Original Paper

Immunoexpression of Mismatch Repair Proteins in a Cohort of Colorectal Cancer Patients

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ABSTRACT: One of the molecular routes of colorectal carcinogenesis is the lack of mismatch repair (MMR) proteins, which may have substantial clinical consequences in predicting therapy success. This study aimed to analyze the expression of the MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), and MutS homolog 6 (MSH6) in a cohort of 91 colorectal cancer (CRC) patients, and to evaluate the relationship between patient clinicopathological characteristics and immunoexpression of these biomarkers. In this study, we obtained the highest scores of the MLH1 immunoexpression in non-mucinous tumors, moderately differentiated lesions, and in stage IV. The highest values of the MSH2 and MSH6 scores were observed in mucinous tumors, and poorly differentiated lesions, in stages II-III, and stages III-IV, respectively. To improve the stratification criteria for targeted oncological therapy and to predict patient outcomes, markers used may help evaluate the aggressiveness of lesions.

KEYWORDS: *Colorectal cancer, adenocarcinoma, mucinous tumors, MMR proteins.*

Introduction

Colorectal cancer (CRC) is a serious global public health concern, accounting for the second-leading cause of cancer-related mortality in industrialized nations and the sixth to seventh-largest cause of cancer-related mortality in developing countries. CRC is one of the most frequent neoplasias globally, attracting the attention of doctors, scientists, and society in general [1,2].

CRC molecular classification has received a lot of attention and scientific development in recent years [3].

There are mutations in tumor suppressor genes and oncogenes causing CRC. Colorectal carcinogenesis occurs via multiple routes, including chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylation (CIMP), with some overlap. MSI is most commonly seen in both hereditary non-polyposis and sporadic CRC [4-8].

MSI is utilized as a molecular marker to detect a faulty deoxyribonucleic acid (DNA) mismatch repair (MMR) system. The MMR pathway, which is involved in the pathogenesis of inherited and sporadic cancer, is one of the best-known molecular pathways. The MMR system helps maintain DNA homeostasis and plays a role in repairing certain types of errors that occur during DNA replication when dividing somatic cells [9].

MSI tumors are distinguished by a rapid accumulation of mutations caused by a deficiency MMR system [10].

Six MMR genes are now known: MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MutS homolog 3(MSH3), postmeiotic segregation increased 1 (PMS1), postmeiotic segregation increased 2 (PMS2), and MutS homolog 6 (MSH6) [11].

MSI is marked by the fact that MLH1 is negative and that the immunomarking positivity is normal because it refers to the normal expression of other proteins in tumor cells.

Although DNA testing is still the benchmark of excellence in the diagnosis of MSI, the College of American Pathologists (CAP) recommends immunohistochemistry (IHC) with a four-antibody panel for initial evaluation, a panel that includes MLH1, MSH2, MSH6, and PMS2, which determines whether or not protein is present or not products.

This study aimed to analyze the expression of MLH1, MSH2, and MSH6, in a cohort of CRC patients, evaluated in the Craiova Reference Center, and to evaluate the relationship between patient clinicopathological characteristics and immunoexpression of these biomarkers involved in CRC progression and invasion.

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Material and Methods

We conducted a retrospective study including 91 patients who underwent surgical resection for CRC, being diagnosed, admitted, and evaluated in the Surgical and 2nd Internal Medicine Clinic, the Clinical Emergency County Hospital Craiova, between October 2020 and October 2022.

Patients aged 18 years or older with a confirmed diagnosis of colon or rectal cancer supported by a histopathological (HP) result containing information on tumor type, grading, and classification in the pathological tumor-node-metastasis (pTNM) system were eligible for the study. Patients with benign colorectal pathology or patients with no pathological data were considered exclusion criteria.

In the Compartment of Pathology of the Clinical Emergency Hospital Craiova, HP, and IHC studies were carried out.

Specimens of the colon or rectum with 10% buffered formalin were used to represent the biological material, processed using the traditional histological approach, and stained with Hematoxylin-Eosin (HE). The lesions were identified using the most recent classification for tumors of the digestive system published by specific commissions of the World Health Organization (WHO) [12].

In this study, the immunoexpression of the MLH1, MSH2, and MSH6, was followed to the patient clinicopathological characteristics.

