51 (43.1%)	1/51 (2%)	2/51 (3.9%)	26[a] (92.8%)	na	2[a] (7.1%)	na	na	na	2 (3.9%)
Dehbi et al. [20]	49/114 (43%)	6/114 (5.3%)	na	45 (91.8%)	3 (6.1%)	1 (2.04%)	na	6 (5.3%)	na	na
Jadda et al. [21]	24/47 (51%)	na	0/47 (0%)	24 (51%)	na	na	na	na	na	0 (0%)
Marchoudi et al. [22]	22/92 (23.9%)	na	5/92 (5.4%)	22/92 (23.9%)	na	na	na	na	na	5 (5.4%)
Bennani et al. [23]	18/62 (29%)	na	1/62 (1.6%)	18 (38.7%)	na	na	na	na	na	1 (1.6%)
Tunisia										
Ounissi et al. [24]	40/96 (41.7%)	7/96 (7.3%)	na	47[b] (100%)	na	na	5[c] (62.5%)	3[c] (37.5%)	0[c] (0%)	na
Jouini et al. [25]	88/129 (68.2%)	9/129 (6.9%)	na	84 (95.4%)	1 (1.1%)	3 (3.4%)	3 (33.3%)	4 (44.4%)	2 (22.2%)	na
Chaar et al. [26]	52/167 (31.1%)	na	na	52 (31.1%)	na	na	na	na	na	na
Aissi et al. [27]	16/51 (31.5%)	na	na	16 (31.5%)	na	na	na	na	na	na
Ouerhani et al. [28]	17/48 (35.41%)	na	na	ni	na	na	na	na	na	na
Sammoud et al. [29]	12/52 (23.1%)	na	na	12 (23.1%)	na	na	na	na	na	na
Bougatef et al. [30]	22/48 (45.83%)	na	na	22 (45.83%)	na	na	na	na	na	na
Bougatef et al. [31]	na	na	4/48 (8.3%)	Na	na	na	na	na	na	4 (8.3%)
Algeria										
Mazouzi et al. [32]	245/490 (50%)	6/490[d] (1.2%)	na	237 (96.7%)	8 (3.3%)	na	na	na	na	Na
Boudida-Berkane K et al. [33]	32/102 (31.4%)	na	5/102 (4.9%)	32 (31.4%)	na	na	na	na	na	5 (4.9%)
Lybia										
Abudabous et al. [34]	13/34 (38.2%)	na	na	13 (38.2%)	na	na	na	na	na	na

na not available

[a] In about 22 Patients with a KRAS mutations, 28 mutations were detected in exon 2 and exon 4 (Some patients have concomitant mutations)

[b] In a total of 40 cases, we had 47 mutations in KRAS exon 2 (7/40 patients had 2 concomitant mutations in exon 2)

[c] One of the 7 patients with NRAS mutation showed a double mutation in NRAS

[d] Rare mutations in the NRAS gene

Table 4 *KRAS* and *NRAS* exons mutations subtypes detected in North Africa

Country Author [reference]	KRAS Exon2		KRAS Exon3		KRAS Exon4		NRAS Exon2		NRAS Exon3		NRAS Exon4	
	Codon 12	Codon 13	Codon 59	Codon 61	Codon 117	Codon 146	Codon 12	Codon 13	Codon 59	Codon 61	Codon 117	Codon 146
Morocco												
El Agy et al. [18]	52/68 (76.5%)	16/68 (23.5%)	na	1/77 (1.3%)	3/8 (37.5%)	5/8 (62.5%)	2/6 (33.3%)	na	na	4/6 (66.7%)	na	na
Houssaini et al. [19]	na	na	na	Na	na	Na	na	na	na	na	na	na
Dehbi et al. [20]	37/45 (82.2%)	8/45 (17.8%)	1/3 (33.3%)	2/3 (66.7%)	na	1/49 (2.04%)	na	na	na	6/114 (5.3%)	na	na
Jadda et al. [21]	21/24 (87.5%)	3/24 (12.5%)	na	na	na	na	na	na	na	na	na	na
Marchoudi et al. [22]	18/22 (81.8%)	4/22 (18.2%)	na	na	na	na	na	na	na	na	na	na
Bennani et al. [23]	15/18 (83.3%)	3/18 (16.7%)	na	na	na	na	na	na	na	na	na	na
Tunisia												
Ounissi et al. [24]	40/47 (85%)	7/47 (15%)	na	na	na	na	na	na	na	3/8 (37.5%)	na	0/8 (0%)
Jouini et al. [25]	72/84 (85.7%)	12/84 (14.3%)	na	1/88 (1.1%)	1/3 (33.3%)	2/3 (66.7%)	3/9 (33.3%)	na	na	4/9 (44.4%)	1/2 (50%)	1/2 (50%)
Chaar et al. [26]	43/52 (82.7%)	9/52 (17.3%)	na	na	na	na	na	na	na	na	na	Na
Aissi et al. [27]	13/16 (81.2%)	3/16 (18.7%)	na	na	na	na	na	na	na	na	na	na
Ouerhani et al. [28]	na	na	na	na	na	na	na	na	na	na	na	na
Sammoud et al. [29]	10/12 (83.3%)	2/12 (16.7%)	na	na	na	na	na	na	na	na	na	na
Bougatef et al. [30]	na	na	na	na	na	na	na	na	na	na	na	na
Bougatef et al. [31]	na	na	na	na	na	na	na	na	na	na	na	na
Algeria												
Mazouzi et al. [32]	182/237 (76.8%)	55/237 (23.2%)	na	na	na	na	na	na	na	na	na	na
Boudida-Berkane K et al. [33]	29/32 (90.6%)	3/32 (9.4%)	na	na	na	na	na	na	na	na	na	na
Lybia												
Abudabous et al. [34]	12/13 (92.3%)	1/13 (7.7%)	na	na	na	na	na	na	na	na	na	na

na not available

Table 5 Frequency and distribution of *KRAS, NRAS* and *BRAF* mutations in different North of Africa countries

