Original Paper

Assessment of mismatch repair deficiency, CDX2, beta-catenin and E-cadherin expression in colon cancer: molecular characteristics and impact on prognosis and survival – an immunohistochemical study

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Abstract

Microsatellite instability (MSI) or the deficiency of mismatch repair (MMR) proteins is one of the molecular pathways of colorectal tumorigenesis and may have important clinical implications in predicting the treatment response. We evaluated the relationship between clinicopathological features and MMR proteins [mutL homologue 1 (MLH1), mutS homologue 2 (MSH2), mutS homologue 6 (MSH6), postmeiotic segregation increased 2 (PMS2)], adhesion molecules (E-cadherin, beta-catenin) and caudal-type homeobox 2 (CDX2) in 31 patients with colon adenocarcinoma, using immunohistochemistry. We also aimed to assess the prognostic value of the studied proteins. MLH1 loss was correlated to PMS2 loss ($p=0.006$) and MSH2 loss ($p=0.023$); MSH2 loss was significantly associated to MSH6 loss ($p=0.011$). Tumors with MSH6 loss, together with tumors with PMS2 loss, covered all the patients with MSI status. We found a significant correlation between MSI tumors and mucinous histological type ($p=0.03$), but no significant associations with other clinicopathological features or with survival rate. There was a significant correlation between E-cadherin expression and differentiation degree ($p=0.018$) and between beta-catenin expression and lymph node invasion ($p=0.046$). No significant association between CDX2 loss and any clinical or pathological features was found ($p>0.05$). No significant differences were identified in overall survival according to E-cadherin, beta-catenin or CDX2 expression ($p>0.05$). In our study, PMS2 loss was significantly correlated with CDX2 loss ($p=0.03$). In conclusion, the molecular analysis of biological markers for colon cancer may be important for patient stratification, in order to select the optimal treatment algorithm. Our results suggest that probably the double panel (MSH6 and PMS2) is enough to detect the MSI status, instead of using the quadruple panel.

Keywords: colon cancer, microsatellite instability, mismatch repair proteins, CDX2, adhesion molecules.

⊡ Introduction

Despite the numerous current diagnostic and therapeutic approaches, colorectal cancer (CRC) is a major public health problem, globally being the third most prevalent cancer in both men and women [1] and the second cause of mortality [1–4].

In Romania, according to *Global Cancer Observatory*, colon cancer was the second in terms of both incidence (13.3%), and mortality (17%), after lung cancer [5].

However, the incidence (0.6% per year) and the number of deaths (18% per year in men and 1.6% per year in women) has declined in the recent years [1, 3], which proves the efficiency of the screening and therapeutic methods and a better understanding of the molecular mechanisms involved in colon carcinogenesis [2, 3].

It is unanimously accepted that CRC is a heterogeneous disease, with various clinical and pathological characteristics, different prognosis, and response to treatment, even in patients with the same tumor, node, metastasis (TNM) stage [6]. This may be due to the various molecular mechanisms implicated in colon tumorigenesis [4].

Two pathways are recognized in colon carcinogenesis: the chromosomal instability (CIN) and microsatellite instability (MSI) [3, 7–10].

MSI occurs when the mismatch repair (*MMR*) genes are inactivated; under normal conditions, these genes repair the spontaneous defects that occur during deoxyribonucleic acid (DNA) replication [11]. Because of *MMR* genes inactivation, the cell becomes more vulnerable to further mutations. These mutations affect the short DNA sequences,

called microsatellite regions [12]. Microsatellites are tandem repetitive sequences of DNA nucleotides [*e.g.*, (CA)n], normally found in the human genome that are susceptible to insertions/deletions mutations during DNA replication [12]. The lack of efficient *MMR* genes enables permanent changes in the length of these repetitive sequences or microsatellites (insertion or deletion of nucleotides), a process called MSI [3, 4, 11–13].

Tumors with MSI are found not only in the colon, but can also occur, at a lower rate, as extracolonic malignancies, particularly endometrial cancer [3, 6, 11, 14–17], breast cancer [11], ovarian cancer [6, 16–18], pancreatic cancer [11], stomach cancer [6, 11, 19], prostate and urinary bladder cancer [6, 11, 20, 21], head and neck squamous cell carcinoma [6], non-small cell lung cancer [20, 22], melanoma [23], etc.

