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Molecular Deciphering of Colorectal Cancer: Exploring Molecular Classifications and Analyzing the Interplay among Molecular Biomarkers MMR/MSI, KRAS, NRAS, BRAF and CDX2 – A Comprehensive Literature Review

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Rezumat

Descrifrarea moleculară a cancerului colorectal: explorarea clasificărilor moleculare și analiza interacțiunilor dintre biomarkerii moleculari MMR/MSI, KRAS, NRAS, BRAF si CDX2 - un review de literatură comprehensiv

Introducere: Cancerul colorectal (CCR) prezintă o diversitate moleculară şi morfologică, implicând alterări genetice, epigenetice şi perturbări ale căilor de semnalizare. Înțelegerea complexității patogenetice a CCR necesită o revizuire cuprinzătoare a literaturii care să sintetizeze cercetările recente privind mecanismele moleculare, biomarkerii convenționali, precum şi cei noi descoperiți, cum ar fi CDX2.

Material and Metodă: Acest review explorează literatura ultimului deceniu şi ghidurile actuale pentru a analiza complexitățile moleculare ale CCR. Analiza se extinde dincolo de biomarkerii tradiționali pentru a-i include pe cei noi descoperiți, precum CDX2, examinând interacțiunea lor cu mecanismele carcinogenetice şi căile moleculare, alături de revizuirea metodologiilor actuale de testare.

Rezultate: O strategie multi-biomarker, care încorporează atât biomarkeri tradiționali, cât şi cei noi descoperiți precum CDX2, este crucială pentru optimizarea managementului CCR. Această

strategie elucidază interacțiunea complexă între biomarkeri şi căile moleculare tumorale, influențând semnificativ evaluarea prognosticului, a strategiilor terapeutice şi deschiderea drumului către o medicină personalizată în CCR.
Concluzii: Acest review propune CDX2 ca biomarker de prognostic în CCR şi subliniază necesitatea unei profilări moleculare amănunțite pentru individualizarea strategiilor de tratament. Prin optimizarea abordărilor terapeutice şi a modului de evaluare a prognosticului în CCR, se marchează un pas înainte către oncologia de precizie, valorificând o înțelegere mai bună a comportamentului tumoral.

Cuvinte cheie: biomarkerii cancerului colorectal, sistemul mismatch repair, instabilitatea microsatelitară, KRAS, NRAS, BRAF, CDX2, clasificarea moleculară a cancerului colorectal

Abstract
Background: Colorectal cancer (CRC) exhibits molecular and morphological diversity, involving genetic, epigenetic alterations, and disruptions in signaling pathways. This necessitates a comprehensive review synthesizing recent advancements in molecular mechanisms, established biomarkers, as well as emerging ones like CDX2 for enhanced CRC assessment.
Material and Methods: This review analyzes the last decade's literature and current guidelines to study CRC's molecular intricacies. It extends the analysis beyond traditional biomarkers to include emerging ones like CDX2, examining their interaction with carcinogenic mechanisms and molecular pathways, alongside reviewing current testing methodologies.
Results: A multi-biomarker strategy, incorporating both traditional and emerging biomarkers like CDX2, is crucial for optimizing CRC management. This strategy elucidates the complex interaction between biomarkers and the tumor's molecular pathways, significantly influencing prognostic evaluations, therapeutic decision-making, and paving the way for personalized medicine in CRC.
Conclusions: This review proposes CDX2 as an emerging prognostic biomarker and emphasizes the necessity of thorough molecular profiling for individualized treatment strategies. By enhancing CRC treatment approaches and prognostic evaluation, this effort marks a step forward in precision oncology, leveraging an enriched understanding of tumor behavior.

Key words: colorectal cancer biomarkers, mismatch repair system, microsatellite instability, KRAS, NRAS, BRAF, CDX2, colorectal cancer molecular classification

Introduction

Globally acknowledged, the importance of early colorectal cancer (CRC) detection cannot be overstated, as it substantially improves survival rates; however, these rates significantly decrease when the disease is diagnosed in stages III and IV. Present-day clinical protocols heavily rely on a multifaceted approach encompassing clinical observations, imaging techniques, and meticulous pathological evaluation of the tumor characteristics for CRC staging, prognosis determination, and informed treatment strategies. Furthermore, the recent incorporation of molecular biology into the field of oncology has brought forth several biomarkers aimed at providing a comprehensive evaluation of CRC. As outlined

in the international guidelines published by the European Society for Medical Oncology (ESMO) and the National Comprehensive Cancer Network (NCCN), the current set of biomarkers evaluated in CRC includes the assessment of the DNA Mismatch Repair (MMR) system coupled with the microsatellite instability (MSI) status, as well as the analysis of the mutational status of KRAS, NRAS and BRAF genes (1-3).

The MMR system is a cellular DNA repair mechanism responsible for correcting errors in DNA replication, maintaining genomic stability and MSI refers to genetic alterations characterized by changes in the length of repetitive DNA sequences (4-8). The MMR system is responsible for rectifying these genetic alterations; however, if this system becomes deficient (dMMR), it results in an accumulation of genetic alterations termed as high MSI (MSI-H) (4-8). Assessing the MMR/MSI status in CRC is crucial because it aids in establishing the prognosis, treatment strategy, and assessing the risk of Lynch syndrome. It serves as a reliable diagnostic, predictive and prognostic biomarker, currently recommended for evaluation via immunohistochemical analysis of the MMR protein expression (1-8).

The KRAS and NRAS genes encode proteins involved in cell signalling pathways regulating cell growth and proliferation (1-3,6). Mutations in these genes, particularly KRAS, are frequent in CRC and are associated with resistence to certain targeted therapies (e.g., anti-EGFR therapy) (1-3,6). Assessing the mutational status of these genes with the help of molecular methods such as Polymerase Chain Reaction (PCR) or Next-Generation Sequencing (NGS) techniques is pivotal in CRC assessment as it guides treatment decisions and aids in predicting patient outcomes (1-3). The BRAF gene is an essential biomarker for CRC assessment as mutations can impact prognosis, specifically the V600E mutation which is associated with poor prognosis (1-3,6). ESMO and NCCN guidelines recommend the evaluation of the BRAF gene mutational status through molecular methods (PCR or NGS) (1-3).

As per current oncological guidelines, a standardized assessment protocol has been recommended for the aforementioned biomarkers, stratified by disease stage. In this protocol, the MMR/MSI status is evaluated in stages II, III and IV of CRC, while the mutational status of KRAS, NRAS and BRAF genes is specifically analyzed in stage IV (1-3).

By integrating molecular testing into the assessment of CRC, it became evident that genetic and epigenetic heterogeneity profoundly impacts tumor behavior, thereby influencing prognosis and treatment response rates to diverse oncological treatment strategies. For this reason, the quest for novel biomarkers gained significance, leading researchers to identify various contenders such as CDX2, a transcription factor mainly found in the nuclei of intestinal epithelial cells, essential for the development and maintenance of the intestinal mucosa (9). Initially utilized as an immunohistochemical marker to determine the intestinal origin of metastases with unknown primary origin, recent studies have revealed its potential to offer valuable prognostic insights for patients diagnosed with CRC (9).

In tandem with the pursuit of novel biomarkers, scientific endeavors are directed towards developing a comprehensive molecular taxonomy of CRC, aimed at assisting clinicians in evaluating patient outcomes and devising personalized therapeutic strategies tailored to the molecular signature of the colorectal tumor (5,8,10). Currently, various molecular classifications are employed for research purposes, including the Consensus Molecular Subtypes (CMS) classification and the Colorectal Cancer Intrinsic Subtypes (CRIS) classification (5,8,10).