For IHC processing, 3μm serial sections were made from the paraffin blocks selected during the HP investigation and applied to slides treated with poly-L-lysine. The slides were then dried for 12 hours at laboratory temperature.

Afterward, the sections were deparaffinized in a benzene bath, thermostated at 58ºC, for 1 hour, then passed through two benzene baths for 10 minutes each. Rehydration was carried out by successively passing the sections through 4 alcohol baths with decreasing concentrations: absolute alcohol, 96%, 80%, and 70%, for 10 minutes/bath, and from the last bath they were placed in distilled water for 10 minutes.

The IHC study was of the type with enzymatic detection using the Labelled Streptavidin-Biotin2 System (LSAB) technique as a working method. The LSAB technique involves the optimal mixing of avidin and biotylinated peroxidase, prepared 30 minutes before use.

The result of the IHC reactions consisted of the visualization of the investigated antigens with the help of DAB chromogen (code 3467, Dako), by coloring them brown.

The obtained sections were examined with a Nikon Eclipse 90i microscope, equipped with a 5-megapixel color camera with cooling, apochromatic plane objectives. The images were acquired at different magnifications using the dedicated Nikon NIS-Elements software.

For each antibody used, we performed in tandem the positive external control on normal tissues that contain the investigated target antigen (positive sections), and the negative external control, which were processed under the same conditions as the lesional specimens.

The monoclonal antibodies used in this study (along with the clone, their source, and the dilution used) were against the MLH1 (Dako, clone NCH38, 1:50 dilution), MSH2 (Abcam, clone SC7893, 1:40 dilution), and MSH6 (Dako, clone 6G11, 1:50 dilution).

For the semiquantitative quantification of the analyzed markers, we used a scoring system, the final staining score (FSS), adapted based on data from the literature, which evaluated the intensity of the reactions and the percentage of marked cells (for each case was analysed 10 microscopic field).

The reactions were considered: intense (score 3), moderate (score 2), and weak (score 1), and the positivity threshold of the reaction was 5% immunostained tumor cells, according to which we established four groups, to evaluate the percentage of labeled cells: score 1 (5-25% cells), score 2 (26-50% cells), score 3 (51-75% cells), and score 4 (over 75% cells). The FSS had values from 1 to 12 and, at intervals 1-4, a low score is set, medium for a score of 6-8, and high if the score was 8-12.

Microsoft Excel was used to manage and process data collected from medical documents for patients. GraphPad Prism 5 trial version (San Diego, CA, USA) was used to analyze the data statistically. Categorical data were reported as a percentage and compared using the Chi-squared or Fisher's Exact test. The difference between the groups was assessed using one-way ANOVA for parametric variables and the Kruskal-Wallis test for non-parametric variables. The statistical threshold was 5%, and for $p \leq 0.05$ values, the results were considered significant.

The ethical aspects of the scientific research were respected, based on the patients' informed agreement. The Ethics Committee of the University of Medicine and Pharmacy of Craiova, No. 257/09.11.2023, approved the study.

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Results

The total number of 91 patients consisted of 39 (42.86%) females and 52 (57.14%) males, aged between 30-89 years, and mean±SD of 68.06±9.39 years. In our study, we had only three (3.30%) patients, males, under the age of 50.

Tumors were identified on the left colon in 52 patients (57.14% of the total), while tumors on the right colon were found in 38 (42.86%). Cases of tumors on the right side of the colon (25 cases, 27.47%) predominated in the female group, while cases of tumors on the left side of the colon (38 cases, 41.76%) predominated in the male group.

Among the 91 samples of colon adenocarcinoma, 14.29% (13 patients) of the tumors identified were mucin-producing colloid adenocarcinomas and for two (2.21%) patients we evidentiated signet ring cell adenocarcinoma (Table 1).

Depending on the tumor grade, most of the tumors were represented by moderately (G2, 53 tumors, 58.24%), and poorly differentiated tumors (G3, 36 tumors, 39.56%) (Figure 1).

According to the tumor stage of CRC [13], most of the patients were tumor stage III with a percentage of 46.71 (34 cases).