MMR genes involved in the development of colorectal MSI tumors are mutL homologue 1 (*MLH1*) gene, mutS homologue 2 (*MSH2*) gene, mutS homologue 6 (*MSH6*) gene or postmeiotic segregation increased 2 (*PMS2*) gene [11, 24]. Inactivation of *MLH1* and *MSH2* accounts for over 90% of deficient MMR (dMMR) cases [25].

The *MMR* genes inactivation can be inherited, in hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome, when germline mutations block the expression of *MMR* genes, usually *MLH1* or *MSH2* (85% of cases). In 12–15% of cases, the genes inactivation can be acquired by epigenetic changes, *e.g.*, in sporadic cancer, when DNA repair genes are inactivated by methylation [3, 4, 6]. *MLH1* hypermethylation is an epigenetic change that could be the main cause of *MMR* genes inactivation in sporadic cancer (70–95% of cases) [4, 6, 11, 12, 26]; it can also be associated, in approximately 50% of cases, with mutations of oncogenic *BRAF V600E* gene [3, 6, 24]. In contrast, in hereditary cancer, there are no *BRAF* mutations, which could be an exclusion criterion for Lynch syndrome [3, 4, 25].

In order to increase the MMR system stability, the *MMR* genes work in pairs, forming heterodimers *MLH1*/ *PMS2* and *MSH2*/*MSH6* [12, 24]. *MLH1* is responsible for the stability of *PMS2*, so, in cases with combined loss of *MLH1* and *PMS2*, the defective gene is *MLH1* [25, 26]. A similar process appears with *MSH2*/*MSH6*, in this situation *MSH2* stabilizing the complex [24, 26]. Isolated loss of *MSH6* reveals defective *MSH6* gene, whereas loss of both protein expression (MSH2 and MSH6) usually indicates that the defect is within *MSH2* [12, 24–26].

The MSI tumor status can be revealed either by molecular genetic techniques, such as polymerase chain reaction (PCR), or by immunohistochemistry, in order to assess the MMR protein expression [3, 27]. The two techniques have been proven to have a high correlation degree [6, 25]; immunohistochemistry is frequently used, since it is more accessible and cost-effective [26].

Tumors that lack MMR proteins are defined as dMMR or MSI tumors, while tumors that express MMR proteins, are regarded as microsatellite stable (MSS) tumors [25, 26].

Many other biological markers, such as caudal-type homeobox 2 (CDX2) or adhesion molecules (E-cadherin, beta-catenin), have been blamed to be involved in colon cancer progression and invasion.

E-cadherin is a transmembrane cell adhesion molecule, important in epithelial cells development and growth. It is responsible for epithelial cell cohesion and is functionally necessary for epithelial tissue integrity [28–30]. Pathological loss of E-cadherin function is associated with epithelial-to-mesenchymal transition (EMT), tumor progression and metastasis [30–32]. Beta-catenin is part of the adherens junctions that binds to cytoplasmatic domain of E-cadherin, thus forming cell-to-cell adhesion. Beta-catenin promotes cell differentiation, proliferation, migration, and invasion [33, 34].

CDX2 is a transcription factor involved in differentiation and proliferation of intestinal epithelial cells, mainly in the distal part of the intestine. Loss of CDX2 expression was reported to be involved in progression of some sporadic CRCs [35–39].

Aim

In the present study, we aimed to analyze the relationship between patient clinicopathological features and immunoexpression of several biological markers, supposed to be involved in colon cancer progression and invasion, such as: MMR proteins (MLH1, MSH2, MSH6, PMS2), adhesion molecules (E-cadherin, beta-catenin), and CDX2. We also aimed to evaluate the correlation between all the analyzed proteins and survival rate.

⌘ Patients, Materials and Methods

A total of 31 patients who underwent surgical resection for colon cancer at the 3rd Surgical Clinic, Prof. Dr. Octavian Fodor Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania, between 2005 and 2006, were included in this study. This is a retrospective study therefore the status of cancer specimens (sporadic or inherited) cannot be specified. No neoadjuvant chemotherapy was applied. The Ethics Committee of Prof. Dr. Octavian Fodor Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, had approved this study.