In this literature review, we aim to analyze in a comprehensive way the recommended biomarkers currently utilized for CRC assessment (MMR/MSI, KRAS, NRAS, BRAF), emphasizing their clinicopathological implications and interactions, testing methodology, as well as their role in the current molecular classifications of CRC. In clinical settings, the

evaluation of these biomarkers is frequently limited qualitatively (methodologically) and quantitatively (incomplete biomarker panel) due to diverse factors, including expertise, technical and/or financial constraints. Additionally, the stratified assessment of biomarker according to disease stage, coupled with the qualitative and quantitative constraints, presents difficulties in elucidating correlations between molecular and clinico-pathological parameters, which could provide a comprehensive and personalized molecular profile for each patient, usable for prognostic and predictive purposes.

We also seek to devote particular attention to the CDX2 protein and its role in colorectal carcinogenesis, as well as its impact on patient prognosis and response to treatment, with the goal of obtaining a thorough comprehension of its prospective utility as a biomarker deserving consideration in the recommended molecular profiling of CRC.

Material and Methods

The study was carried out utilizing the Google Scholar, ScienceDirect, PubMed, and Elicit databases (accessed as of February 5, 2024), employing various combinations of keywords to retrieve research articles published within the past decade addressing the subject of molecular pathology and biomarkers in CRC: "colorectal cancer" or "CRC" or "colorectal tumor", "biomarkers" or "novel biomarkers", "MSI" or "MSS" or "MMR/MSI" or "microsatellite instability", "MMR" or "Mismatch Repair", "dMMR/MSI-H" or " pMMR/MSS", "Lynch syndrome", "BRAF" or "BRAFV600E", "RAS" or "KRAS" or "NRAS", "CDX2", "signaling pathway", "molecular mechanisms", "molecular classification", "Immunohistochemistry", "PCR" or "Polymerase Chain Reaction", "NGS" or "Next Generation Sequencing", "Liquid Biopsy", "CMS" or "Consensus Molecular Subtypes", "CRIS" or "CRC intrinsic subtypes".

Furthermore, the utilization of artificial intelligence-based platforms such as Litmaps® has aided in efficiently identifying and clustering related research studies based on topics of interest, thereby simplifying the literature review process.

The inclusion criteria for this review encompassed: 1) Studies published in English; 2) Full-text studies; 3) Studies from the past 10 years; 4) Clinical studies/clinical trials; 5) Literature reviews; 6) Systematic reviews and meta-analyses; and 7) Current international guidelines in medical oncology and pathology regarding the management of colorectal cancer, such as the ones published by ESMO and NCCN. The exclusion criteria consisted of: 1) Studies outside the scope of interest of this review; and 2) Case presentations.

By using the designated search platforms and applying the aforementioned inclusion and exclusion criteria, we have identified 70 full-text studies published in English between 8 December, 2017 and January 23, 2024, encompassing original research articles, literature reviews, systematic reviews, meta-analyses, and pertinent international guidelines concerning the researched subject (*Fig. 1*). The synopses of these studies are outlined in the subsequent sections.

Results

The MMR and Microsatellite Instability Status in Colorectal Cancer

Exploring the Relationship between the MMR System and Microsatellite Instability

Microsatellite regions, also known as STR (Short Tandem Repeats) or SSR (Simple Sequence Repeats), are scattered throughout the human genome (approximately 3%) and consist of repetitive short sequences of DNA bases (ranging from 1 to 5 nucleotides in length), located in the chromosome telomeres of both coding and non-coding DNA regions (4-8). These errors that occur in the telomer region, represented by insertion-deletion loops or mispairing of bases, are corrected by the MMR (Mismatch Repair) system during DNA replication (4-8). The cellular MMR system, consisting of protein complexes (MLH1-PMS2 and MSH2-MSH6), is tasked with rectifying

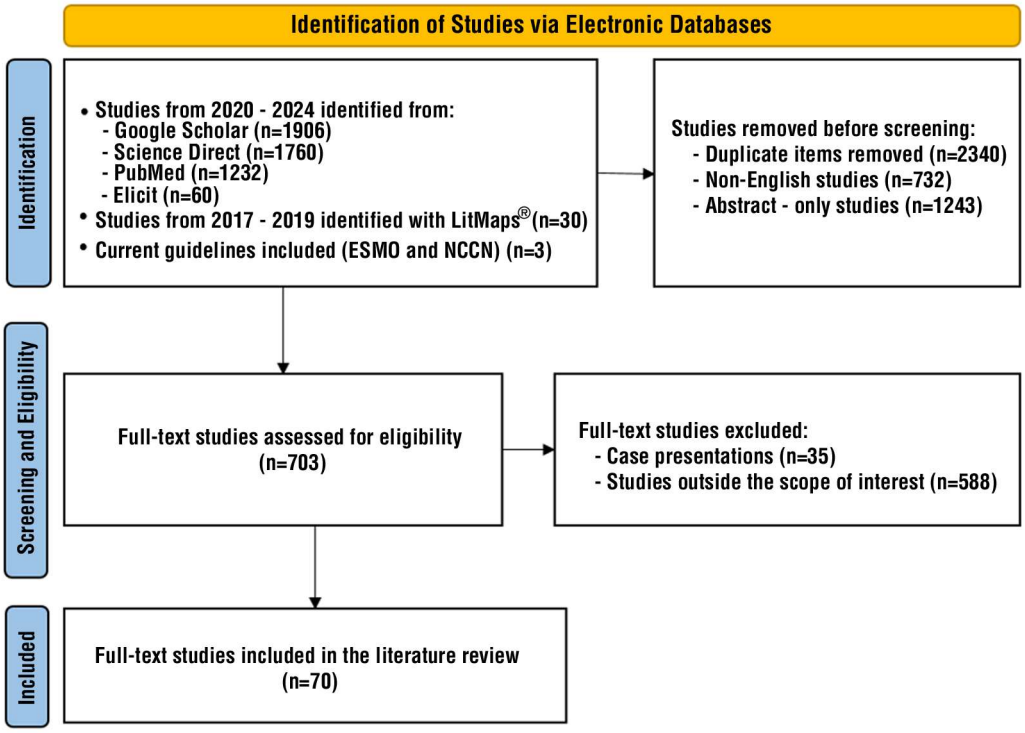


Figure 1. Literature flow diagram of the study selection process. ESMO, European Society for Medical Oncology. NCCN, National Comprehensive Cancer Network.

the aforementioned errors by recognizing and removing them, subsequently restoring DNA continuity (4,11). A dysfunctional MMR system (dMMR) results in the buildup of abnormal DNA sequences, identified as high microsatellite instability (MSI-H), consequently leading to a hypermutational state within the affected cells, responsible for approximately 15% of CRC cases (4-8,9).

The phenomenon of dMMR involves the inactivation of a gene within this system through germ-line mutations, somatic mutations, epigenetic modifications, or the simultaneous presence of these mechanisms (4). Lynch syndrome, an autosomal dominant inherited syndrome predisposing to the development of CRC, is characterized by germ-line mutations in the MMR genes MLH1 or MSH2; alternatively, it can be caused by the germ-line deletion of the EpCAM gene (epithelial cell adhesion molecule), leading to the suppression of MSH2 as a consequence of its proximity to the EpCAM gene (4,6). The sporadic forms of CRC with MSI-H status (representing approximately 12% of sporadic CRC) largely develop (about 95%) through epigenetic modifications, specifically through sporadic hypermethylation of the MLH1 gene promoter (5-7).