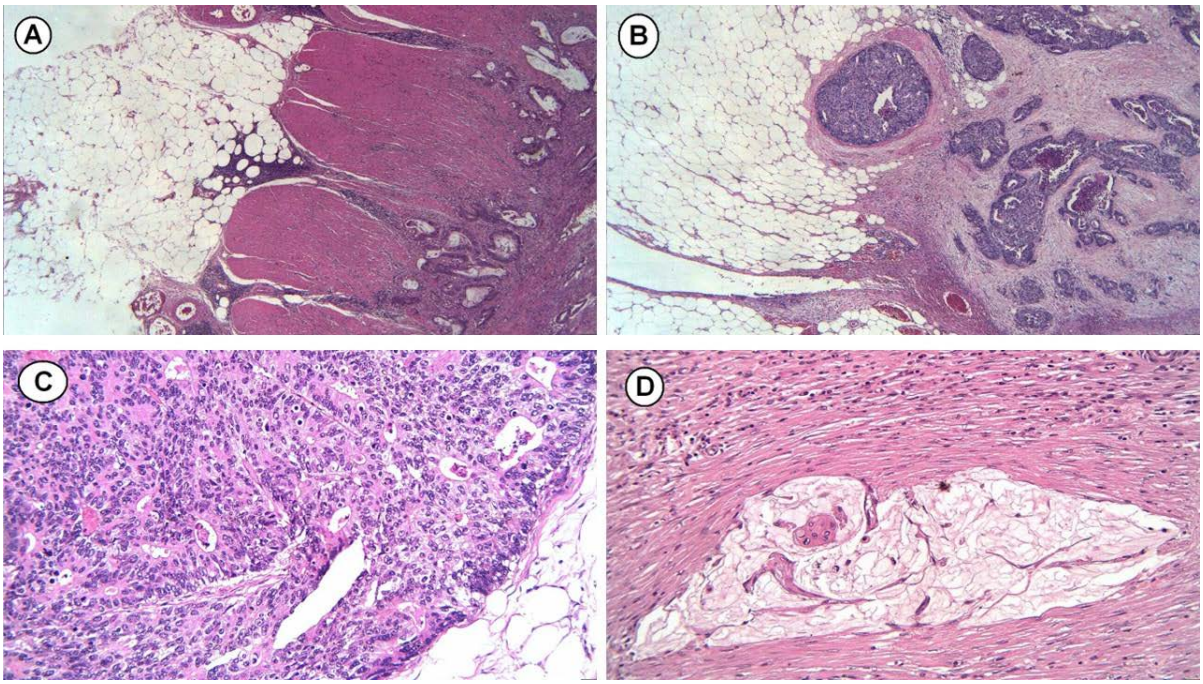

Figure 1. Colon adenocarcinoma (col HE): A. G1-well-differentiated adenocarcinoma with subserosal invasion, (x4); B. G2-moderately differentiated adenocarcinoma with invasion into the serosa and adjacent adipose tissue, (x4); C. G3-poorly differentiated adenocarcinoma, (x20); D. Mucinous adenocarcinoma, with mucus stains and cells in a "signet ring" with invasion into the muscularis propria, (x20).

Immunoexpression of MLH1

MLH1 was identified in 71 cases (78.02%) of investigated colorectal adenocarcinoma, with localization at the nuclear level. The negative cases belonging to 16 non-mucinous tumors and 5 mucinous colorectal adenocarcinoma (of high grade, most of them in stage I tumor).

In our study group, for the whole lot analyzed, the mean percentage of immunolabeled cells was 45.35±17.52, the intensity of IHC reactions was considered variable, and an FSS with a mean value of 4.39.

The strongest reactions were reported in non-mucinous adenocarcinomas, with the mean percentage of immunolabeled cells of 59.32±15.6, variable intensity, and a mean FSS value of 6.78 (Figure 2 A,D).

Mucinous colorectal adenocarcinomas showed a mean percentage of immunolabeled cells of 10.3±15.4, weak and moderate intensity of the reactions and the FSS having a mean value of 2.00.

We discovered that the G2-moderately differentiated lesions had greater scores, a mean percentage of immunolabeled cells of 61.18±14.75, with moderate intensity and a mean FSS value of 7.26 (Figure 2B).