Both histopathological and immunohistochemical (IHC) studies were performed in the Department of Pathology, Prof. Dr. Ion Chiricuță Institute of Oncology, Cluj-Napoca.

Firstly, the 10% formalin-fixed tissue samples were embedded in tissue microarray (TMA) paraffin blocks, cut in 5 μm thick sections, and stained with Hematoxylin–Eosin (HE) for routine examination. The microscopic analysis was performed by two anatomopathologists and the histological type was established according to the 2010 *World Health Organization* (WHO) *Classification of Tumours of the Digestive System* [40, 41]. We evaluated the tumor location, histological type, tumor differentiation degree, lymph node metastasis, perineural and vascular invasion, tumor necrosis, local invasion, peritumoral inflammatory infiltrate, etc. Tumor stage was established according to *American Joint Committee on Cancer* (AJCC, 8th edition) classification of colon cancer [42]. Mucinous adenocarcinoma was diagnosed if the tumor had more than 25% mucus pools.

Subsequently, the IHC determinations were carried out, using the Streptavidin–Biotin complex. IHC used primary monoclonal antibodies against: MLH1 (Novocastra, clone ES05, 1/100 dilution), PMS2 (Novocastra, clone M0R4G,

1/100 dilution), MSH2 (Novocastra, clone 25D12, 1/100 dilution), MSH6 (Novocastra, clone PU29, 1/100 dilution), beta-catenin (Novocastra, clone 17C2, 1/80 dilution), E-cadherin (Novocastra, clone 36B5, 1/80 dilution), and CDX2 (Novocastra, clone EP25, 1/50 dilution).

Assessment of protein expression

The sections were examined with an Olympus CKX 41 microscope.

Loss of MMR protein expression was considered when nuclear staining was completely absent in the tumor cells. The positive internal control was considered the nuclear staining in normal colonic mucosa, stromal cells, and lymphocytic infiltration. The normal expression of all four MMR proteins (MLH1, MSH2, MSH6, and PMS2) in tumor cells was considered to be MSS.

For beta-catenin, the positive control was the membranous staining in normal colonic mucosa, whereas in the tumor cells there was an aberrant nuclear/ cytoplasmatic expression. E-cadherin was identified as a normal membranous expression in normal colonic mucosa (positive control). In tumor cells, E-cadherin stained as a diffuse cytoplasmic and membranous signal. To evaluate the beta-catenin and E-cadherin immunostaining, we assessed the percentage of positive cells out of 100 visible cells in the microscopic field, while the intensity score represented the average staining intensity (0 – negative, 1 – weak, 2 – intermediate, and 3 – strong) [28].

Nuclear CDX2 expression was seen in normal and neoplastic colonic mucosa. All the colonic tumors that had a nuclear expression of CDX2 were scored CDX2 positive, and the tumors that lacked nuclear CDX2 expression were scored CDX2 negative.

The median survival time for the studied group was 32.26 ± 8.73 months. Survival data was obtained using the *National Administrative Databases* (*Computerized Population Register Service*).

Statistical analysis

For statistical analysis, version 25 for Widows of the Statistical Package for the Social Sciences (SPSS) statistical processing program was used. The normality of data distribution was assessed with Kolmogorov–Smirnov test. The association between qualitative variables was observed using χ^2 (*chi*-squared) test, Fisher's exact test, and relative risk (RR). Non-parametric tests for correlations (Spearman,

Mann–Whitney *U*-test for independent samples) were used for the variables which did not follow a normal distribution. For the description of the data contingency tables, frequency, average and standard deviation were used. The *p*-value was considered significant when ≤0.05.

Results

The total number of 31 patients included 15 (48.39%) men and 16 (51.61%) women; the age ranged from 19 to 76 years, with a mean age of 63 ± 11.71 years. Five (16.12%) patients were less than 50 years. Among the 31 samples of colon adenocarcinomas, 48.39% were located in the right colon, 67.74% were intestinal type, and 32.26% were considered mucinous type (with more than 25% mucus pools). Most of the tumors (48.39%) were moderately differentiated, 29.03% were well differentiated, and 22.58% of tumors were poorly differentiated.