The Clinicopathological Characteristics of the MMR/MSI Biomarker in Colorectal Cancer

According to recent studies, dMMR/MSI-H phenotype is often encountered in young patients with tumors located in the right colon, exhibiting poor cellular differentiation, and a tumor-infiltrating lymphocyte-rich environment (5,9,12). A crucial aspect to note is that despite the association of this phenotype with a favorable prognosis, these tumors demonstrate aggressive characteristics, frequently penetrating the serosa (pT4 stage),

exhibiting a low differentiation grade, and being linked with histological subtypes such as mucinous and signet-ring cell types (9,13).

Methods Used in Assessing the Microsatellite Instability Status in Colorectal Cancer

The MSI status can be evaluated through genetic and molecular methods such as PCR, by comparing the nucleotide lengths between the tumor microsatellite regions and those from the corresponding normal tissue, using a panel of five markers recommended by the National Cancer Institute (NCI) in the USA (2 markers for mononucleotide repeats – BAT25 and BAT26, and 3 markers for dinucleotide repeats – D5S346 and D2S123) (6,11). According to the Bethesda guideline developed by the NCI in 1997, CRC has been classified into three phenotypes using the panel of five markers: 1) MSI-H when 2 or more markers exhibit instability; 2) MSI-L (low microsatellite instability) when only 1 marker shows instability; 3) MSS (microsatellite stable) when all the markers are stable (6,7,11). Currently, the European Society for Medical Oncology (ESMO) and the revised 2004 Bethesda guidelines have eliminated the MSI-L classification due to its overlap with MSS, while the 2024 edition (version V.1.2024) of the NCCN guideline maintains the term MSI-L, considering it from a clinical point of view synonymous with MSS (1,7,14). Additionally, upon observing the heightened specificity and sensitivity of mononucleotide markers in contrast with dinucleotide markers, both the revised 2004 Bethesda guidelines and the 2019 ESMO guidelines recommended, as a standard clinical practice, the adoption of a PCR analysis panel comprised solely of mononucleotide markers named the Pentaplex PCR system, incorporating BAT-25, BAT-26, NR-21, NR-24, and NR-27 markers (11,14,15).

Next-Generation Sequencing (NGS) emerges as a more precise genetic and molecular approach for evaluating the MSI status, capable of simultaneously analyzing thousands of STR or SSR regions along with the tumor mutational burden (TMB), and according to the guidelines of the College of American Pathologists (CAP) published in 2022, it is recommended as a validation test following MSI status evaluation via PCR (7,16,17).

Methods Used in Assessing the Mismatch Repair System in Colorectal Cancer

Immunohistochemistry is the method most commonly used to evaluate the expression of the MMR proteins (MLH1, PMS2, MSH2, and MSH6), and the lack of expression of any of these proteins is often correlated with a deficiency in the MMR system (6,11). The assessment of MMR system proteins via immunohistochemistry may face technical challenges, often occurring in the pre-analytical phase, and biological obstacles, which can result in findings that do not accurately reflect the true MSI status (6,14). For a more comprehensive grasp on how the MMR system is evaluated, it's important to consider that this DNA repair mechanism involves homodimeric complexes, including MSH2-MSH3-MSH6, MLH1-MLH2-MLH3, and PMS1-PMS2, functioning as active hetero-dimers, initiated sequentially in a cascade manner (MLH1-PMS2 and MSH2-MSH6) (6,11). Therefore, the lack of expression of a single MMR protein can compromise the formation of active heterodimers, resulting in the impairment of the entire system or, conversely, the phenotypic expression of the absent primary homodimer can be taken over by a secondary partner (MSH3 instead of MSH6, MLH3, or PMS1 instead of PMS2) (14). One of the biological challenges in immunohistochemical assessment of MMR proteins stems from this genetic interdependence, leading to false-positive results if all four proteins are not included in the evaluation (14). As a result, the current ESMO guidelines recommend conducting immunohistochemical analysis for all four MMR proteins, with PCR validation of the findings in cases where a single member of a heterodimer complex is absent or uncertainties arise regarding the findings (14). Given that the assessment of the MMR protein status is used for Lynch syndrome

screening, a second biological challenge that can generate false-positive results, inconsistent with the actual deficiency of the MMR system, involves missense mutations that disrupt the catalytic function but not the structure of the MMR genes, which can only be evaluated through genetic and molecular methods (6,14). DNA polymerase mutations can also generate false-negative results in immunohistochemical testing of MMR proteins, as they do not directly affect the MSI status despite potentially disrupting the MMR system's DNA repair function (18).

The Prognostic and Predictive Implications of the MMR/MSI Biomarker in Colorectal Cancer

According to current guidelines, the MMR/MSI status serves as a crucial biomarker for diagnosis, prognosis, and prediction of therapeutic response in colorectal cancer (1-315). Screening for hereditary syndromes such as Lynch syndrome or familial adenomatous polyposis (FAP) by assessing the MMR/MSI status is imperative in individuals with a familial predisposition to colorectal cancer (1,2). Lynch syndrome, prevalent in 2%–4% of CRC cases, arises from germ-line mutations in MMR genes or in the EpCAM gene (1). Upon identifying the absence of MLH1 protein expression through immunohistochemistry, the subsequent step in patient pre-therapeutic management involves assessing the status of the BRAF gene, as the presence of BRAF mutation serves to exclude Lynch syndrome (1,7). Alternatively, if there is a lack of MSH2 protein expression, the next step is to evaluate the status of the EpCAM gene using immunohistochemical or genetic and molecular methods such as PCR or NGS (1,7).

Regarding the prevalence of the dMMR/MSI-H phenotype, researchers have found that it varies depending on the stage of the disease, being higher in stages II and III (approximately 15%) compared to stage IV CRC, where only 4%–5% of patients exhibit dMMR/MSI-H status (5).

Currently, in stages II and III of the disease, assessing the MMR/MSI status is recommended because it is useful from a predictive perspective, providing relevant information on whether to administer adjuvant chemotherapy or not (1-3). Research indicates that for patients with stage II or III CRC and dMMR/MSI-H status, the administration of fluoropyrimidines (e.g., 5-FU, capecitabine, tegafur) is discouraged, whereas those with pMMR/MSS CRC often exhibit a favorable response to 5-fluorouracil treatment (1,2,8). In terms of prognostic implications for MMR/MSI status, the results of the PETACC-3 trial suggest that patients with colorectal cancer diagnosed in stages II and III exhibiting dMMR/MSI-H status have a reduced risk of metastasis, supporting the idea that this phenotype may imply a favorable prognosis (1,4). Additionally, it has been noted that adjuvant chemotherapy is generally not recommended for patients with dMMR/MSI-H status in stages II or III (2,8). These patients may undergo oncologic surveillance, whereas individuals with pMMR/MSS status typically encounter an unfavorable prognosis without adjuvant chemotherapy (8).

For a long period of time, advanced CRC (metastatic) with dMMR/MSI-H status was associated with an unfavourable prognosis and resistance to standard chemotherapy, particularly in cases with BRAF gene mutations (5,6,19). The integrative multi-omic approach has succeeded in changing the paradigm regarding the negative prognosis of dMMR/MSI-H metastatic CRCs, showing that the significant mutational burden (TMB) associated with this phenotype leads to the formation of a large number of neoantigens, thus triggering a strong immune response in the tumor microenvironment (increased tumor-infiltrating lymphocytes – TILs) (4–6,8). In MSI-H tumors, the beneficial impact of the immune response is offset by the membrane expression of PD-L1, triggering the PD-1/PD-L1 immune suppression pathway, which can be countered through PD-1 inhibitor immunotherapy (4–6,8). Therefore, in the metastatic phase of CRC, current guidelines recommend predictive assessment of the

MMR/MSI status to determine whether immunotherapy should be added to the treatment regimen or not (1,3,8).