Poorly-differentiated tumors had a mean percentage of immunolabeled cells of 67.6±31.6, variable intensity and a mean FSS value of 6.8. High-differentiated tumors had a mean percentage of 38.5±41.5 immunolabeled cells, the intensity was predominantly weak and moderate and the mean FSS value was 4.41.

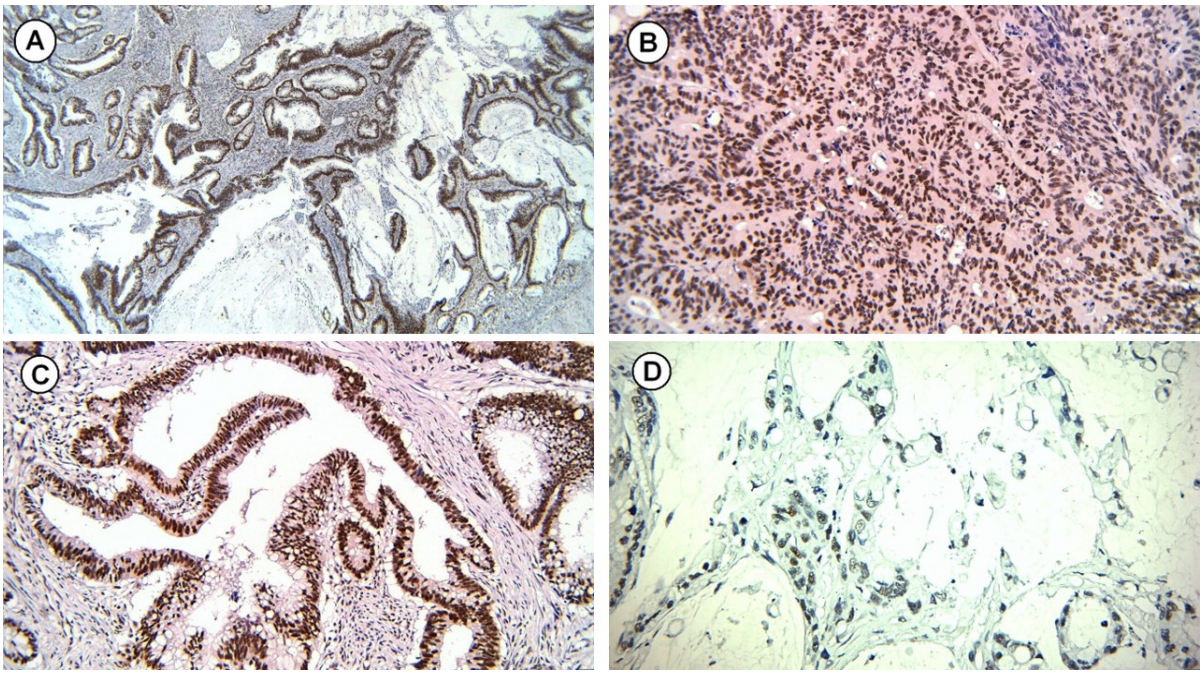

Figure 2. MLH1 immunohistochemical staining in colon cancer:
A. mucinous adenocarcinoma (x20); B. G2 non-mucinous colon adenocarcinoma (x20);
C. G3 non-mucinous colon adenocarcinoma (x20); D. mucinous adenocarcinoma (x20).

Table 1. The relation between the HP parameters and the expression of the MLH1, MSH2, and MSH6

Parameters/ No. Cases/ *p*-value		**FSSm MLH1**	**FSSm MSH2**	**FSSm MSH6**
HP type	Non-Mucinous (68)	6.78	8.00	7.74
	Mucinous (23)	2.00	11.40	10.00
	p-value	p<0.0001	0.002	0.056
Tumor grade	G1 (18)	6.80	8.50	7.50
	G2 (30)	7.26	9.07	9.03
	G3 (43)	4.41	11.00	11.25
	p-value	0.005	0.006	0.043
Tumor stage	I (19)	5.75	6.53	7.07
	II (26)	7.05	9.85	7.96
	III (34)	5.20	9.53	8.58
	IV (12)	7.88	7.00	9.56
	p-value	0.024	0.058	0.055

Note: MLH1: MutL homologue 1; MSH2: MutS homologue 2; MSH6: MutS homologue 6; Tumor grade: G1-well differentiated; G2-moderately differentiated; G3-poorly differentiated; HP: Histopathological; FSSm: Final staining score mean values; p-value <0.05 was considered statistically significant.