Regarding the tumor stage, according to the TNM staging of CRC [42], most of the patients (54.8%) were TNM stage III and 9.67% of patients had distant metastases. Lymph node metastases were detected in 58.06% of cases; both venous and perineural invasion were observed in 22.58% of cases. Tumor necrosis and peritumoral lymphocytic infiltrate were present in 38.70%, respectively 45.16% of cases. Local invasion in nearby organs was detected in only 6.45% of cases. The follow-up period was 40 months; the median survival time was 32.26 ± 8.73 months.

IHC analysis of MMR proteins detected a loss of expression for one or more MMR proteins in 26.81% (8/31 patients). Among all patients with dMMR tumors, individual or combined loss for MLH1, MSH2, MSH6 and PMS2 proteins was noticed in 75% (6/8 patients), 50% (4/8), 50% (4/8), and 62.5% (5/8), respectively (Figures 1–4).

Combined loss of MLH1 and PMS2 (MLH1/PMS2 loss status) was detected in 62.5% (5/8 patients) of dMMR tumors and combined loss of MSH2 and MSH6 (MSH2/MSH6 loss status) in 75% (6/8 patients) of MSI tumors. Loss of MSH6, without MSH2 loss was seen in 25% (2/8 patients) MSI patients, but we did not find any tumors with isolated loss of PMS2, without MLH1 loss. Tumors with MSH6 loss, together with tumors with PMS2 loss, covered all the dMMR tumors.
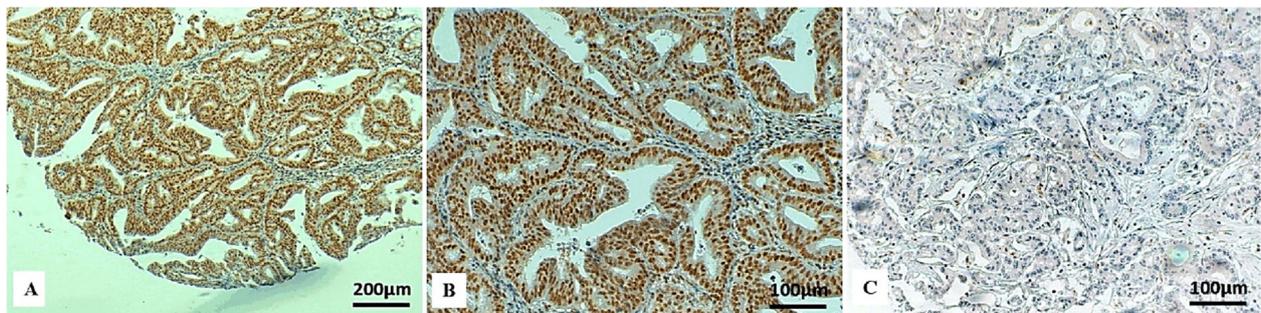

