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ORIGINAL ARTICLE

MOLECULAR AND IMMUNOHISTOCHEMICAL BIOMARKERS IN COLORECTAL CARCINOMA - A SINGLE CENTER STUDY

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ABSTRACT

Objective: Colorectal cancer is the third most common malignancy in the world and among the most frequent causes of cancer-related death. Our study aimed to evaluate the molecular profile of the patients diagnosed with colorectal carcinoma at Clinical Hospital Acibadem-Sistina in Skopje.

Materials and methods: This study is retrospective-prospective, conducted at the Department of histopathology and cytology, at Clinical Hospital Acibadem-Sistina in Skopje. Tissue samples from surgical material from 152 patients diagnosed with CRC were processed for molecular and immunohistochemical analysis. KRAS and BRAF mutations were analyzed, and MMR status was obtained. In 90 metastatic cases, evaluation of HER2 and PDL-1 expression was performed on a tissue microarray.

Results: Among 152 analyzed patients diagnosed with colorectal carcinoma, the majority were males (98, 64.47%) compared to females (54, 35.53%). The mean age was 68.4±11.3 years; the median age was 70 years. KRAS/NRAS mutations were detected in 47(31%) of patients, BRAF mutations in 11(7%) patients, and mismatch repair gene deficiency (MMRd) was found in 15(10%) of patients. HER2 positive expression was present in 36(40%) of patients, and 17(19%) of patients showed PDL-1 expression. In the group of 17 PDL1-positive tumors, a cutoff of more than 1% positive tumor cells was detected in 10 cases, more than 10% tumor cells in 4 cases, and more than 50% tumor cells in 3 cases. From 36 HER2 positive cases, 32(32,5%) were with score 2+, and 4(4,4%) with score 3+.

Conclusions: Continued research into molecular mechanisms and biomarkers holds the promise of further improving CRC outcomes through personalized and effective interventions.

Keywords: colorectal carcinoma, biomarkers, KRAS, BRAF, HER2, PDL-1

INTRODUCTION

Colorectal cancer (CRC) is one of the most common and lethal malignancies worldwide. Its development and progression are influenced by a complex interplay of genetic, epigenetic, and environmental factors. Recent advancements in molecular biology have provided a deeper understanding of the mechanisms underlying CRC and have paved the way for novel diagnostic, prognostic, and therapeutic approaches. These molecular strategies have become pivotal in improving patient outcomes [1].

Molecular Pathogenesis of CRC

CRC typically arises through the stepwise accumulation of genetic alterations. The adenoma-carcinoma sequence, which accounts for the majority of CRC cases, involves mutations in key oncogenes and tumor suppressor genes. The earliest event is often a mutation in the APC gene, leading to aberrant activation of the Wnt/β-catenin signaling pathway. This is followed by activating mutations in the KRAS gene, which promote uncontrolled cell proliferation. Inactivation of the tumor suppressor TP53 and mutations in genes involved in TGF-β signaling, such as SMAD4, occur at later stages of tumor progression.

Another molecular pathway implicated in CRC is the microsatellite instability (MSI) pathway, characterized

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by defective DNA mismatch repair (MMR) systems. This pathway accounts for approximately 15% of CRC cases and is associated with hypermutability, leading to a distinct molecular and clinical phenotype. Lynch syndrome, a hereditary form of CRC, is strongly linked to MSI [2].

Additionally, the CpG island methylator phenotype (CIMP) represents an epigenetic mechanism contributing to CRC. Hypermethylation of promoter regions in genes such as MLH1 silences critical tumor suppressors, further driving carcinogenesis.

Molecular Biomarkers in CRC

The identification of molecular biomarkers has revolutionized CRC management. These biomarkers play roles in early detection, prognosis, and treatment selection.

Diagnostic biomarkers such as circulating tumor DNA (ctDNA) and cell-free DNA (cfDNA) are emerging as non-invasive tools for CRC detection. Liquid biopsies analyzing these biomarkers offer the potential for early diagnosis and monitoring of disease progression [3].