Gene	Exon	Codon	Amino acid	Nucleotide	Protein	Morocco	Tunisia	Algeria	Lybia
KRAS	2	12	G12D	c.35G > A	p.Gly12Asp	19 (24.7%) [18] 7 (14.3%) [20] 9 (50%) [23] 7 (29.2%) [21] 11(50%) [22]	12(25.5%) [24] 17(19.3%) [25] 22(42.3%) [26] 8(50%) [27] 5(41.7%) [29]	14(43.7%) [33]	6(46.1%) [34]
KRAS	2	12	G12V	c.35G > T	p.Gly12Val	17 (22.1%) [18] 18 (36.7%) [20] 3(16.7%) [23] 10(41.7%) [21] 2(9.1%) [22]	12(25.5%) [24] 25(28.4%) [25] 13(25%) [26] 3(25%) [29]	3(9.4%) [33]	4(30.8%) [34]
KRAS	2	12	G12C	c.34G > T	p.Gly12Cys	9 (11.7%) [18] 4 (8.2%) [20] 1 (4.2%) [21] 2(11.1%) [23] 3(13.6%) [22]	3(6.4%) [24] 12(13.6%) [25] 4(7.7%) [26] 1(6.2%) [27] 1(8.3%) [29]	2(6.2%) [33]	2(15.4%) [34]
KRAS	2	12	G12S	c.34G > A	p.Gly12Ser	4 (8.2%) [20] 1(5.6%) [23]	2(4.3%) [24] 17(19.3%) [25] 2(12.5%) [27]	2(6.2%) [33]	na
KRAS	2	12	G12A	c.35G > C	p.Gly12Ala	5 (6.5%) [18] 3(6.1%) [20] 1 (4.2%) [21] 2(9.1%) [22]	8(17%) [24] 2(12.5%) [27] 1(8.3%) [29]	8(25%) [33]	na
KRAS	2	12	G12R	c.34G > C	p.Gly12Arg	2 (2.6%) [18] 1(2.0%) [20] 2 (8.33%) [21]	3(6.4%) [24] 1(1.1%) [25] 4(7.7%) [26]	na	na
KRAS	2	13	G13D	c.38G > A	p.Gly13Asp	13(16.9%) [18] 7 (14.3%) [20] 3 (12.5%) [21] 3(16.7%) [23] 4(18.2%) [22]	7(14.9%) [24] 12(13.6%) [25] 9(17.3%) [26] 3(18.7%) [27] 2(16.7%) [29]	3(9.4%) [33]	1(7.7%) [34]
KRAS	2	13	G13V	c.38G > T	p.Gly13Val	2(2.6%) [18]	na	na	na
KRAS	2	13	G13R	c.37G > C	p.Gly13Arg	1(1.3%) [18]	na	na	na
KRAS	2	13	G13C	c.37G > T	p.Gly13Cys	1(2.0%) [20]	na	na	na
KRAS	3	59	A59T	c.175G > A	p.Ala59Thr	1(2.0%) [20]	na	na	na
KRAS	3	59	A59G	c.176C > G	p.Ala59Gly	na	na	na	na
KRAS	3	61	Q61H	c.183A > C	p.Gln61His	na	na	na	na
KRAS	3	61		c.183A > G		1(2.0%) [20]	na	na	na
KRAS	3	61	Q61L	c.182A > T	p.Gln61Leu	1(1.3%) [18] 1(2.0%) [20]	na	na	na
KRAS	3	61	Q61R	c.182A > G	p.Gln61Arg	na	na	na	na
KRAS	3	61	Q61E	c.181 C > G	p.Gln61Glu	na	1(1.1%) [25]	na	na
KRAS	4	117	K117N	c.351A > C	p.Lys117Asn	3(3.9%) [18]	1(1.1%) [25]	na	na
KRAS	4	146	A146T	c.436G > A	p.Ala146Thr	3(3.9%) [18] 1(2.0%) [20]	21(2.3%) [25]	na	na
KRAS	4	146	A146P	c.436G > C	p.Ala146Pro	1(1.3%) [18]	na	na	na
KRAS	4	146	A146V	c.437C > T	p.Ala146Val	1(1.3%) [18]	na	na	na
NRAS	2	12	G12S	c.34G > A	p.Gly12Ser	na	1(11.1%) [25]	na	na
NRAS	2	12	G12C	c.34G > T	p.Gly12Cys	na	na	na	na
NRAS	2	12	G12R	c.34G > C	p.Gly12Arg	na	na	na	na
NRAS	2	12	G12D	c.35G > A	p.Gly12ASP	2(33.3%) [18]	2(22.2%) [25]	na	na
NRAS	2	12	G12V	c.35G > T	p.Gly12Val	na	na	na	na
NRAS	2	12	G12A	c.35G > C	p.Gly12Ala	na	na	na	na
NRAS	2	13	G13S	C.37G > A	p.Gly13Ser	na	na	na	na
NRAS	2	13	G13C	c.37G > T	p.Gly13Cys	na	na	na	na
NRAS	2	13	G13R	c.37G > C	p.Gly12Arg	na	na	na	na
NRAS	2	13	G13D	c.38G > A	p.Gly13Asp	na	na	na	na

Table 5 (continued)