Figure 1 – *Immunohistochemical staining for MLH1 in colon cancer: (A) Positive nuclear immunostaining for MLH1 in tumor cells and normal intestinal mucosa (upper right corner) (×100); (B) Strong nuclear immunoreactivity for MLH1 in tumor cells (×200); (C) MLH1 loss in tumor cells, with positive internal control of stromal lymphocytes (×200). MLH1: mutL homologue 1.*
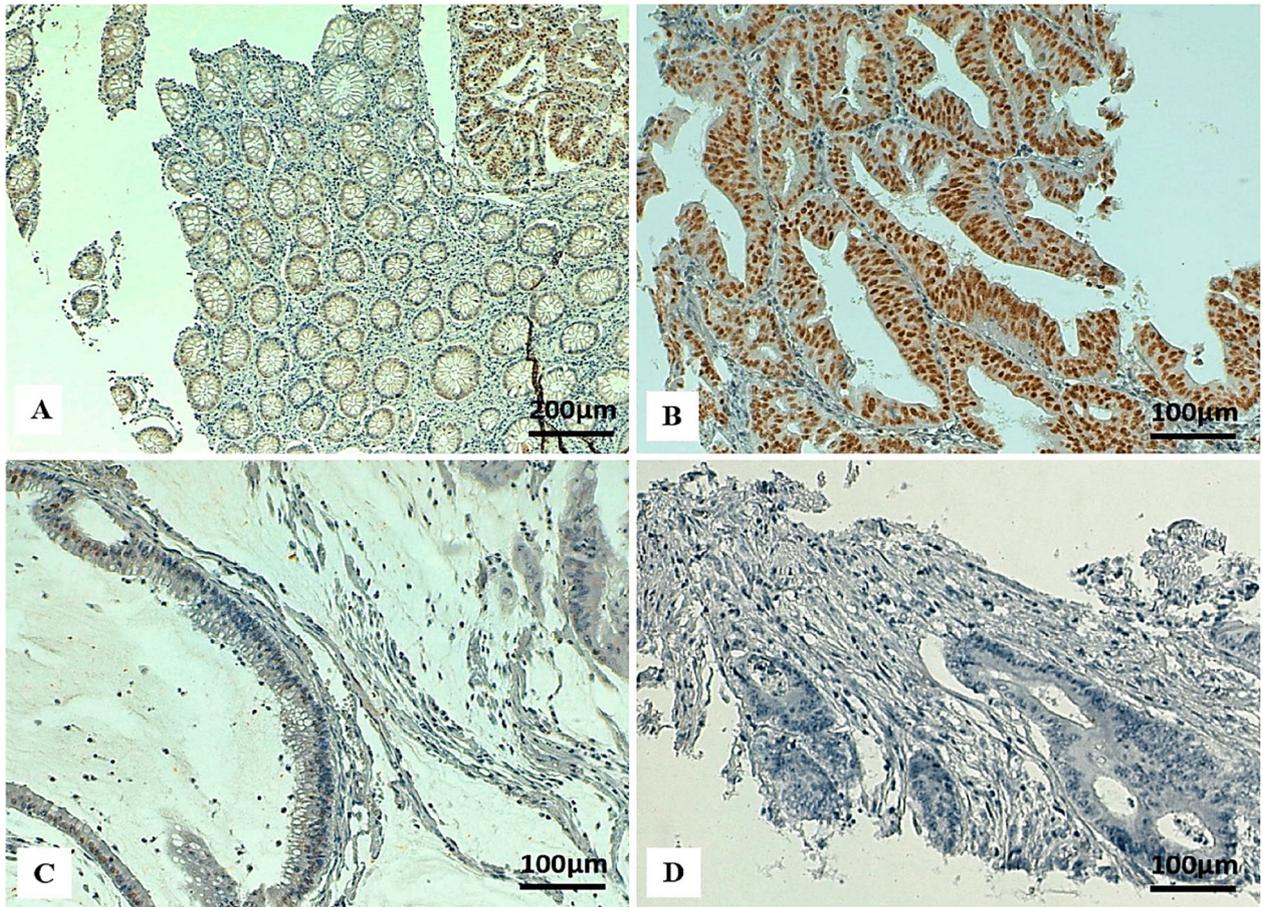
Figure 2 – *Immunohistochemical staining for MSH2 in colon cancer: (A) Positive nuclear immunostaining for MSH2 in normal intestinal mucosa and in tumor cells (upper right corner) (×100); (B) Strong nuclear immunoreactivity for MSH2 in tumor cells (×200); (C) Low intensity of nuclear immunostaining for MSH2 in tumor cells (×200); (D) MSH2 loss in tumor cells (×200). MSH2: mutS homologue 2.*
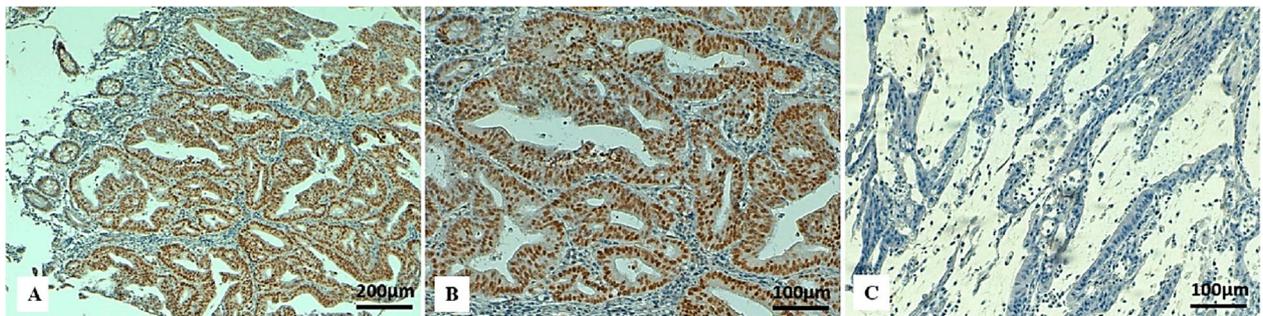