In summary of current oncologic guidelines, evaluating the MMR/MSI status serves as a diagnostic biomarker in patients with a familial history of colorectal cancer to identify hereditary syndromes such as Lynch syndrome or FAP. Moreover, this biomarker acts as a predictive biomarker in metastatic disease to guide immunotherapy decisions, and in stages II and III of the disease to offer prognostic and therapeutic predictive insights.

The Role of All RAS (KRAS, NRAS) and BRAF Biomarkers in Colorectal Cancer

Exploring the Relationship between All RAS and BRAF Biomarkers

The Mitogen-Activated Protein Kinase (MAPK) signaling cascade, also known as the RAS/RAF/MEK/ERK pathway, plays a fundamental role in cellular homeostasis (20–22). The activation of this signaling pathway is initiated by the activation of membrane-bound receptor tyrosine kinases (RTKs) by the Epidermal Growth Factor (EGF), which leads to the activation of the intracellular RAS GTPase family (HRAS, KRAS, NRAS), subsequently inducing the dimerization of the RAF kinase family (ARAF, BRAF, and CRAF) (22). In turn, RAF initiates the activation of the MEK (serine/threonine kinase) - ERK (serine/tyrosine/threonine kinase) cascade, leading to the nuclear translocation of ERK kinase, where it stimulates a series of transcription factors responsible for the expression of specific genes involved in cellular processes such as proliferation, differentiation, cell survival, and apoptosis (22). Normally, MAPK inactivation occurs through a feedback mechanism that inhibits RAS activity by dephosphorylating BRAF residues (Thr599 and Ser602) and phosphorylating inhibitory sites in BRAF, thereby disrupting RAS binding or RAF dimerization (22). In colorectal carcinogenesis, this cascade is dysregulated by the generation of mutations in the essential genes of the MAPK signaling pathway (RAF and RAS), leading to disruption of cellular homeostasis (22).

The family of RAS proteins, known as GTPases (enzymes that facilitate the breakdown of GTP into GDP and inorganic phosphate), act as switches, converting extracellular signals such as growth factors, differentiation factors, or other mitogens into intranuclear transcription factors, thereby regulating cellular homeostasis (20,23). Therefore, when mutations occur in RAS genes, cells become predisposed to cancer invasion (20,23). In CRC, the KRAS gene (mutations in this gene are responsible for approximately 45% of metastatic CRCs and 15%–37% of early-stage CRCs) and NRAS gene (less frequently mutated) undergo somatic point mutations (missense) involving the alteration of a single nucleotide within the DNA sequence, with a predilection for exon 2, in codons 12 and 13 for the KRAS gene and exons 2, 3, and 4 for the NRAS gene (5,6,20).

Regarding the RAF protein family (intracellular serine/threonine kinase family), the most clinically significant mutations occur in the BRAF gene (accounting for approximately 10% of metastatic CRCs), particularly those in exon 15, codon 600, resulting in the substitution of valine with glutamic acid (BRAF V600E mutation) (5,6,20).

Through the application of NGS techniques, a new subset of BRAF mutations, termed non-V600 atypical BRAF mutations, has been identified, representing approximately 2% of metastatic CRCs (6,20,24,25). This discovery has led to the classification of BRAF mutations into three classes: 1) Class I (monomeric) – BRAF V600D/E/K/R mutations characterized by strong kinase activity, activating the MAPK pathway in the absence of an extracellular signal; 2) Class II (dimeric – BRAF-CRAF) – non-V600 BRAF mutations (at codons 464, 469, 601, and 597) characterized by moderate to high kinase activity, thus associated with weaker activation of the MAPK pathway; 3) Class III (RAS-dependent) – non-V600 BRAF mutations (at codons 466, 594, and 596) with low or absent kinase activity, unable to activate the MAPK pathway in the absence of an extracellular signal (6,22,26).

Mutations in the KRAS, NRAS, and BRAF genes cannot individually induce carcinogenesis, requiring the presence of numerous other oncogenic factors (6,25).

The Clinicopathological Characteristics of the All RAS (KRAS, NRAS) and BRAF Biomarkers in Colorectal Cancer

The primary genetic mutations driving colorectal carcinogenesis are somatic mutations in the RAS and BRAF genes, typically occurring independently of each other (27). Although uncommon, concurrent KRAS and BRAF mutations can arise, particularly in patients exhibiting the pMMR/MSS phenotype, potentially exacerbating or hastening the progression of CRC (25,27).

The correlations between all RAS mutations and other socio-demographic and clinicopathological parameters (sex, age, tumor location, histological subtype, and cellular differentiation grade) are controversial, with discrepant results possibly attributed to ethnic differences, various detection techniques, and the lack of multicenter studies on larger patient samples (28). Certain studies have indicated a correlation between KRAS gene mutations, particularly those occurring at codon 13, and regional lymph node invasion (N) as well as the mucinous subtype (20,25, 28). So far, there is no consensus among researchers regarding the association between KRAS mutation and the location of the primary tumor (20,28). NRAS-mutated tumors are less prevalent and there is relatively limited research available compared to studies on KRAS and BRAF-mutated tumors (20,28). Certain studies indicate that in contrast to tumors with BRAF mutations, those harboring RAS mutations exhibit a metastatic pattern predominantly involving the liver, lungs, and peritoneum, with a notably increased incidence observed in the lungs and peritoneum among KRAS-mutated tumors (20,25).

The current research offers more detailed insights into BRAF V600E-mutated tumors, indicating a higher occurrence among female patients, the elderly, and a correlation with right-sided colon localization, mucinous subtype, and low differentiation grade (5,20,21, 25,26,29). Additionally, BRAF V600E mutations are associated with a higher frequency of peritoneal and distant lymph node metastases (25,29). Last but not least, the BRAF V600E mutation is linked to the hypermutational phenotype (dMMR/MSI-H) and does not correlate with Lynch syndrome, thus assisting in ruling out a genetic predisposition to CRC (21,25,26).

Concerning tumors associated with non-V600 BRAF mutations, they are observed more commonly in younger male patients, predominantly found in the left colon, exhibiting a histological differentiation grade ranging from moderate to poor and peritoneal metastases are rarely seen in advanced scenarios (6,26). Moreover, these tumors are often linked with RAS mutations and only rarely associated with the hypermutational phenotype (dMMR/MSI-H) (6,26).

Methods Used in Assessing the Status of All RAS and BRAF genes in Colorectal Cancer

According to the 2024 NCCN guidelines, there is no standardized protocol for examining all RAS and BRAF mutations, with testing methodologies potentially differing across laboratories based on factors such as available technology, expertise level, and resources (1). Although it is a more cost-effective option, immunohistochemistry is not considered the gold standard for identifying BRAF V600E and RAS mutations (missense mutations) because recent studies have highlighted discrepancies when the results were compared with those obtained through other methods such as PCR or DNA sequencing (30,31). The most readily available methods for the most accurate detection of these mutations are DNA amplification techniques (PCR-based methods) and DNA sequencing methods (Sanger sequencing, NGS) (1).

Over the years, the Sanger sequencing method has been considered the gold standard in genotyping, despite being expensive and

time-consuming, as it can do a step-by-step analysis of DNA sequences (32,33). Due to advancements in medical technology, new generations of DNA sequencing techniques, referred to as Next-Generation Sequencing (NGS), have been integrated into clinical practice (33). NGS techniques (WES – Whole Exome Sequencing and WGS – Whole Genome Sequencing) allow for swift and cost-effective multi-gene analysis by concurrently sequencing millions of DNA fragments, identifying epigenetic variations and various DNA replication errors (base substitutions, insertions, deletions, changes in copy number, and genetic rearrangements), thus profoundly influencing therapeutic decisions (32,33). However, the integration of NGS into clinical practice presents challenges due to the absence of standardized methodologies for data processing and interpretation (1,33).