Gene	Exon	Codon	Amino acid	Nucleotide	Protein	Morocco	Tunisia	Algeria	Lybia
NRAS	2	13	G13V	c.38G > T	p.Gly13Val	na	na	na	na
NRAS	2	13	G13A	c.38G > C	p.Gly13Ala	na	na	na	na
NRAS	3	59	A59T	c.175G > A	p.Ala59Thr	na	na	na	na
NRAS	3	59	A59G	c.176 C > G	p.Ala59Gly	na	na	na	na
NRAS	3	61	Q61K	c.181C > A	p.Gln61Lys	2(33.3%) [18] 3(50%) [20]	1(11.1%) [25]	na	na
NRAS	3	61		c.181A > G		1(16.7%) [20]	na	na	na
NRAS	3	61	Q61R	c.182A > G	p.Gln61Arg	2(33.3%) [20]	na	na	na
NRAS	3	61	Q61L	c.182A > T	p.Gln61Leu	2(33.3%) [18]	na	na	na
NRAS	3	61	Q61H	c.183A > T	p.Gln61His	na	3(33.3%) [25]	na	na
NRAS	3	61	Q61E	c.181C > G	p.Gln61Glu	na	na	na	na
NRAS	4	117	K117N	c.351G > C	p.Lys117Asn	na	1(11.1%) [25]	na	na
NRAS	4	117	K117N	c.351G > T	p.Lys117Asn	na	na	na	na
NRAS	4	146	A146T	c.426G > A	p.Ala146Thr	na	1(11.1%) [25]	na	na
NRAS	4	146	A146V	c.437C > T	p.Ala146Val	na	na	na	na
BRAF	15	600	V600E	1799 T > A	p.Val600Glu	2(3.9%) [19] 5(5.4%) [22] 1(1.6%) [23]	4(8.3%) [31]	5(4.9%) [33]	na

na not available

patient. *NRAS* mutations were more common in codon 61, accounting for 48.6%. The most common mutations were G12D in exon 2 and Q61K in exon 3 and accounted for 40% and 35.3% of CRC patients, respectively. In *BRAF* gene, the overall frequency of the *BRAF* V600E mutation was 2.8%.

Our review of mutation prevalence among CRC patients in North Africa identified a significant difference in prevalence of both *RAS* (*KRAS* and *NRAS*) and *BRAF* mutations. Interestingly, our finding showed that the *KRAS* mutation rate was different with more investigation that therefore the geographic location and ethnicity/race can impact on the prevalence of *KRAS* mutation in CRC patients. The *KRAS* mutation frequency was 23.9% to 51% in a Moroccan population [18–23], 23.1% to 68.2% in a Tunisian population [24–30], 31.4% to 50% in an Algerian population [32, 33], and 38.2% in a Libyan population [34]. This broad range of reported *KRAS* mutation frequencies may be related with diverse ethnic background. Arabs and Berbers make up the overwhelming majority of the population throughout the North Africa today. However, throughout its history, North Africa has been the site of invasions and migratory waves of ethnic groups such as Phoenicians, Romans, Vandals, Arabs, Ottomans and Europeans.

Worldwide, a persistent finding in the literature is the substantial variation in *KRAS* mutation frequency between race/ethnic groups. In Nigerian, the frequency of CRC with *KRAS* mutations is 21% [38]. In Asian populations, *KRAS* mutations vary from 37.9% (Japan) to 52.7% (China) [39, 40], while Middle Eastern population reported frequencies of 32% (Saudi Arabia) and 48% (Iraq) [41, 42]. Data from USA showed *KRAS* mutation rate slightly higher among African American compared to Caucasians (47% versus 43%) [43]. Studies performed in Latin America report *KRAS* mutation prevalence ranging from 13% (Colombia and Venezuela) to 40% (Chile) [44]. The European data showed mutation rate in Greece (29%), Netherland (37%), Germany (39%) and Slovenia (46%), Spain (48%) [43, 45, 46]. In Turkey, the mutation frequency for the *KRAS* mutation gene has been reported to be in the range of 11% to 49.1% [47–51]. Turkey is known from the geographical location between Europe, the Middle East and the Caucasus region. Thus, Turkey is comprised of many ethnic groups with European, Middle Eastern, Caucasian or Asian origins. This difference can be primarily attributed to ethnicity [51]. The *KRAS* mutation prevalence may also vary in the same population. Studies from Japan with homogeneous ethnic groups found a wide range of *KRAS* mutation frequencies (9–71%) [52, 53]. Studies with heterogeneous ethnic groups, such as studies from USA, also reported various frequencies of *KRAS* mutation (14–83%) [54, 55]. The finding of novel *KRAS* and *BRAF* gene mutations in cancerous tissue obtained from Saudi CRC patients were typical of those observed elsewhere, and may be attributed to environmental factors and/or racial/ethnic variations due to genetic differences in Saudi Arabia [56, 57].

In agreement with COSMIC, G12D and G12V mutation frequencies were the most frequent mutations

among all CRC patients with *KRAS* mutation in North Africa. However, there are important differences among North Africa populations. In Morocco and Tunisia there is a higher prevalence of G12D mutation (≈50%) [22, 27]. G12D mutation accounts for about 41% of all the G12 mutations [58, 59]. Generally, *KRAS* mutations are predominantly found in codon G12 and mutations in codon 12 diminish both inherent and GAP-mediated hydrolysis without affecting the rate of nucleotide exchange, except for G12C, which exhibits GTPase activity similar to that of wild type [60]. G12C has become a promising target for novel strategies to treat *KRAS*-mutant CRC [61], adding up a new role for routine *KRAS* testing. A series of novel inhibitors that act against G12C in its GDP-bound state have been developed (such as Sotorasib and Adagrasib) and provides new therapeutic strategies to improve patient outcomes.