Prognostic biomarkers as mutations in BRAF, particularly the V600E mutation, are associated with poor prognosis in CRC patients. Similarly, MSI-high tumors generally have a better prognosis but show limited response to conventional chemotherapy.

Predictive biomarkers are molecular markers that guide personalized treatment approaches. For example, patients with RAS-mutant tumors do not benefit from anti-EGFR therapies such as cetuximab and panitumumab. Conversely, MSI-high tumors are responsive to immune checkpoint inhibitors, making MSI status a key determinant in immunotherapy eligibility [4-9].

Targeted Therapies and Personalized treatment

Molecular approaches have led to the development of targeted therapies tailored to specific genetic and molecular alterations in CRC. Anti-EGFR Therapy Cetuximab and panitumumab are monoclonal antibodies targeting the epidermal growth factor receptor (EGFR). These agents are effective in patients with wild-type RAS genes.

Patients with BRAF-mutant CRC can benefit from combination therapies involving BRAF inhibitors, MEK inhibitors, and anti-EGFR agents.

MSI-high/mismatch repair-deficient (dMMR) tumors exhibit high mutational loads, making them responsive to immune checkpoint inhibitors such as pembrolizumab and nivolumab [10-14].

Our study aimed to evaluate the molecular profile of the patients diagnosed with colorectal carcinoma at Clinical Hospital Acibadem-Sistina in Skopje.

MATERIALS AND METHODS

This study is retrospective-prospective, conducted at Department of histopathology and cytology, at Clinical Hospital Acibadem-Sistina in Skopje. Representative tumor samples from formalin-fixed paraffin-embedded (FFPE) tissue from surgical material of 152 patients diagnosed with CRC were processed. Informed consent was obtained from all the patient or their relatives.

Molecular analysis

The DNA from all FFPE specimens was extracted using the SaMag 12 automated nucleic acid extractor (Sacace Biotechnologies, Como, Italy) with the SaMag FFPE DNA extraction kit, following the manufacturer's protocol instructions. The quantification of DNA was obtained using Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). KRAS and BRAF/NRAS mutations were analyzed on Cobas z 480, PCR for automated amplification using the KRAS and BRAF/NRAS mutation test (LSR).

Immunohistochemical analysis

The 4 μm-thick sections of the tumor tissue were processed immunohistochemically using an automated procedure in the Ventana BenchMark Ultra Advanced staining system. Antibodies from the colorectal cancer panel were used (Table 1).

Analysis of mismatch repair proteins (MLH1, PMS2, MSH2, MSH6) was evaluated to determine whether there was a nuclear signal in all as MMR proficient (MMRp), and if it was lost in one or more proteins as MMR defi-

Table 1. Immunohistochemical stains

Antibody(rtu)	Clone	Producer	Type of signal
MLH1	M1	Ventana, USA	nuclear
PMS2	A16-4	Ventana, USA	nuclear
MSH2	G219-1129	Ventana, USA	nuclear
MSH6	SP93	Ventana, USA	nuclear
PDL1	SP263	Ventana, USA	membranous
HER2	4B5	Ventana, USA	membranous

cient (MMRd). Additional PCR or NGS analysis was not conducted for protein alterations.

Immunohistochemical analysis for PDL-1 and HER2 was performed only on metastatic cases, 90 in number, on a tissue microarray. PD-L1 expression was assessed at cut-offs of 1-10%, 10-50%, and 50-100% of positive tumor cells. Diagnostic evaluation of HER2 in colorectal cancer is not yet standardized and is based on a modification of the already accepted one for gastric cancer (0,1+, 2+, 3+).