Figure 3 – *Immunohistochemical staining for MSH6 in colon cancer: (A) Positive nuclear immunostaining for MSH2 in normal intestinal mucosa (left upper corner) and in tumor cells (×100); (B) Strong positive nuclear immunoreactivity for MSH6 in tumor cells (×200); (C) Absence of nuclear immunostaining for MSH6 in tumor cells in a mucinous colon cancer (×200). MSH6: mutS homologue 6.*
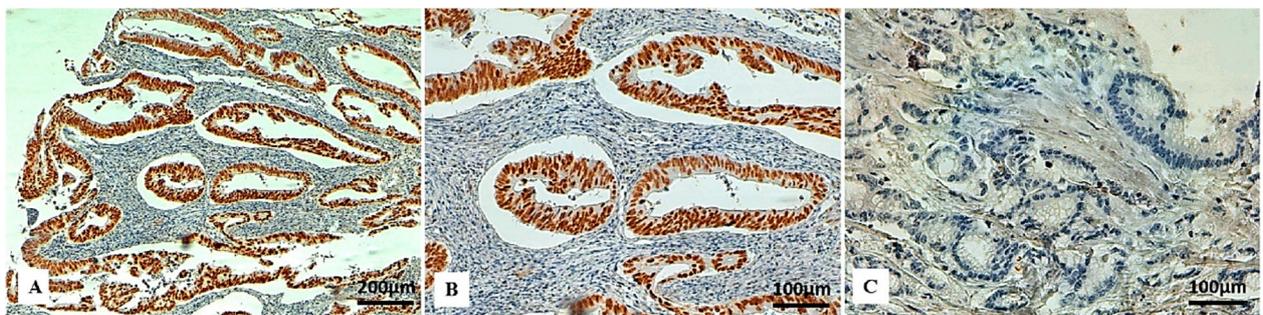

Figure 4 – *Immunohistochemical staining for PMS2 in colon cancer: (A) Positive nuclear immunostaining for PMS2 in tumor cells (×100); (B) Strong positive nuclear immunostaining for PMS2 in tumor cells (×200); (C) PMS2 loss in tumor cells (×200). PMS2: Postmeiotic segregation increased 2.*

The relationship between individual loss of MMR protein immunoexpression and clinicopathological parameters is presented in Table 1. There was no significant relationship between isolated loss of MMR proteins and the clinicopathological features, such as: gender, tumor location, histological type, tumor stage, tumor grade, perineural invasion, vascular invasion, tumor necrosis, local invasion, peritumoral inflammatory infiltrate ($p>0.05$) (Table 1). Relation between age and loss of MLH1 was at the limit of statistical significance ($p=0.066$), tumors with loss of MLH1 being more frequent in young patients (under the age of 50). Correlation between lymph node metastasis (pN) and individual MMR protein loss was not statistically significant, but near-marginal significance for MSH2 loss ($p=0.163$) and PMS2 loss ($p=0.181$).

No significant correlation was found between the individual expressions of MMR proteins and survival rate, but tumors with loss of MSH6 showed a tendency towards a better survival ($p=0.131$) (Table 1).