The aspects that seem to play an important role in the incidence of *KRAS* gene mutations in CRC in North Africa populations are the change in dietary patterns and nutrient intakes. In Morocco, The major pattern of nutritional change includes a large increase in the consumption of high calorie diets and fatty foods. Consumption of high amounts of these foods was associated with increased body weight, BMI, and risk of overweight and obesity. Between 2001 and 2014, the consumption of high-fat diets increased following the intake of oils which increased on average by 5.4 L (22.4 L against 17.02 L per person per year) [62]. Significant associations were found between the highest intakes of red meats, cold meats, sausages and the risk of CRC in a case–control study on dietary risk factors for CRC in Morocco [63]. High levels of animal protein, acrylamide foods, and low levels of vitamin A consumption have been shown to be associated with increased risk of CRC tumors with *KRAS* mutations [64]. Polyunsaturated fatty acids (PUFA) may be positively associated with CRC risk by potentially generating G > A transitions in the *KRAS* oncogene in Moroccan population. El Asri et al., showed that an increase in the consumption of PUFA above 16.9 g/day was associated with an increase in the presence of *KRAS* mutations (Odds Ratio OR = 2.48, 95% Confidence Interval CI = 1.22–4.96) as compared to the reference group whose consumption was less than 16.9 g [65]. A high intake of PUFA, in particular linoleic acid, may be an important dietary risk factor for *KRAS* mutated colon tumors, possibly by generating G > A transitions or G > T or G > C transversions in the *KRAS* oncogene [66]. S Deoula et *al.*, showed that consumption of red meat was positively associated with colon cancer (OR = 1.23, 95% CI = 1.05–1.44) and CRC risk in Moroccan patients (OR = 1.14, 95% CI = 1.02–1.27) [67]. In Tunisia, the CRC incidence has increased markedly from 1994 to 2009, and it is suggested that if no interventions are implemented it is going to double by 2024. It is relevant to highlight that an upward trend in obesity largely explains the increasing incidence of CRC in Tunisian population. The trend of the incidence of obesity had increased from 10.9% in 1998 to 26.9% in 2016 [68] and 80% of Tunisian aged over 15 years old did not consume enough fruits and vegetables per day [69]. A high total day meat consumption (> 100 g) was significantly associated with a high risk of CRC compared to low consumption (< 50 g) in Tunisian population [70]. The growth of meat consumption is likely to increase in Morocco and Tunisia. Between 1971 and 2020, total production of meat of Morocco grew substantially from 205,387 to 1.45 million tonnes rising at an increasing annual rate that reached a maximum of 23.12% in 1987 and then decreased to 2.70% in 2020. Production of meat of Tunisia increased from 19,400 thousand tonnes in 1971 to 41,600 thousand tonnes in 2020 growing at an average annual rate of 2.19%. In Algeria, occupational exposures showed a significant link with an increased risk of CRC, as did obesity, alcohol consumption, and passive smoking. Yogurt, cereals, sugar, butter, and margarine consumption were significant protective factors, while cheese, dried fruits, red meat, juice, and fizzy drink consumption was associated with increased CRC risk [71]. The World Health Organization (WHO) and International Agency for Research on Cancer (IARC) declared that over-intake of red meat and/or processed meat increases CRC risk [72]. A recent Global Health Data Exchange review indicated that red and processed meat intake accounts for 1.77% and 1.18%, respectively, of worldwide CRC mortality, respectively [73]. The World Cancer Research Fund International Continuous Update Project showed that red meat or processed meats and alcohol consumption increase CRC risk and that food containing dietary fibre and dairy products decrease the CRC risk [74].

Red meat consumption may increase colon cancer risk by inducing the endogenous production of N-nitroso compounds and their precursors, which may induce *KRAS* mutations [70, 71]. Analysis of 900 CRCs with whole exome sequencing and epidemiologic annotations revealed an alkylating mutational signature that was associated with red meat consumption and distal tumor location, as well as predicted to target *KRAS* G12D/G13D [72]. Heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) formed during high-temperature meat cooking are also an important pathway for environmental colon cancer [73, 74]. HCAs and PAHs have been found to be mutagenic after

they are metabolized by specific enzymes in the body. Previous studies have identified that the activity of these enzymes may be relevant to the cancer risks associated with exposure to these compounds [75, 76].

Interestingly, the use of antibiotics such as Fluoroquinolone and the third-generation cephalosporins was increasing in North Africa [77]. An increasing of antibiotic consumption was associated with an increasing CRC risk, particularly when used commonly [78] and colon cancer pathogenesis across all age-groups in a large population-based case–control study of patients with early onset-CRC [79]. Antibiotics are known to influence the gut microbiome [80, 81], which has been implicated in CRC or tumor progression, possibly through bacterial involvement in nutrient metabolism and direct interaction with gut mucosa [82]. In the context of CRC, bacteria have been shown to play a role in cell signaling [83–85]. In a *KRAS*-specific context, the role of *KRAS* signaling on the tumor microbiome is still to be determined, even though, it is accepted that the exposure to bacterial can shape the development of CRC of which *KRAS* can be one major player [86, 87]. Nevertheless, in *KRAS*-driven CRCs, it has been shown that genotoxic stress and some other factors, including metabolites produced by the microbiota, can facilitate genetic and epigenetic changes leading to carcinogenesis [88]. A priori, computational modeling is a promising approach for studying genotype-to-metabolic-phenotype relations or microbe–microbe and host–microbe metabolic interactions in CRC [89]. Microbiome modulation is one of the most prospective new strategies in medicine to improve the gut health in individuals and is likely to play a key role in the outcome of colorectal surgery in North Africa.

Modifiable dietary and lifestyle risk factors could prevent most cases of CRC and. Up to 47% of CRC cases could be prevented by staying physically active, maintaining a healthy body weight and eating a healthy diet [90]. In North Africa, dietary and lifestyle factors are one of the most promising approaches to reduce the occurrence and progression of CRC. Dietary and lifestyle factors may not only play a role in causing mutations and epigenetic modifications, but also in enhancing tumor growth in tissues that have already acquired specific epigenetic aberrations. There may be direct causal associations between diet and lifestyle factors and molecular changes in CRC, and establishing this is important for prevention strategies, and increasing the ability to better predict disease progression and prognosis [91].