RESULTS

The study analyzed tissue materials from 152 patients diagnosed with colorectal carcinoma, who were mostly males, 98(64.47%), vs females, 54(35.53%), and the age of the patients ranged from 34 to 89 years; the mean age was 68.4±11.3 years (Table 2). The analyzed patients in terms of postoperative disease stage showed that the most commonly diagnosed are in stage IIIB, 35.53% and 90 cases (59,21%) are metastatic (Stage III and IV) (Table 3).

In this cohort of patients, KRAS/NRAS mutations were detected in 47(31%) of patients (Figure 1), BRAF mutations in 11(7%) patients (Figure 2), mismatch repair gene deficient (MMRd) were found in 15(10%) of patients (Figure 3).

Table 2. Demographic characteristics of the patients

variable	n (%)
Gender	
female	54 (35.53)
male	98 (64.47)
Age groups	
≤50 years	15 (9.87)
>50 years	137 (90.13)
Age/years	
(mean ± SD)(min- max)	(68.37 ± 11.3)(34 – 89)
median (IQR)	75.5(62 – 11.32)

Table 3. Distribution of the patients according to postoperative Stage

variable	n(%)
STAGE	
0	4(2,62)
I	9(5,92)
II A	35(23,02)
II B	2(1,32)
II C	2(1,32)
III A	1(0,66)
III B	54(35,53)
III C	32(21,05)
IV A	7(4,61)
IV C	6(3,95)

HER2 and PDL1 expression were analyzed in metastatic cases only, 90 in count, on a tissue microarray. HER2 positive expression was present in 36(40%) of patients (Figure 4) and 17(19%) of patients showed PDL-1 expression (Figure 5). Immunohistochemical analysis for HER2 score 3+ and PDL-1 with membranous positivity in more than 50% of tumor cells is shown in Figure 6. From 36 HER2 positive cases, 32(32,5%) were with score 2+, and 4(4,4%) with score 3+ (Figure 7). In the group of 17 PDL1-positive tumors, a cutoff of more than 1% positive tumor cells was detected in 10 cases, more than 10% in 4 cases, and more than 50% in 3 cases (Figure 8).