While PCR-based techniques are more economically viable than genomic sequencing technologies and produce results on par with those from NGS, their efficiency is diminished by lower result sensitivity, and additionally, their capacity to detect a wide range of mutations is inferior to that of NGS (31,34).

Apart from the methodologies employed in assessing mutational status, careful consideration should be given to the specimen type utilized in performing these tests. The primary materials used for molecular and genetic evaluation of colorectal tumors are tissue samples fixed with formalin and embedded in paraffin (FFPE), yet they are suboptimal for such tests due to the fragmentation and chemical alteration of the extracted nucleic acids (32). In recent years, there has been significant interest in an alternative method of tumor specimen collection called liquid biopsy, which retrieves circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs) (35,36). The specimens obtained in this manner are optimal for tumor genotyping, facilitating a thorough and comprehensive analysis of DNA sequences, with the additional benefit of being able to perform molecular analyses in advanced stages when tissue sampling through surgical interventions is not feasible (35,36). Another significant aspect is that samples acquired via liquid biopsy, particularly ctDNA, tackle a challenge in tumor molecular analysis - tumor heterogeneity - and also facilitate the real-time tracking of disease advancement (35-37).

The Prognostic and Predictive Implications of the All RAS and BRAF Biomarkers in Colorectal Cancer

Oncologists consider the RAS status crucial as a predictive factor for therapy selection, as it can predict resistance to anti-EGFR treatment, making this therapy unsuitable for patients with RAS-mutated tumors (1,6,20). The prognostic impact of RAS mutations is weaker compared to that of BRAF mutations, with studies indicating that only in the metastatic stage of the disease can RAS mutations negatively influence overall survival (OS) and disease-free survival (DFS) rates (6,9). For patients with RAS wildtype tumors, the choice between anti-EGFR or anti-VEGF therapy is determined by the location of the primary tumor; thus, anti-EGFR therapy is preferred for left-sided colon tumors, while anti-VEGF therapy is more effective for right-sided colon tumors (8,20).

The BRAF V600E mutation is known as a significant biomarker of negative prognosis in the metastatic stage of the disease, as evidenced by several trials (e.g., COIN, CAIRO, CAIRO 2, PRIME, CRYSTAL, FIRE 3, and OPUS), resulting in a reported median survival of 10–16 months regardless of the therapeutic strategy (chemotherapy and/or surgical - metastasectomies) (6,8,20,26). However, research has shown that this pattern is only observed in patients with pMMR/MSS phenotype, while the hypermutational status (dMMR/MSI-H) nullifies the unfavorable prognosis (21,26).

In stages II and III of CRC, studies suggest that the BRAF V600E mutation is associated with a poor prognosis, impacting the survival rate following potential recurrence, with no notable differences in DFS rates observed between patients with the BRAF mutation

and those without (BRAF wildtype) (1,26). Another significant observation in the disease's early stages is that having the hypermutational status (dMMR/MSI-H) alongside the BRAF V600E mutation leads to enhanced DFS compared to individuals with the pMMR/MSS phenotype (26).

The predictive impact of the BRAF V600E mutation continues to be a matter of debate. Studies on the impact of anti-EGFR therapy in patients with this mutation show varying results, with some indicating a slight improvement in overall median survival, therefore the inclusion of anti-EGFR therapy in the treatment plan is not currently considered contraindicated (5,20, 21,26,29).

In light of the therapeutic efficacy seen with BRAF inhibitors like Vemurafenib in metastatic melanoma patients harboring the BRAF V600E mutation, researchers have attempted to study its effects in metastatic CRC; nonetheless, the outcomes have been less than optimal (5,25,26).

However, there are studies and trials examining a potential benefit of dual therapy with MEK inhibitors and BRAF inhibitors, as well as triple therapy with MEK inhibitors, BRAF inhibitors, and anti-EGFR antibodies in metastatic CRC associated with BRAF V600E mutation, aiming to simultaneously target multiple molecular pathways involved in carcinogenesis (6,8,25,38).

Currently, anti-VEGF treatment remains the established therapy for patients with the BRAF V600E mutation, whereas immunotherapy could be a beneficial choice for those presenting a hypermutational phenotype (dMMR/MSI-H) (5,21,26).

Non-V600 mutations offer a significantly improved prognosis compared to BRAF V600E mutations, with Class III showing resistance to anti-EGFR therapy due to its RAS-dependence, contrasting with the second class, which might respond positively to this treatment (6,20,24,26).

The current NCCN and ESMO guidelines strongly recommend genotyping all RAS mutations (KRAS and NRAS) and the BRAF V600E mutation only in the metastatic stage of the disease, regardless of the specimen's origin (metastasis/primary tumor) (1-3).

The Role of CDX2 Biomarker in Colorectal Cancer

Unraveling the Significance of the CDX2 Biomarker in Colorectal Cancer

CDX1, CDX2, and CDX4 transcription factors are proteins found in the nuclei of intestinal epithelial cells, encoded by the CDX gene family (Caudal-Related Homeobox Genes), and play vital roles in the embryonic development of the digestive tract and in cellular differentiation (9,39).

During embryogenesis, each gene has a distinct function in the development of the gastrointestinal and other endoderm-derived tissues, with CDX2 playing a crucial role in both the development and upkeep of the intestinal epithelium through the activation of genes regulating intestinal identity and enterocyte maturation, including SI, CA1, and MUC2 (9,39,40). By orchestrating a network of genes to maintain gastrointestinal balance, CDX2 aids in fortifying intestinal barrier integrity, regulating cellular flux, and thereby preventing the onset of inflammatory bowel diseases (39,41). In in vivo studies, researchers have shown that CDX2 deletion leads to the onset of chronic inflammatory response, characterized by the accumulation of macrophages in the intestinal tissue (40). Furthermore, it appears that the CDX2 gene may exert a tumor-suppressive function, as suggested in a recent study conducted by Yu et al., which demonstrated that this gene reduces tumor cell proliferation in CRC by inhibiting the Wnt/ß-catenin signaling pathway (9,41–44).

The activation of the CDX2 gene results from the synergistic action of multiple intercellular signaling pathways, including Wnt/ß-catenin, MAPK, HNF, and GATA (39).

According to recent research, its inhibition appears to be regulated by the transcription factors SOX2 and SOX9 (39). Current studies

indicate that the loss of CDX2 expression is rarely associated with genetic mutations and, instead, occurs through epigenetic mechanisms such as CIMP, via hypermethylation of the CDX2 gene promoter region, which is rich in CpG islands (44-46).

Throughout time, the immunohistochemical panel consisting of CDX2, CK20, and CK7 markers has been utilized to determine the primary origin of tumors of unknown source, with a positive CK20, negative CK7, and positive CDX2 outcome profile affirming their colorectal origin (39,41). The expression of the CDX2 gene initiates in the duodenum and peaks in intensity in the distal small intestine and proximal colon, with complete absence in the distal rectum, where CDX1 gene expression appears to be strong (39).