Beside established anti-CRC treatment, better understanding of the causality of CRC can be established by combining epidemiology and genetic/epigenetic on CRC etiology. This approach may be able to significantly reduce the burden of disease in North Africa population. Furthermore, the government should develop policy on CRC prevention and public health programs which may serve as a feasible setting to increase public awareness on lifestyle risk factors.

Abbreviations

ASCO: American Society of Clinical Oncology; ASR: Age-Standardized Incidence Rates; BMI: Body Mass Index; CI: Confidence Interval; COSMIC: Catalogue Of Somatic Mutations In Cancer; CRC: Colorectal Cancer; EGFR: Epidermal Growth Factor Receptor; ERK: Extracellular-signal-Regulated Kinase; GDP: Guanosine Bi-Phosphate; GLOBOCAN: Global Cancer Observatory: CANCER TODAY; GTP: Guanosine Tri-Phosphate; HCAs: Heterocyclic Amines; HDI: High Human Development Index; HRAS: Harvey Rat Sarcoma; IARC: International Agency for Research on Cancer; KRAS: Kirsten Rat Sarcoma; MAPK: Mitogen-Activated Protein kinase; MEK: Mitogen-Activated Protein kinase kinase; NRAS: Neuroblastoma Rat Sarcoma; OR: Odds Ratio; PAHs: Polycyclic Aromatic Hydrocarbons; PUFA: Polyunsaturated fatty acids; RAF: Rapidly Accelerated Fibrosarcoma; RAS: Rat Sarcoma; SSA: Sub-Saharan Africa; UAE: United Arab Emirates; USA: United States of America; WHO: World Health Organization.

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Authors' contributions

MJ, AL, and WB have conceived the study, exploited data, coordinated and drafted the paper. SB and SEZ participated in the designed. TM, HEA, RA, RT, HEH, LB and, SE generated data and involved in data analyses. AAA, AB, MM, MRT, YS, IAL, MI, AEN, SEK, and AB have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the [Prevalence and Patterns of Mutations in RASRAFMEKERKMAPK Signaling in North Africa Tables] repository.Pub med (Open access): https://pubmed.ncbi.nlm.nih.gov/. Science Direct (Open access): https://www.sciencedirect.com/. Google Scholar (Open access): https://scholar.google.com/.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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Publisher's Note

Discussion
Conclusion

VII. Discussion

Colorectal cancer poses a significant global health burden due to its high morbidity and mortality rates. It stands as a leading cause of cancer-related deaths worldwide, with millions of new cases and reported deaths annually *(Xi and Xu 2021)*.

Managing CRC presents a challenge primarily due to its high mortality rate. This is largely attributed to its aggressive metastatic potential and often late-stage diagnosis. The 5-year survival rate for CRC, while showing improvement in recent years, remains relatively low, hovering around 65% across all stages. However, it is crucial to note that the survival outlook varies significantly depending on the tumor stage at the time of diagnosis. The stage at diagnosis is one of the most critical predictors of survival, with 5-year relative survival rates ranging from a promising 91% for localized disease to a more concerning 14% for cases with distant metastasis *(Siegel et al., 2023)*.

Advanced stage (III and IV) diagnoses of CRC still prevail, accounting for about 35% and 21% of cases, respectively. This underscores the urgent need for enhanced screening methods and early detection strategies to improve treatment success rates and patient outcomes *(AlZaabi et al., 2022)*.

The development of CRC is a complex multistep process involving numerous genetic alterations and disrupted signaling pathways. These genomic aberrations play a central role in promoting oncogenic phenotypes, resulting in uncontrolled cellular growth and tumor formation. Key oncogenes and tumor suppressor genes frequently implicated in CRC include TP53, KRAS, PIK3CA, and BRAF, among others *(Hasbullah and Musa 2021)*.

CRC arises from a sequence of genetic and epigenetic changes that drive the transformation of normal colonic epithelial cells into malignant tumors. Among the key genetic alterations, mutations in the RAS gene and the BRAF gene have emerged as critical drivers of CRC pathogenesis.

The choice to study the RAS gene in the context of CRC research is driven by its central role in CRC pathogenesis. The RAS gene encodes GTPase proteins crucial for regulating cell growth, differentiation, and survival. Mutations in RAS genes lead to constitutive activation of RAS proteins, resulting in aberrant signaling and uncontrolled cell proliferation. Understanding the genetic alterations within the RAS gene is of paramount importance due to several factors. First, RAS mutations are among the most frequent oncogenic events in CRC, with a prevalence of approximately 40-50% of cases. These mutations are associated with aggressive disease progression, resistance to targeted therapies, and poorer patient outcomes. Investigating the landscape of RAS mutations can provide valuable insights into the underlying mechanisms of

CRC development, metastasis, and therapeutic resistance. Moreover, the choice to study RAS mutations is underpinned by their clinical relevance. The presence of RAS mutations in CRC patients can significantly impact treatment decisions. For example, the mutation status of the RAS gene affects the response to anti-epidermal growth factor receptor (EGFR) therapies, a class of targeted treatments commonly used in CRC management. Patients with RAS mutations are less likely to respond to these therapies, making accurate mutation detection essential for personalized treatment strategies. *(Mesti et al., 2023)*.

The most prominent mutations in KRAS, exceeding 90% of all KRAS mutations, are those at codon 12 (G12A, G12V, G12S, G12R, G12C, and G12D). Subsequent mutations affecting codon 13 (G13D and G13C), codon 61 (Q61L, Q61R, Q61H), codon 146 (A146T, A146V, A146P), and codon 117 (K117N) also contribute to the spectrum of KRAS mutations found in CRC (*Serebriiskii et al., 2019)*. Among these mutations, those in exon 2 are the most frequent in CRC, with glycine 12 (G12) mutations exhibiting the highest prevalence and diversity. Notably, G12D stands out as the most common, accounting for approximately 41% of all G12 mutations, followed closely by G12V and G12C at approximately 28% and 14%, respectively *(Meng et al., 2021)*.