In terms of tumour stage, stage I adenocarcinomas showed a mean percentage of immunolabeled cells was 45.81±21.22, the intensity of IHC reactions was considered moderate, and a mean FSS value of 5.75. In the other stages II, III, and IV, the mean percentage of immunolabeled cells was 57.35±18.71, 44.72±22.6, and 60.55±24.37, with the intensity of the reactions predominantly moderate, and mean FSS values of 7.05, 5.20, and 7.88, respectively (Table 1).

MLH1 immunoexpression was statistically associated with HP type (p<0.0001, $\chi 2$ test), tumor grade (p=0.005, Kruskal-Wallis test), and tumor stage (p=0.024, Kruskal-Wallis test). The highest FSS mean values of MLH1 were recorded for non-mucinous tumors, moderately differentiated lesions, and in stage IV.

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Immunoexpression of MSH2

MSH2 was identified in 91.21% (83 cases) of investigated colorectal adenocarcinoma, the immunohistochemical staining being present at the nuclear level in the tumor cells. The negative cases belonging to 6 non-mucinous tumors and 2 mucinous tumors, with well differentiated grade, in stage I tumor.

In our study group, for the whole lot analyzed, the mean percentage of immunolabeled cells was 78.27±17.39, the intensity of IHC reactions was considered variable, and an FSS with a mean value of 9.70.

The strongest reactions were reported in mucinous adenocarcinomas, with a mean percentage of immunolabeled cells of 81.11±14.13, variable intensity, and a mean FSS value of 11.40 (Figure 3A,D).

Non-mucinous adenocarcinomas showed a mean percentage of 97.8±14.5 immunolabeled cells, variable intensity and a mean FSS value of 8.00.

We discovered that the G3-poorly differentiated lesions had greater values, a mean percentage of immunolabeled cells of 79.21±13.76 with intense intensity of reactions and a mean FSS value of 11.00 (Figure 3C).

In cases of moderately differentiated, the mean percentage of immunolabeled cells was 95.6±20.8, the reactions were predominantly intense and a mean FSS value of 9.07. High-differentiated adenocarcinomas presented a mean percentage of 85.7±35.6 labeled tumor cells, the reactions were intense and moderate, and thee mean FSS value was 8.5.