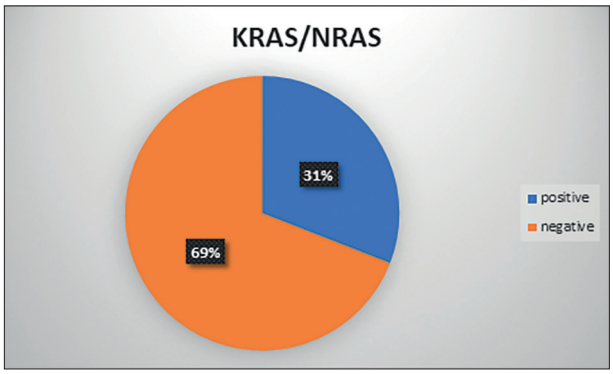

Figure 1. KRAS/NRAS mutations

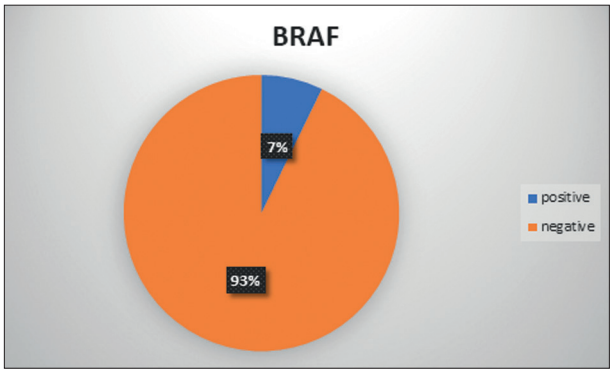

Figure 2. BRAF mutations

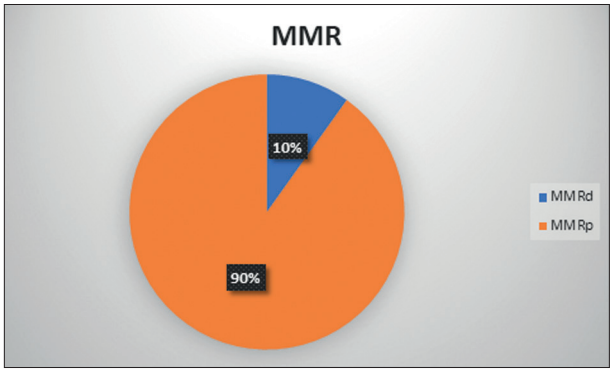

Figure 3. MMR status

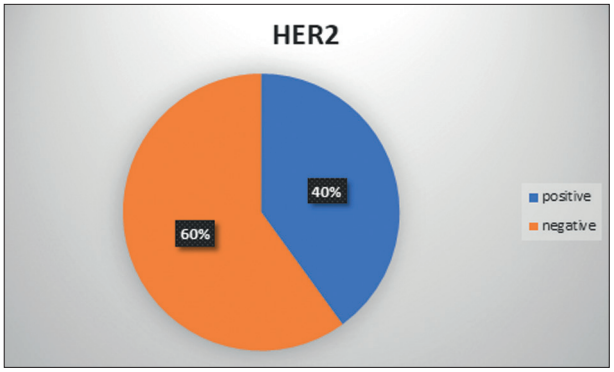

Figure 4. HER2 expression in metastatic cases

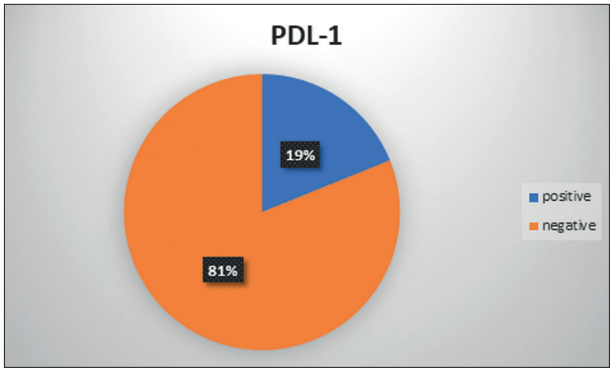

Figure 5. PDL-1 expression in metastatic cases

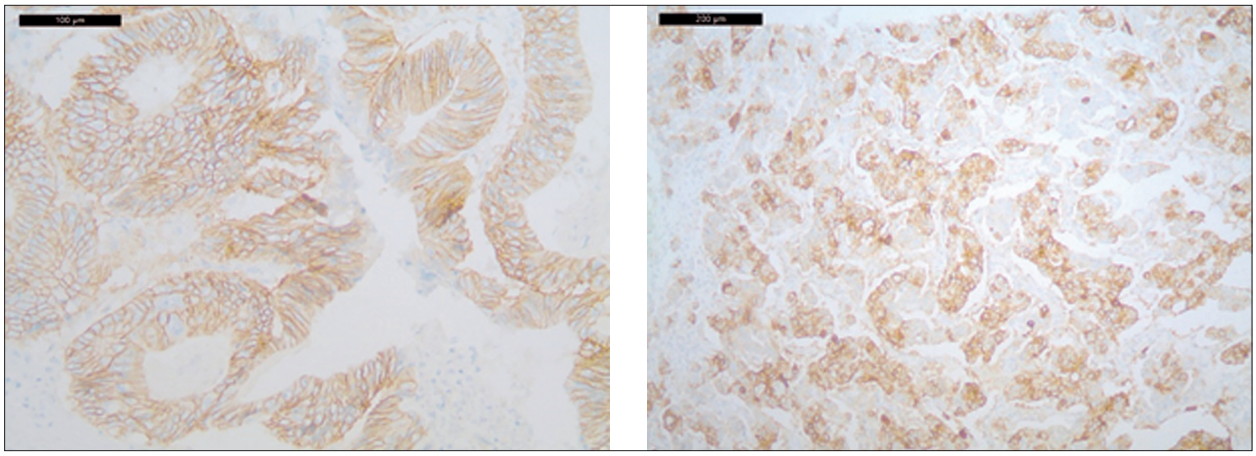

Figure 6. Immunohistochemical analysis showing HER2 score 3+ and PDL-1 score >50%