Within the intricate landscape of CRC genetics, the choice to investigate the RAS gene is underscored by its connection to downstream effectors like the BRAF gene. BRAF functions as a crucial component of the MAPK signaling pathway, regulating vital processes such as cell proliferation and survival. Mutations in the BRAF gene are present in around 5-15% of CRC cases, particularly the V600E mutation, lead to the constitutive activation of BRAF protein, promoting tumor growth and exacerbating the oncogenic potential of CRC. Importantly, BRAF mutations are intrinsically linked to a distinct CRC subtype characterized by microsatellite instability (MSI), which in turn impacts disease prognosis and treatment response. Understanding the interplay between RAS and its downstream targets, like the BRAF gene, is vital for unraveling the intricate molecular mechanisms underlying CRC pathogenesis *(Caputo et al., 2019)*.

In recent years, significant progress has been made in the development of targeted therapies and treatment inhibitors for CRC. These therapies aim to specifically target the molecular and genetic alterations driving tumor growth, thereby offering more effective and potentially less toxic treatment options. Some of the notable treatment inhibitors used in the management of CRC include EGFR inhibitors, BRAF inhibitors, anti-VEGF therapy, and immune checkpoint inhibitors. Early detection through regular screening and lifestyle modifications can further contribute to improved outcomes. However, challenges remain,

especially in cases with advanced disease and resistance to existing treatments. Further research, including investigating novel treatment combinations and expanding the use of immunotherapy, is vital to improving patient outcomes and ultimately reducing the impact of CRC on global health *(Shuford et al., 2020)*. Additionally, promoting preventive measures and public health initiatives will be essential in reducing the incidence and burden of this disease.

To comprehensively elucidate the landscape of KRAS mutations in CRC, it becomes imperative to consider regional variations and their connections to demographic and clinicopathological factors. Our two studies, conducted in North Africa and the Middle East, contribute valuable insights into the prevalence and patterns of KRAS mutations within these specific geographic regions. By dissecting the mutation frequencies and specific alterations observed in these regions, we shed light on the interplay of genetic and environmental factors in shaping the mutation landscape of CRC.

The comprehensive examination of mutations within the RAS/RAF/MEK/ERK/MAPK signaling pathway in CRC across the North African region provides valuable insight into the complex interplay between genetics, ethnicity, and environmental factors. The diverse mutation frequencies identified in various North African countries highlight the potential impact of local dietary habits and antibiotic consumption on CRC pathogenesis. Our study not only sheds light on the specific mutational patterns within this pathway but also emphasizes the need to consider both genetic and environmental factors when understanding CRC development.

The analysis of mutations within the RAS/RAF/MEK/ERK/MAPK pathway in North African CRC patients has yielded compelling insights into the genetic landscape of the disease. Notably, KRAS mutations were detected in 41.7% of the CRC tumors, with distinct distribution across exons. Within KRAS exon 2, the G12D mutation emerged as the most frequently identified mutation. Moreover, NRAS mutations were identified in 3.2% of tumor samples, predominantly in exons 2 and 3. In contrast, BRAF mutations were less prevalent, with the V600E mutation being the most commonly observed.

A notable strength of this study is its contextualization within the broader global landscape of CRC mutations. Comparison of mutation frequencies across diverse populations highlights the significant influence of ethnicity on the prevalence of specific mutations. In North Africa, the prevalence of KRAS mutations ranged from 23.9% to 51%, with variability attributed to diverse ethnic backgrounds. This finding aligns with global studies that have reported substantial variation in KRAS mutation frequencies among different racial and ethnic groups. For instance, Asian populations exhibited mutation frequencies ranging from 37.9% (Japan) (*Kawazoe et al., 2015)* to 52.7% (China) *(Fang et al., 2018)* whereas the Middle Eastern

population showed frequencies of 32% (Saudi Arabia) (Zekri et al., 2012) to 48% (Iraq) *(Al-Allawi et al., 2012)*. These disparities emphasize the intricate interplay between genetics, ethnicity, and CRC susceptibility.

The study's focus on North Africa also underscores the role of dietary and environmental factors in CRC pathogenesis. Changes in dietary habits, including increased consumption of high-calorie and fatty foods, have been linked to CRC risk. Notably, consumption of red meat, processed meats, and high-fat diets has shown associations with an elevated risk of CRC harboring KRAS mutations. The increased use of antibiotics in the region has also been implicated in CRC risk, with the gut microbiome potentially mediating the interaction between antibiotics, mutations, and CRC development *(Mint Sidi Deoula et al., 2020).*

Meanwhile, the Middle East study brings to light a distinct mutation pattern characterized by the prominence of G12D mutation, among others. Intriguingly, the prevalence of KRAS mutations in the Middle East seems to be higher than in Caucasians but lower than in Asians, further accentuating the intricate interplay of genetic and environmental factors in CRC pathogenesis. The Middle East study's findings echo those from North Africa, underscoring the significance of G12D and G12V mutations, which are consistent with results from broader populations. Importantly, these findings not only expand our knowledge of the mutation patterns in the RAS/RAF/MEK/ERK/MAPK pathway but also emphasize the need to consider both genetic and environmental factors in unraveling the complexities of CRC development.
The findings reveal a KRAS mutation frequency of 37.09%, aligning closely with reported data from various Asian countries like China, Japan, and Taiwan, as well as from Western countries such as the United States, France, and the United Kingdom. However, variations emerge when comparing these results with those from Germany, Italy, Turkey, India, Pakistan, Morocco, Egypt, Thailand, and Korea, highlighting the substantial global heterogeneity in KRAS mutation frequencies.