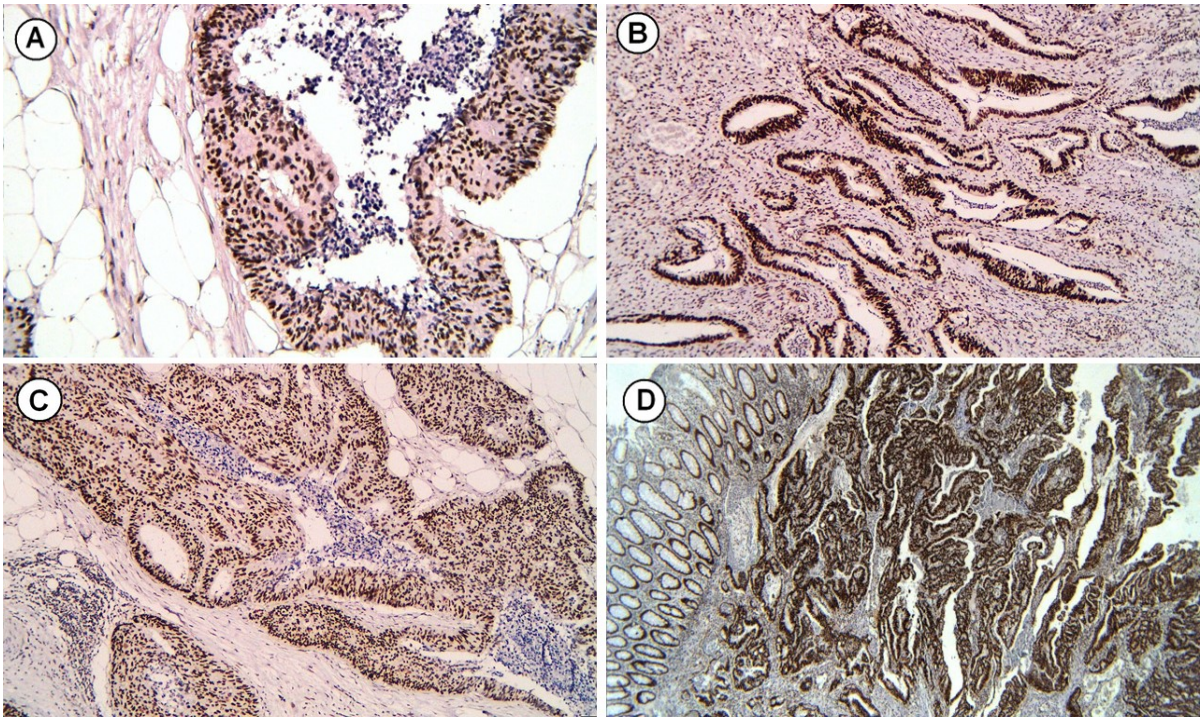

Figure 3. MSH2 immunohistochemical staining in colon cancer:
A. G2 non-mucinous colon adenocarcinoma (x20); B. G1 non-mucinous colon adenocarcinoma (x10);
C. G3 non-mucinous colon adenocarcinoma (x10); D. G2 non-mucinous colon adenocarcinoma (x4).

For tumor stages II and III, the mean percentage of marked cells was 75.81±21.22, and 68.65±20.77, the intensity of reactions was intense, and mean FSS values of 9.85 and 9.53, respectively (Table 1).

In tumor stages I and IV, the immunomarking was identified in all tumor cells, the intensity was variable and moderate, and the mean FSS values of 6.53, respectively 7.00.

MSH2 immunoexpression was statistically associated with HP type (p=0.002, $\chi 2$ test), tumor grade (p=0.006, Kruskal-Wallis test), instead, in relation to tumor stage, borderline significant values were recorded (p=0.058, Kruskal-Wallis test). The highest FSS mean values of MSH2 were recorded for mucinous tumors, poorly differentiated lesions, and in stages II-III.

Immunoexpression of MSH6

MSH6 was identified in 85 cases (93.41%) of investigated colorectal adenocarcinoma, the immunohistochemical staining being present at the nuclear level in the tumor cells. The negative cases belonging to 6 non-mucinous tumors, of moderate and high differentiated adenocarcinomas, included in stage I.

In our study tumors, the mean percentage of immunolabeled cells was 61.77±14.89, the reactions presented intense intensity of reactions, the FSS having a mean value of 8.87 (Table 1).

The strongest reactions were reported in mucinous adenocarcinomas, with a mean percentage of immunolabeled cells of 74.33±17.45, intense intensity of reactions, and mean FSS value of 10.00 (Figure 4A,D).

Non-mucinous adenocarcinomas presented a mean percentage of 97.8±14.5 immunomarked tumor cells, variable intensity and the mean FSS value was 7.74.

We discovered that the G3-poorly differentiated lesions had greater values, a mean percentage of immunolabeled cells of 81.44±18.23, with intense intensity of reactions, and a mean FSS value of 11.25 (Figure 4C).

Moderate and well differentiated tumors had a mean percentage of 95.6±20.8 and 89.2±31.4 immunolabeled tumor cells, variable intensity of reactions and the mean FSS value was 9.03, respectively 7.50.