Exploring the Relationship between CDX2 and Clinicopathological as well as Molecular Parameters

There are numerous studies that have demonstrated that the lack of CDX2 protein expression is correlated with aggressive behavior of CRC, advanced TNM stage, poor histological differentiation grade, as well as a low overall survival rate (9,41,43,44,47). Due to this rationale, we advocate for the inclusion of CDX2 expression assessment among the recommended biomarkers for prognostic evaluation in colorectal cancer. Recent studies have found no significant correlation between CDX2 expression and socio-demographic factors such as sex and age, indicating a potential influence of ethnic diversity (48). Additionally, the absence of CDX2 expression is often observed in right-sided colonic tumors, consistent with the abundant expression of this gene in healthy epithelial tissue of the right colon (41,43,49). Researchers also suggest that the CDX2 gene status plays a pivotal role in shaping the tumor's evolution in terms of invasiveness (44). Recent studies have demonstrated that tumors with decreased or absent CDX2 expression are linked to advanced grades of invasiveness, including pT3 (limited to the serosa) and pT4 (invasion of the serosa) (43,44). The level of CDX2 expression (negative, low, or positive) is also associated with both the grade of tumor differentiation and the histological subtype of colorectal tumors, with current research suggesting that negative CDX2 expression is linked to poorly differentiated mucinous adenocarcinomas (43,50). Regarding the status of perineural and lymphovascular invasion, there are conflicting views regarding the correlation between CDX2 expression and these histopathological parameters (43,50).

Apart from the link observed between the negative expression of CDX2 and the CIMP-H phenotype, research also indicates connections with BRAF V600E mutations, serrated precancerous lesions, and diverse MMR/MSI patterns (39,41,44,47,51). The studies conducted by Slik et al. and Konukiewitz et al. suggest that in cases where the absence of CDX2 expression is associated with a pMMR/MSS phenotype, the overall survival rate is lower compared to those with a dMMR/MSI-H phenotype (41,52). Furthermore, some studies report that the absence of CDX2 expression also contributes to the epithelial-to-mesenchymal transition (EMT) process by being associated with the loss of E-cadherin protein expression (41,53). Few studies support a connection between KRAS gene mutation and loss of CDX2 expression, with some suggesting that such correlation indicates an unfavorable prognosis (44,54).

As stated in the previous subsection, the activation or suppression of the CDX2 gene is facilitated by various signaling pathways, one of which involves hepatocyte nuclear factor (HNF), mediated by the transcription factors HNF1α and HNF4α (39). Additionally, studies have shown that HNF1α and HNF4α play a crucial role in liver development, and their inhibition contributes to the onset of liver carcinogenesis (55).

Considering these aspects, it can be hypothesized that the absence of CDX2 expression in relation to the disruption of the HNF signaling pathway may correlate with the occurrence of colorectal liver metastases.

Methods Used in Assessing the Expression of CDX2 in Colorectal Cancer

As mentioned in the preceding subsections, the CDX2 biomarker is commonly employed in current practice to confirm or exclude colorectal origin in metastases of unknown source, given its strong expression in the nucleus of intestinal epithelial cells (39,41,43,56).

Regarding the role of CDX2 as a prognostic biomarker, recent studies have reported a lack of CDX2 expression in 10% to 30% of all colorectal cancers (9,56).

Currently, the assessment of CDX2 status is performed through immunohistochemistry using monoclonal anti-CDX2 antibodies from various companies, yielding qualitative but semi-quantitative results, with no standardized scoring method for CDX2 expression yet established (24,43,44,48,56). Researchers have attempted various auxiliary methods to generate qualitative, quantitative, and reproducible results regarding CDX2 status, such as specific statistical methods, various digital analysis platforms, or artificial intelligence platforms (39,57,58).

Considering the aforementioned aspects, we believe that a standardized methodology for assessing CDX2 as a prognostic biomarker in colorectal cancer should be developed through comprehensive clinical studies that analyze and compare the results obtained via the existing testing methods (e.g., immunohistochemistry and genotyping), and also examine the relationship between the cost and effectiveness of these methods (39,57). The development of such a standardized methodology will ease the incorporation of this biomarker into clinical practice for prognostic assessment in CRC.

The Prognostic Implications of CDX2 in Colorectal Cancer

According to the meta-analysis by Tomasello et al., CDX2 presence in CRC predicts a favorable prognosis across all disease stages, halving the risk of death compared to cases where this protein is absent, with a 70% reduction in mortality risk observed specifically in stages II and III (59). Furthermore, tumors with CDX2 expression exhibit a 52% higher rate of progression-free or recurrence-free survival than those lacking this protein (59).

In line with the previous paragraph and with the extensive research investigating the relationship between CDX2 expression and various clinical, paraclinical, histopathological, and molecular parameters, the lack of CDX2 expression in CRC serves as a negative prognostic factor across all oncologic stages, often linked with BRAF V600E gene mutation, variable MMR/MSI patterns, and CIMP-H phenotype (39,41,44,47,51). Currently, the NCCN and ESMO guidelines do not acknowledge CDX2 as a prognostic biomarker in colorectal cancer, irrespective of the disease stage.

The Interactions between Biomarkers and Molecular Pathways in Colorectal Cancer

Exploring the Interrelationship between Molecular Pathways and Signaling Pathways Involved in Colorectal Carcinogenesis

Colorectal sporadic carcinogenesis is an extremely complex process involving the interaction of external oncogenic factors such as diet and unhealthy lifestyle with internal oncogenic factors like intestinal inflammation and oxidative stress (29,61). This interaction disrupts cellular functions and leads to the progressive accumulation of various genetic and/or epigenetic abnormalities (29,61).

Consequently, the signaling pathways that regulate cellular balance are impacted, resulting in the progression of normal glandular epithelial tissue to benign dysplasia and later to invasive malignant dysplasia (29,61).

Comprehension of the carcinogenic process has unveiled two significant carcinogenic pathways: the traditional adenoma-carcinoma model and the serrated pathway, originating from the precancerous lesion type (polyp) (23,29,61). The progressive mutational process known as the adenoma-carcinoma model involves the chromosomal instability mechanism which leads to the inactivation of the

APC tumor suppressor gene (23,29,61). The inactivation of the APC gene disrupts the Wnt/ß-catenin signaling pathway, causing the intestinal mucosa to transform into conventional adenomatous polyps (tubular, villous, or tubulo-villous), subsequently leading to additional genetic mutations (e.g., KRAS, SMAD4, and TP53) and the consequent dysregulation of other signaling pathways (MAPK, PI3K, and TGF-ß), ultimately culminating in the development of colorectal adenocarcinoma (23,29,61). Conversely, the serrated pathway is identified by the existence of serrated polyps, which, because of the epigenetic mechanism involved in their development (hypermethylation of gene promoter regions abundant in CpG islands) and the linkage of this pathway with BRAF mutations, exhibit a heightened potential for malignant progression and also possess a less favorable prognosis (29,61,62).

The molecular mechanisms underlying colorectal carcinogenesis are categorized according to genetic instability, represented by the chromosomal instability pathway, and epigenetic instability, encompassing the deficient MMR system pathway and the hypermethylation mechanism affecting promoter regions of genes abundant in CpG islands (23,29).

The intercellular signaling pathways normally act synergistically to maintain cellular homeostasis, contributing to processes such as growth, proliferation, differentiation, survival, and cellular apoptosis (65). Genetic and/or epigenetic alterations can impact these pathways, potentially disturbing the normal function of essential molecules responsible for regulating cellular processes, including ligands binding to cell surface receptors, specific intracellular proteins (such as kinases, GTPases, phosphatases), or target genes associated with these pathways (65).