Comparing the results from the Middle East with those obtained from North Africa, intriguing disparities come to light. In the Middle East, the prevalence of KRAS mutations was 37.09%, while in North Africa, it was higher at 41.7%. The distribution of specific mutations also exhibited variations, with G12D and G12V being the most common in both regions. Additionally, NRAS mutations were found in 3.3% of tumor samples in the Middle East and 3.2% in North Africa. These observations highlight the dynamic nature of mutation landscapes and their potential regional differences.

Furthermore, the prevalence of the BRAF V600E mutation in the Middle East was 2.6%, consistent with results obtained from North Africa, where it was also 2.8%. The frequency of

BRAF mutations varies widely across different populations, with the lowest incidence observed in Taiwan (1%) *(Hsieh et al., 2012)* and the highest in the Netherlands and the USA (19.8% and 21.8%, respectively) *(Clancy et al., 2013)*. This convergence suggests that specific mutation patterns might transcend regional boundaries, indicating potential common molecular pathways in CRC pathogenesis.

Our third paper significantly advances the discourse by delving into the prevalence, distribution, and clinical implications of KRAS and NRAS mutations within the context of Moroccan CRC patients. This study stands as a pivotal link between the intricate landscape of molecular alterations and their potential clinical ramifications. Notably, an astounding discovery emerges from our comprehensive dataset, encompassing a cohort of 80 CRC patients, wherein the prevalence of RAS mutations reaches a substantial 57.5%. This finding marks a distinct departure from the observations of prior Moroccan studies, underscoring the significance of our research.

The underlying driver behind this remarkable insight rests in our meticulous choice of mutation detection methodologies. To enhance our ability to capture these genetic aberrations with heightened precision, we embraced the cutting-edge Pyrosequencing technology. Celebrated for its exceptional sensitivity, Pyrosequencing empowers us to detect even the subtlest mutations embedded within the RAS gene. More than that, its real-time quantitative capabilities equip us with a powerful tool to decipher complex genetic alterations, a trait particularly valuable when dealing with clinical samples *(Shen and Qin, 2012)*.

Pyrosequencing's proficiency in handling degraded DNA a recurring challenge in formalin-fixed paraffin-embedded (FFPE) tissues – constitutes a game-changer in our study. The DNA degradation that often plagues FFPE specimens can compromise the quality and quantity of extracted genetic material, consequently impacting downstream analyses. Our successful application of Pyrosequencing to these samples not only circumvents this hurdle but also unlocks a wealth of accurate mutation data that might otherwise remain elusive.

By harnessing the precision and sensitivity intrinsic to Pyrosequencing, our investigation achieves a profound understanding of the distribution and prevalence of RAS mutations within our cohort. This understanding, however, gains further depth when contextualized within the broader landscape of existing research. When comparing our results with those of other studies, both within Morocco and across North Africa, intriguing patterns emerge.

A striking trend becomes apparent when juxtaposing our findings against the backdrop of previous Moroccan studies. The prevalence of KRAS and NRAS mutations within our cohort

surpasses the ranges reported by earlier investigations. In particular, our observed rates of KRAS mutations (56.2%) and NRAS mutations (8.8%) stand notably higher than those documented in prior Moroccan research. The disparities between these figures underscore the need for methodological precision, as well as the influence of sample quality, in achieving accurate mutation assessment. No BRAF gene alterations were found.

Noteworthy are the insights derived from examining mutation patterns within specific exons. In our study, the majority of KRAS gene mutations cluster within exon 2, with the most frequent mutation being G12D, followed by G12C, G12S, and G12V. Similarly, our observations regarding NRAS mutations indicate a predilection for exon 2, with G13A and Q61H mutations being the most prevalent. These findings align with previous Moroccan studies in some aspects, while diverging in others.

As we broaden our horizon to encompass the North African region, additional intriguing observations unfold. Algeria, for instance, exhibits variations in KRAS mutation prevalence, ranging from 31.3% to 51.2% among CRC patients (Boudida-Berkane et al., 2016; Mazouzi et al., 2017). Notably, exon 2 mutations remain dominant, with codon 12 mutations – especially G12D – being the most frequent. A similar trend emerges in Tunisia, where KRAS mutation frequencies span 23.1% to 75.2%, primarily concentrated within exon 2. Moreover, Tunisian studies point to an intricate interplay between KRAS and NRAS mutations, emphasizing the complex mutational landscape *(Sammoud et al., 2012; Karim et al., 2011; Ines et al., 2014; Jouini et al., 2019; Aissi et al., 2013; Ouerhani et al., 2013).*

The scenario is no less intriguing in Libya, where KRAS codon 12/13 mutations representing 38.2% , are present in a significant proportion of CRC patients. G12D and G12V dominate codon 12, while G13D prevails in codon 13. These variations not only highlight the regional diversity in mutation profiles but also underscore the complex interplay of genetic factors that contribute to CRC susceptibility *(Aboudabous et al., 2021).*

Beyond North Africa, our findings echo the global trend of variable mutation frequencies across different races and ethnicities. For instance, while Non-Hispanic Black patients exhibit a higher KRAS mutation rate, Non-Hispanic White patients demonstrate a different pattern. This interplay of genetic factors and race emphasizes the need to consider ethnicity in deciphering the molecular underpinnings of CRC *(Bien et al., 2021).*

Yet, amidst these diverse insights, a recurring theme emerges – the interplay between lifestyle and dietary habits. Our study draws attention to the potential links between the incidence of KRAS gene mutations and lifestyle factors, such as sedentary behavior, dietary choices, and nutritional intake. The rise of high-calorie diets, increased consumption of fatty

foods, and shifts in dietary habits form a backdrop against which we observe the elevated prevalence of KRAS mutations *(Dolatkhah et al., 2018)*.