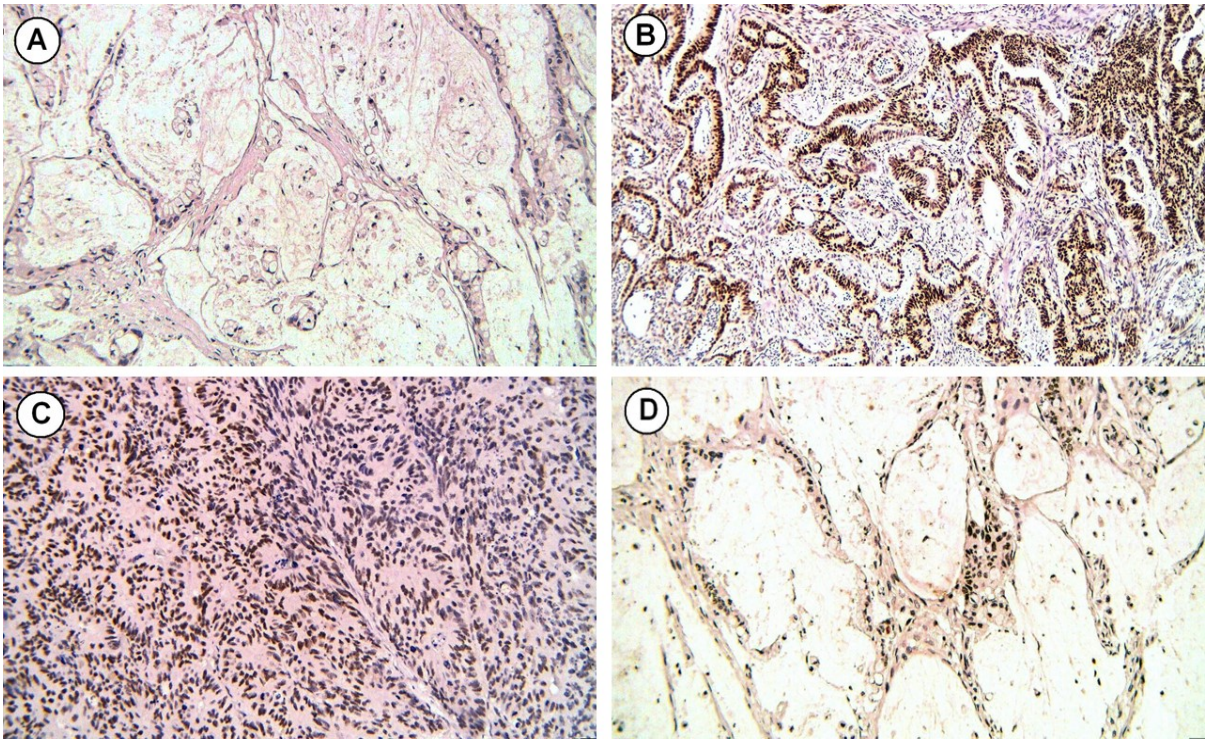

Figure 4. MSH6 immunohistochemical staining in colon cancer:
A. G1 mucinous colon adenocarcinoma (x20); B. G1 non-mucinous colon adenocarcinoma (x10);
C. G3 non-mucinous colon adenocarcinoma (x20); D. G1 mucinous colon adenocarcinoma (x20).

In terms of tumour stage, stage IV adenocarcinomas showed a greater mean percentage of immunolabeled cells of 75.11±23.46, the intensity of reactions was intense, and a mean FSS value of 9.56. Tumors classified in stage I and III presented a mean percentage of immunolabeled cells of 89.6±30.9 and 92.8±26.7, and for stage II the immunomarking was present in all tumor cells, the intensity of reactions of was variable for all stages. The mean FSS value was 7.07, and 8.58 for stage I and III tumors, and 7.96 for stage II tumors.

MSH6 immunoexpression was statistically associated with tumor grade ($p=0.043$, Kruskal-Wallis test), instead, in relation to HP type and tumor stage, borderline significant values were recorded ($p=0.056$, $\chi 2$ test, and $p=0.055$, Kruskal-Wallis test, respectively). The highest FSS mean values of MSH6 were recorded for mucinous tumors, poorly differentiated lesions, and in stages III-IV.

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Discussion

CRC has long been regarded as a single illness with distinct clinical stages and phenotypes described by neoplastic cell differentiation grade and structural growth pattern [14].

Several studies have shown that MLH1 and MSH2 play an important role, with mutations in both genes resulting in complete loss of function leading to tumor growth predominantly located in the proximal colon, and in sporadic MSI-positive tumors, hypermethylation was considerably more common [15-17].

Because gene mutation is the primary cause of decreased MLH1 and MSH2 expression, detecting those two genes is vital for understanding the etiology of sporadic CRC since they are the most important components of the MMR system.