The most important intercellular signaling pathways, each with a distinct set of target genes associated with colorectal carcinogenesis, include: 1) The MAPK pathway associated with KRAS, NRAS, and BRAF genes; 2) The Notch pathway associated with NOTCH1/2, HES1, and HEY1/2 genes; 3) The PI3K/AKT/mTOR pathway associated with PIK3CA, PTEN, and AKT1 genes; 4) The TGF-ß pathway associated with SMAD2 and SMAD4 genes; and 5) The Wnt/ß-catenin pathway associated with APC, CTNNB1, and AXIN2 genes (62,65,66). Research has shown that the heterogeneity of colorectal tumors stems from the synergistic collaboration of the five signaling pathways, where disruption of one may lead to dysregulation of others, thus contributing to the diverse molecular landscape of CRC (62,66).

Molecular Classifications of Colorectal Cancer and Their Implementation in Clinical Practice

Classifying CRC into two carcinogenic models (the conventional adenoma-carcinoma model and the serrated pathway), which encompass the molecular mechanisms CIN, MMR, and CIMP, fails to fully encapsulate the diversity of these tumors (23,62).

Therefore, around 2015, an international consortium of researchers developed a comprehensive molecular classification known as CMS (Consensus Molecular Subtypes), based on multi-omic and clinical analysis of this type of cancer, which can be used for the development of personalized therapies, as well as for assessing prognosis and treatment response (8,23,29,62,67).

The CMS1 category (the immune subtype) represents approximately 14% of CRC and is characterized by a hypermutational phenotype (MSI-H), CIMP-H (hypermethylation, BRAF mutations), low prevalence of somatic copy number alterations (SCNA), hyperactivation of the Wnt/ß-catenin signaling pathway, and high TILs (5,23,29,36,62,68).

From a clinicopathological perspective, CMS1 tumors are frequently encountered in females and typically occur in the right colon, with a tendency towards poor histological differentiation (29).

The CMS2 category (the canonical subtype) represents about 37% of CRC instances and is defined by the CIN mechanism, a high incidence of SCNA, clearly delineated epithelial histological characteristics, and the

hyperactivation of the Wnt/ß-catenin and MYC signaling pathways (5,23,29,36,62,68). Additionally, this category is associated with overexpression of EGFR and its ligands AREG and EREG, as well as mutations in the ERBB2 (HER2), TP53, APC, and RAS genes (68). CMS2 tumors are frequently found in the left colon and rectum (68). This category has been linked to a favorable prognosis across all disease stages (36,67). Moreover, certain clinical trials have indicated that for stage IV CRCs, CMS2 tumors might benefit from anti-VEGF therapy as part of first-line chemotherapy, resulting in enhanced survival outcomes (29,36).

The CMS3 category (the metabolic subtype) accounts for approximately 13% of CRC cases and is defined by the CIN mechanism, CIMP-L with variable MSI status, KRAS mutations, low prevalence of SCNA, metabolic hyperactivity (glutaminolysis and lipidogenesis), hyperactivation of the let-7 microRNA pathway, as well as the Wnt/ß-catenin and MYC signaling pathways (5,23,29,36,62,68). This category also presents a favorable prognosis, and like CMS2, in stage IV CRC, it could benefit from anti-VEGF as first-line chemotherapy (29,36).

The CMS4 category (the mesenchymal subtype) represents approximately 23% of CRC cases and is characterized by the MSS phenotype, high prevalence of SCNA, significant amount of intratumoral stromal and immune cells infiltration, neoangiogenesis, hyperactivity of genes involved in the epithelial-to-mesenchymal transition (EMT) process, and hyperactivity of the TGF-ß signaling pathway (5,23,29,36,62,68). CMS4 tumors are commonly located in the distal colon and rectum and are associated with serrated precancerous lesions (62,68). Of all four categories, CMS4 has the least favorable prognosis regardless of the disease stage (29,36,67). The FIRE3 trial showed that these patients might benefit from anti-EGFR chemotherapy (29).

Regarding the correlation between CDX2 status and CMS classification, studies have indicated that tumors lacking CDX2 expression are often associated with CMS1 and CMS4 subtypes (39,69). The CMS classification has generated a new stratification of colorectal tumors based on the characteristics of the tumor microenvironment: CMS1 is associated with an abundant immune cell infiltrates within the tumor, CMS2 and CMS3 are characterized by well-defined epithelial tissue, while CMS4 stands out for its significant stromal tissue infiltration (10,62,70). This observation prompted the development of a new classification known as the Colorectal Cancer Intrinsic Subtypes (CRIS) (10,62,70).

CRIS employs criteria such as tumor morphology and the expression of tissue-specific genes to gain a broader understanding of the microenvironment of colorectal tumors, utilizing preclinical models such as cell lines and patient-derived xenografts (PDX) (10,70).

Taking into account the heterogeneity of CRC, in recent decades, researchers have formulated comprehensive molecular classifications using multi-omic databases, aiming to pinpoint precise molecular subgroups applicable in current clinical practice for prognostic and predictive purposes (62,70). An outstanding example is the molecular classification of breast cancer, successfully achieved through the i-SPY-2 trial, which has subdivided this type of cancer into distinct molecular subgroups, thereby facilitating the customization of therapeutic plans based on biomarkers such as ER, PR, HER2, and ki67 (62,70).

Over the past 12 years, researchers have developed a range of classification systems for colorectal cancer, aiming to construct precise models based on molecular biomarkers that can be used for prognostic purposes and, furthermore, for treatment personalization (70).

The CMS and CRIS classifications currently used solely for scientific purposes represent the most comprehensive molecular models (10,62,70). The official integration of the CMS and CRIS molecular classifications into clinical practice entails the development of a standardized methodology by incorpo-

rating them into large-scale retrospective and prospective clinical trials with comprehensive multi-omic databases (8,62,70).

Discussions

In this comprehensive literature review, we conducted a thorough analysis of the main biomarkers endorsed by contemporary oncological guidelines (MMR/MSI phenotypes and the mutational status of the all RAS and BRAF genes) for assessing patients diagnosed with CRC, shedding light on their contributions to the molecular pathogenesis, correlations with frequently employed clinicopathological parameters, testing methodologies, and recent breakthroughs. Additionally, we introduced a novel biomarker, the CDX2 protein, which exhibits promising prognostic capabilities and offers the advantage of a simplified assessment through immunohistochemical methods, in contrast to conventional biomarkers such as the BRAF gene, which requires PCR or NGS techniques for testing *(Fig. 2)*.

Lastly, we delved into the complex molecular mechanisms driving colorectal carcinogenesis, shedding light on intricate molecular interactions to deepen out comprehension of biomarker dynamics. Recent scientific progress has shown that exploring molecular interactions within both inter- and intracellular signaling pathways, influencing diverse cellular functions, represents a crucial first step in comprehending the molecular mechanisms driving colorectal carcinogenesis. This understanding extends to the evaluation and interpretation of biomarkers, considering their interplay and contextual significance in conjunction with other clinicopathological parameters, thereby aiding in the refinement of existing molecular classifications of CRC or in the development of new ones suitable for clinical use. Therefore, we advocate for the continued research and discovery of new signaling pathways implicated in the etiology and progression of colorectal cancer, with a particular emphasis on elucidating how they interact, aiming to identify potential new therapeutic targets (novel biomarkers) that could contribute to obtaining a broader understanding of tumor behavior.