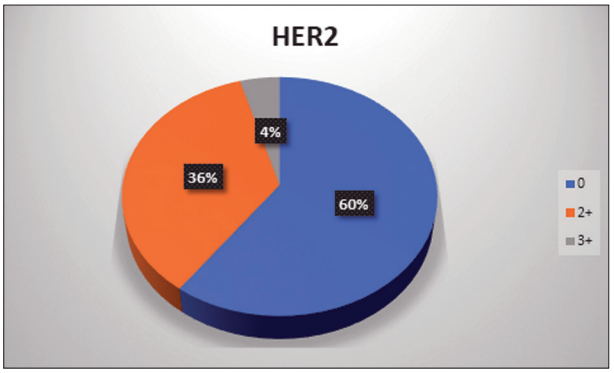

Figure 7. HER2 distribution by score

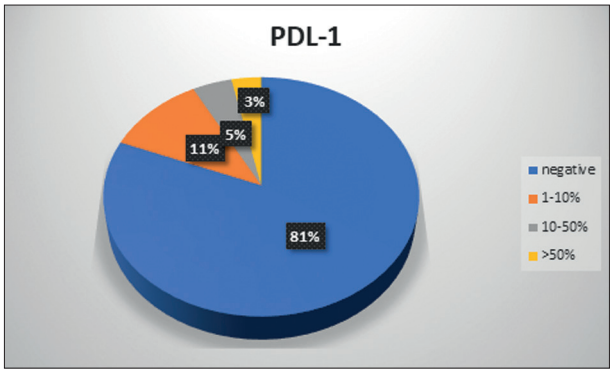

Figure 8. PDL-1 distribution by score

DISCUSSION

KRAS Mutations in Colorectal Cancer

KRAS (Kirsten rat sarcoma viral oncogene homolog) is a proto-oncogene encoding a small GTPase protein that plays a critical role in the RAS/MAPK signaling pathway. This pathway regulates cell proliferation, differentiation, and survival. Mutations in the KRAS gene, present in approximately 40% of CRC cases, lead to constitutive activation of the RAS protein, resulting in uncontrolled cell growth and tumor progression. In our study there were 31% of cases that showed KRAS mutations. The most frequent KRAS mutations in CRC occur at codons 12 and 13 in exon 2, with less common mutations in codons 61 and 146. KRAS mutations are associated with poor prognosis and resistance to anti-EGFR (epidermal growth factor receptor) therapies, such as cetuximab and panitumumab. Consequently, KRAS mutation testing is standard practice to guide treatment decisions, ensuring that only patients with wild-type KRAS tumors receive anti-EGFR therapy [15,16].

BRAF Mutations in Colorectal Cancer

BRAF (B-Raf proto-oncogene, serine/threonine kinase) is another critical component of the RAS/MAPK pathway. Mutations in the BRAF gene are present in approximately 10–15% of CRC cases, with the V600E mutation being the most common. Our cases with BRAF mutations were 7% of the analyzed cases. This mutation leads to constitutive activation of BRAF, driving tumor growth and progression. BRAF V600E mutations are associated with a distinct clinical and molecular phenotype, including poor prognosis, with reduced overall survival compared to BRAF wild-type tumors, a higher likelihood of occurring in the right colon, frequent co-occurrence with microsatellite instability (MSI) and CIMP (CpG island methylator phenotype). In metastatic CRC, BRAF-mutated tumors exhibit resistance to standard chemotherapy and anti-EGFR therapy when used alone [17-19]. However, combination therapies targeting BRAF, MEK, and EGFR have shown efficacy in this subset of patients. For example, the BEACON CRC trial demonstrated improved outcomes with a combination of encorafenib (a BRAF inhibitor), binimetinib (a MEK inhibitor), and cetuximab[20].