IHC was employed in this investigation to detect MLH1, MSH2, and MSH6 immunoexpression in all patients who had undergone surgery since it was more accurate, quicker, and less expensive than other methods of detecting MMR status. As a result, IHC staining for MMR is now routinely performed in practically all institutions following surgery in the pathology department.

In our study, we aimed to analyze the expression of the MLH1, MSH2, and MSH6 in a cohort of CRC patients, and to evaluate the relationship between patient clinicopathological characteristics and immunoexpression of these biomarkers.

Methylation of the MLH1 promoter was frequently detected in elderly female patients, some of whom were often older than 70 years [18].

In our study, out of a total of 91 patients, there were 39 (42.86%) female patients with an average age of 69.05±9.32 years.

We identified in most cases with colorectal adenocarcinoma the MLH1 (71 cases, 78.02%), the MSH2 (83 cases, 91.21%), and the MSH6 (85 cases, 93.41%). These findings are consistent with those reported by other authors [19-23]. Zhao et al. [20], observed in a study that the MLH1, MSH2, and MSH6 expression was at 70.7, 71.7, and 33.7% of stage II colon cancer tissues, respectively. Also, the MLH1, MSH2, and MSH6 expression was higher in stage III tumors, at 90.2, 80.3, and 60.7% respectively.

In our study, we observed that the MLH1, MSH2, and MSH6 immunoexpression had a mean FSS value of 7.05, 9.85, and 7.96 in stage II, and 7.88, 7.00, and 9.56 in stage IV colon cancer tissues, and revealed a significant association in relation to the tumor stage. The differences in immunoexpression between tumor stages were statistically significant for all biomarkers investigated. There were significantly higher values in stage II and IV non-mucinous tumors than in stage I and III tumors (mean FSS value of 7.05 and 7.88 vs. 5.75 and 5.20, $p<0.05$, $\chi 2$ test) for MLH1; significantly higher in stage II and III non-mucinous tumors, than in stage I and IV tumors (mean FSS value of 9.85 and 9.53 vs. 6.53 and 7.00, $p<0.05$, $\chi 2$ test) for MSH2; significantly higher in stage III and IV non-mucinous tumors, than in stage I and II tumors (mean FSS value of 8.58 and 9.56 vs. 7.07 and 7.96, $p<0.05$, $\chi 2$ test) for MSH6. A study published by Zhao et al. communicated similar expressions for MLH1 and MSH6 and were significantly higher in stage III colon cancer than in stage II tumors ($\chi 2=8.225$ and 10.798, respectively; $p<0.05$) [20].

Our results indicate that the highest values of the MLH1 scores are observed in non-mucinous tumors, and moderately differentiated lesions, in contrast to the MSH2, and MSH6 scores that have the highest values in mucinous tumors and poorly differentiated lesions. These results are in line with a number of previous studies. Lanza et al. [25], for example, found that individuals with MLH1/MSH2-positive carcinomas were younger, had tumors in the right colon, had infrequent nodal metastases, were bigger, and had poorly differentiated or mucinous histology. An MMR expression system was found in 15% of stage II and III colon cancers in another study by Sinicrope et al [26], and the MMR phenotype was substantially linked with higher tumor stage, proximal site, poor or undifferentiated histology, female sex, and older age. The MLH1 and MSH6 protein expression was found to be substantially related to big, poorly differentiated tumors with extraserosal invasion and uncommon lymph node metastases in Zhao et al.'s study [20].

Our statistical study of the immunoexpression of MLH1, MSH2, and MSH6 demonstrated a strong association with HP type ($p<0.05$, $\chi 2$ test), regarding the type of adenocarcinomas, the strongest reactions were observed in mucinous tumors. Previous studies have shown the same results, the isolated expression of MLH1 or MSH2 was associated with mucinous differentiation [27-30].

Conclusions

In this study, we obtained the highest scores of the MLH1 immunoexpression in non-mucinous tumors, moderately differentiated lesions, and in stage IV.

The highest values of the MSH2 and MSH6 scores were observed in mucinous tumors, poorly differentiated lesions, and in stages II-III, and stages III-IV, respectively.

To improve the stratification criteria for targeted oncological therapy and to predict patient outcomes, markers used may help evaluate the aggressiveness of lesions.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest.

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