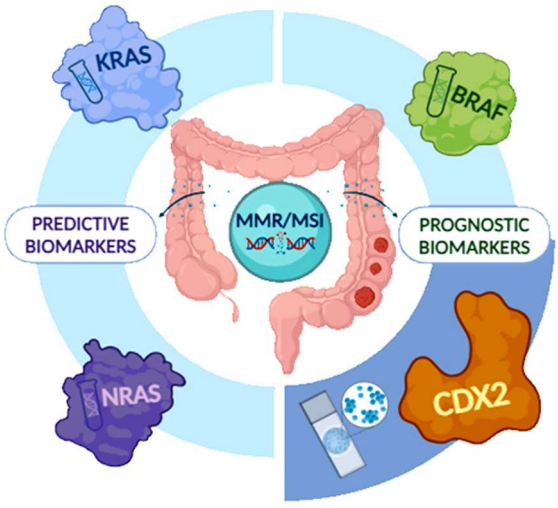


Figure 2. Interplay of Predictive and Prognostic Biomarkers in Colorectal Cancer: Emphasizing Established and Novel Biomarkers

In current clinical settings, the initial molecular assessment typically begins with the immunohistochemical analysis of the MMR protein expression, primarily due to its swift evaluation process and cost-effectiveness. When MMR protein expression indicates proficiency, it is typically assumed that there is no high microsatellite instability without direct assessment of the MSI status using genetic and molecular testing methods. Recent studies have highlighted the potential for false-positive results in immunohistochemical MMR testing, particularly in cases involving missense mutations in associated genes or false-negative results when DNA polymerase mutations are implicated. This underscores the inadequacy of relying solely on immunohistochemical MMR testing to precisely determine the relationship between the DNA repair system status and the MSI status. Furthermore, considering recent studies on the benefit of immunotherapy in cases of advanced (metastatic) CRC with MSI-H phenotype, and in accordance with the latest recommendations from ESMO and NCCN, we consider MSI and MMR testing to

be indispensable in all stages of the disease.

Concerning the KRAS biomarker, its predictive utility exceeds its prognostic relevance. Recent studies suggest that in association with tumor localization, customized therapeutic approaches can be formulated for individuals harboring KRAS-wildtype tumors. Furthermore, albeit infrequent, concurrent KRAS and BRAF mutations may arise, especially among patients manifesting a pMMR/MSS phenotype, potentially resulting in an adverse prognosis. This emerging scenario necessitates in-depth exploration of the interplay between these mutations and the exploration of prospective treatment modalities.

Researchers have identified that, in addition to the BRAF V600E mutation known to be associated with an unfavorable prognosis in advanced stages of CRC, there are also other atypical non-V600 BRAF mutations which, according to recent studies, show a favorable prognosis and may benefit from customized treatment strategies based on their specific mutation class. Additionally, researchers propose that combining MAPK and BRAF inhibitors may positively affect therapeutic response in BRAF V600E-mutated CRCs diagnosed at an advanced stage. Lastly, current research indicates that patients harboring BRAF V600E mutation and high microsatellite instability often exhibit a positive response to immunotherapy and that the dMMR/MSI-H phenotype may counteract the negative impact of this mutation. Therefore, we advocate for assessing the BRAF biomarker alongside MSI /MMR status in advanced CRCs. Additionally, we support investigating potential therapeutic strategies applicable in this context.

Concerning the assessment of MSI, KRAS, NRAS and BRAF biomarkers, it is crucial to establish a standardized and economically viable methodology for their evaluation, taking into account the quality of the specimen utilized (FFPE tumor specimens or liquid biopsy) and integrating novel genotyping techniques. Recent studies have highlighted that assessing these biomarkers using modern DNA sequencing techniques, such as NGS, provides rapid results with higher sensitivity and specificity compared to other methods like PCR-based techniques, Sanger sequencing, and conventional immunohistochemistry.

Molecular testing has become an integral component of routine clinical practice, playing essential roles in CRC screening, diagnosis, prognostic evaluation, and treatment decision-making. Moreover, there are several ongoing clinical trials aimed at validating various targeted therapies, immunotherapies, and combination treatments guided by specific biomarkers associated with CRC, prompting the scientific community to make extraordinary efforts to discover more such molecular targets. One of the extensively researched novel biomarkers is the CDX2 protein, a transcription factor primarily located in the nuclei of intestinal epithelial cells, commonly utilized as a marker of intestinal origin for metastases of unknown primary origin. Numerous studies revealed its significant role in colorectal carcinogenesis, influencing tumor invasiveness, microenvironmental adaptation, and metastatic progression through modulation of diverse signaling pathways and direct engagement in the EMT process. Moreover, researchers have indicated potential links between CDX2 gene alterations and the CIMP mechanism, often associated with aggressive tumor behavior. Its rapid and cost-effective evaluation via immunohistochemical methods could facilitate seamless assessment across all stages of CRC, contrasting with the BRAF biomarker, recommended to be evaluated solely in the fourth stage of CRC for prognostic purposes due to the complexity of the testing methods involved. Acknowledged as a prognostic biomarker for CRC by the scientific community, CDX2 currently lacks a standardized immunohistochemical scoring system, leading to the utilization of diverse cut-off scoring models to evaluate its involvement in colorectal carcinogenesis. Therefore, we advocate, alongside the scientific community, for the development of a standardized testing methodology for CDX2. This endeavor aims to facilitate the integration of this biomarker into clinical practice and enhance

its acknowledgement as a prognostic indicator in international oncological guidelines.

Strenghts and Limitations

This literature review's strengths lie in its comprehensive examination of established and emerging colorectal cancer (CRC) biomarkers, notably introducing CDX2 as a significant prognostic marker. By delving into the molecular intricacies of CRC and the interplay between biomarkers and molecular pathways, it advances the understanding of CRC's pathogenesis and supports the shift towards personalized medicine. Highlighting the integration of multi-biomarker strategies, it significantly contributes to refining CRC management and prognostic evaluation.

In terms of this review's limitations, it is important to note that while it encompasses a broad spectrum of biomarkers and molecular pathways in CRC, the fast-evolving nature of molecular oncology might extend beyond the scope of the analyzed studies. Additionally, while it addresses the current testing methodologies and novel approaches, including NGS and liquid biopsies, variations in technology and expertise across different laboratories might affect the generalizability of our findings. This review also acknowledges potential discrepancies in the results due to ethnic diversity, sample size, and multicenter collaboration limitations. Despite these challenges, our analysis aims to provide a foundational understanding, anticipating future integration of emerging biomarkers and molecular classifications into clinical practice.

Conclusions

In conclusion, we emphasize the importance of conducting a comprehensive molecular assessment of all biomarkers delineated in international guidelines, particularly the MMR/MSI in all stages of CRC, as well as the BRAF and all RAS biomarkers, to gain a deeper insight into tumor behavior and accurately interpret results in the context of clinical and pathological characteristics, thereby tailoring treatment accordingly. The incorporation of novel biomarkers, such as the CDX2 protein, into CRC assessment can fortify the framework of predictive, prognostic, and personalized medicine, which relies heavily on interdisciplinary therapeutic approaches tailored to each patient. Furthermore, the adoption of innovative testing methodologies such as NGS, utilizing high-quality specimens like those obtain from liquid biopsy, promises to yield results with enhanced specificity and sensitivity, obtained from easily accessible patient samples, facilitating real-time disease monitoring. Lastly, a thorough assessment of CRC biomarkers will facilitate the establishment of an optimal database for investigating the association between these biomarkers and socio-demographic, as well as clinicopathological parameters. This endeavor seeks to refine the existing molecular taxonomy of CRC and promote the commencement of clinical investigations aimed at validating prospective therapeutic approaches tailored to molecular subtypes of the disease.

Author's Contributions

Conceptualization, A.C.I.P.; methodology, A.C.I.P. and C.M.A.; formal analysis, A.C.I.P., A.C. and A.S.D.; data curation, A.C.I.P and A.C. writing - original draft preparation, A.C.I.P.; writing - review and editing, A.C.I.P., A.C., D.M.M., A.P., C.V. and R.V.; supervision, C.M.A., D.A.C., R.N. and A.B. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the research, authorship, and/or publication of this article.

Data Availability Statement

No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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