To sum up, CRC represents a formidable global health challenge due to its distressing morbidity and mortality rates. The urgency to unveil its intricate mechanisms is driven by the gravity of its impact on public health. Genomic anomalies, particularly those manifesting within the RAS and BRAF genes, occupy a central role in the intricate tapestry of CRC pathogenesis. These genetic aberrations wield substantial influence over disease progression trajectories and determine the efficacy of therapeutic interventions.

The realm of targeted therapies emerges as a beacon of hope, holding the promise of more precise and effective treatment options. However, the road to successful management remains riddled with obstacles, notably the formidable challenge of treatment resistance. The dynamic interaction between genetic mutations, signaling pathways, and the evolving tumor microenvironment creates a complex landscape that necessitates continuous adaptation in therapeutic approaches.

The comprehensive scrutiny of the prevalence and distribution of KRAS and NRAS mutations across diverse geographical regions forms an essential pillar of our research agenda. This endeavor not only broadens our comprehension of the molecular panorama underpinning CRC but also underscores the undeniable influence of genetic and environmental factors in shaping disease profiles. The utilization of cutting-edge techniques like pyrosequencing empowers us with the precision and sensitivity required to unravel the intricacies of these mutations, even in the context of degraded DNA samples.

By exploring the intricate changes in genes and using advanced technologies, our research aims to better understand the genetics of CRC. This understanding helps us create personalized treatments that match each person's unique genetic makeup. As we venture into the unknown aspects of CRC genetics, we're not just learning more about science we're also finding new ways to help patients feel better. Our combined efforts are geared towards lessening the challenges posed by CRC, improving the lives of many individuals, and making a positive impact on global health. As we move forward, it is advisable to conduct future studies using larger sample sizes. This will provide a clearer picture of how often the KRAS, NRAS, and BRAF genetic variations occur and how they influence the disease.

VIII. Conclusion

CRC remains a significant global health concern marked by substantial morbidity and mortality rates. Within the intricate genetic landscape of CRC, the pivotal role of RAS mutations, including those at codon 12 (G12A, G12V, G12S, G12R, G12C, and G12D) and codon 13 (G13D), comes to the forefront. These mutations drive uncontrolled cellular growth, disrupt signaling pathways, and fuel tumor formation. Notably, G12D and G13D mutations emerge as prevalent drivers, underscoring their profound influence on the development of CRC. CRC remains an unrelenting global health challenge, characterized by its pervasive impact on morbidity and mortality rates. Within the intricate genetic mosaic that underlies CRC, the pivotal role of RAS mutations takes center stage, unraveling a complex narrative of oncogenesis. Among these mutations, those occurring at codon 12 (including G12A, G12V, G12S, G12R, G12C, and G12D) and codon 13 (notably G13D) stand as formidable drivers, orchestrating uncontrolled cellular growth, distorting crucial signaling pathways, and fueling the relentless formation of malignant tumors. The prevalence of G12D and G13D mutations, eminent in this scenario, serves as a poignant testament to their profound influence in sculpting the trajectory of CRC development.

While targeted therapies present promising strategies for improved treatment, the persistent challenge of treatment resistance calls for ongoing adaptation in therapeutic approaches. The intricate interplay between genetic mutations, signaling pathways, and the evolving tumor microenvironment adds layers of complexity to CRC pathogenesis, underscoring the need for continued research and innovative solutions.

In light of these insights, it is imperative to expand the horizon of our studies. A comprehensive exploration of RAS mutations, including G12D and G13D, as well as other genetic variations like NRAS and BRAF, across diverse geographical regions should be pursued with larger sample sizes. This approach will provide a more nuanced understanding of the prevalence, distribution, and clinical implications of these mutations, thereby paving the way for tailored and personalized treatment strategies. By delving deeper into the intricate genetic tapestry of CRC, we can collectively strive to mitigate the challenges posed by this disease, making a meaningful impact on global health.

IX. Perspectives

Looking into the future, CRC research presents exciting directions for exploration, particularly concerning changes in the RAS and BRAF genes. It's essential to conduct large-scale studies encompassing diverse populations. This will help us understand how common these gene mutations are and how they function. Ultimately, it can lead to personalized treatment strategies that suit each patient's unique situation.

A crucial challenge is uncovering why certain treatments become ineffective, especially when it comes to targeted therapies. By delving into the reasons behind treatment resistance, we can develop innovative combinations of therapies. This approach has the potential to significantly improve patient outcomes.

However, our focus extends beyond gene mutations alone. We must also investigate the dynamic relationship between these gene changes, such as RAS and BRAF mutations, and the surrounding tumor environment. This exploration may open doors to fresh and improved approaches to cancer treatment. In our pursuit of understanding these gene changes, advanced tools like pyrosequencing have emerged as invaluable assets. They enable accurate detection of mutations within the RAS gene, providing us with precise insights.

Furthermore, a comprehensive analysis of genes and other alterations, empowered by these advanced techniques, holds the promise of revealing new biomarkers and targets for therapeutic intervention. The bridge between foundational discoveries and practical application in clinical settings is crucial. This connection ensures that the knowledge gained from research is translated into real benefits for patients. Prevention is a key aspect of CRC management. Prioritizing lifestyle changes and early screening initiatives can significantly lower the incidence of CRC and reduce its impact on individuals and society.

Uniting researchers globally amplifies our potential, hastening significant advancements in CRC research and treatment. The path forward involves diverse approaches: studying gene changes, utilizing advanced techniques, and promoting joint endeavors. This propels our understanding, driving effective treatments and prevention worldwide. The ultimate aim is to create a positive impact on a global scale in the realm of CRC.

X. References

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