Mismatch Repair (MMR) Deficiency/Microsatellite Instability (MSI)

The MMR system is responsible for correcting DNA replication errors. Deficiency in MMR (dMMR) leads to the accumulation of errors, particularly in repetitive DNA sequences known as microsatellites, resulting in microsatellite instability (MSI). MSI is present in approximately 15% of CRC cases and is classified as high (MSI-H) or low/stable (MSI-L/MSS).

MMR deficiency can be sporadic or hereditary, with the latter being a hallmark of Lynch syndrome, a hereditary cancer predisposition syndrome caused by germline mutations in MMR genes such as MLH1, MSH2, MSH6, and PMS2. MSI-H tumors are associated with: a distinct clinical phenotype, including right-sided location, mucinous histology, and a high tumor mutational burden (TMB), better prognosis in early-stage CRC compared to MSS tumors, and resistance to fluoropyrimidine-based chemotherapy when used as monotherapy.

Importantly, MSI-H/dMMR status is a predictive biomarker for response to immune checkpoint inhibitors (ICIs) such as pembrolizumab and nivolumab. MMRd in our study were 10% of the analyzed cases. These therapies exploit the high neoantigen load of MSI-H tumors, enhancing immune recognition and tumor elimination [21-26].

HER2 Amplification in Colorectal Cancer

HER2 (human epidermal growth factor receptor 2) is a receptor tyrosine kinase involved in cell growth and differentiation. HER2 overexpression or amplification is observed in approximately 2–5% of CRC cases, primarily in RAS/BRAF wild-type tumors. HER2 amplification is associated with poor prognosis and resistance to anti-EGFR therapy. However, it also represents a targetable alteration. HER2-targeted therapies, such as trastuzumab and dual HER2 inhibition with trastuzumab and lapatinib, have shown promising results in HER2-positive metastatic CRC [27-31]. Clinical trials such as HERACLES and MyPathway have demonstrated the efficacy of these therapies, leading to durable responses in selected patients. HER2 status is determined using immunohistochemistry (IHC) and in situ hybridization (ISH)[32,33]. Accurate testing is crucial for identifying patients who may benefit from HER2-targeted therapies.

PD-L1 Expression in Colorectal Cancer

Programmed death-ligand 1 (PD-L1) is an immune checkpoint protein expressed on tumor cells and immune cells within the tumor microenvironment. It interacts with the PD-1 receptor on T-cells, suppressing immune activation and promoting immune evasion.

In CRC, PD-L1 expression is variable and more commonly observed in MSI-H tumors due to their high mutational burden and immunogenicity. PD-L1 expression serves as a predictive biomarker for response to ICIs. While ICIs such as pembrolizumab and nivolumab are approved for MSI-H/dMMR CRC, their efficacy in MSS CRC is limited due to the immune "cold" nature of these tumors. Strategies to enhance the efficacy of ICIs in MSS CRC include combination therapies with VEGF inhibitors, chemotherapy, or novel immune modulators.

PD-L1 testing in CRC is less standardized compared to other cancers, such as non-small cell lung cancer. Efforts are ongoing to develop robust assays and explore the predictive value of PD-L1 in CRC [34-36].

CONCLUSION

Molecular approaches to CRC have significantly advanced our understanding of its pathogenesis and transformed its clinical management. Identifying molecular subgroups in colorectal cancer is critically important for advancing specialized and personalized treatment approaches because of tumor heterogeneity, targeted therapy, avoiding ineffective treatment, predicting prognosis, clinical trial selection, and precision oncology. Continued research into molecular mechanisms and biomarkers holds the promise of further improving CRC outcomes through personalized and effective interventions.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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