Table 1 – *Association between individual loss of MMR protein immunoexpression and clinicopathological parameters*

		N (%)	MLH1 loss Mean [%]	p-value	MSH2 loss Mean [%]	p-value	MSH6 loss Mean [%]	p-value	PMS2 loss Mean [%]	p-value
Age [years]	<50	5 (16.12)	42.66	0.066	45.21	0.654	49.38	0.984	37.81	0.830
	>50	26 (83.87)	18.13		49.56		50.33		40.50	
Gender	Female	16 (51.61)	31.56	0.682	41.77	0.358	40.62	0.188	40.94	0.800
	Male	15 (48.39)	29.97		53.22		59.67		37.17	
Tumor site[1]	Right	15 (48.39)	37.50	0.247	53.00	0.401	47.67	0.654	44.00	0.446
	Left	16 (51.61)	24.50		41.98		51.88		34.53	
Histological type[2]	Intestinal	21 (67.74)	33.54	0.262	47.98	1.00	58.57	0.356	41.96	0.493
	Mucinous	10 (32.26)	28.53		46.76		42.65		36.76	
Tumor grade[3]	G1	9 (29.03)	40.25	0.210	48.96	0.623	52.50	0.858	49.38	0.230
	G2	15 (48.39)	23.91		42.50		51.88		31.25	
	G3	7 (22.58)	35.71		56.43		42.14		45.36	
pN	N0	13 (41.93)	27.46	0.945	57.82	0.163	39.20	0.238	47.88	0.181
	N1	11 (35.48)	29.55		47.73		35.63		28.64	
	N2	7 (22.58)	38.93		27.14		31.43		39.29	
Vascular emboli	Absent	24 (77.41)	29.69	1.00	46.18	0.729	53.13	0.317	37.40	0.473
	Present	7 (22.58)	34.57		51.19		38.57		45.00	
Perineural invasion	Absent	24 (77.41)	29.69	1.00	47.64	0.945	49.17	0.872	41.35	0.444
	Present	7 (22.58)	34.57		46.19		52.14		31.43	
Lymphocytic response	Absent	17 (54.83)	29.85	0.830	52.84	0.316	48.24	0.922	38.38	0.953
	Present	14 (45.16)	31.93		40.60		51.79		40.00	
Tumor necrosis	Absent	19 (61.3)	24.47	0.367	46.75	0.889	46.84	0.617	41.05	0.857
	Present	12 (38.7)	40.79		48.19		54.58		36.04	
Metastasis	Absent	28 (90.32)	32.12	0.826	43.45	0.55	49.29	0.777	37.23	0.285
	Present	3 (9.67)	18.33		63.33		55.00		56.67	
Local invasion	Absent	29 (93.54)	28.26	0.129	47.13	1.00	50.17	0.727	38.53	0.520
	Present	2 (6.45)	67.50		50.00		45.00		47.50	
TNM staging [41]	I	2 (6.45)	17.50	0.995	55.00	0.202	55.00	0.801	45.00	0.226
	II	9 (29.03)	33.00		50.74		58.89		50.28	
	III	17 (54.8)	33.38		38.24		43.53		29.41	
	IV	3 (9.67)	18.33		83.33		55.00		56.67	
Survival status	Present	24 (77.41)	33.24	0.701	44.20	0.564	56.09	0.131	38.59	1.00
	Absent	7 (22.58)	25.00		52.86		30.71		39.29	

MLH1: mutL homologue 1; MMR: Mismatch repair; MSH2: mutS homologue 2; MSH6: mutS homologue 6; *N*: No. of cases; PMS2: Postmeiotic segregation increased 2; TNM: Tumor, node, metastasis. [1]Tumor site: right colon location – cecum, ascending colon; left colon location – transverse segment to the sigmoid. [2]Mucinous type: more than 25% mucus pools. [3]Tumor grade: G1 – well differentiated; G2 – moderately differentiated; G3 – poorly differentiated. *p*-value <0.05 was considered statistically significant.

When comparing the relationship between MSS or MSI status and clinicopathological parameters (Table 2), we found a statistically significant correlation between MSI tumors and histological type ($p=0.03$). Patients with MSI tumors had a high RR to develop mucinous tumors [RR: 2.045, 95% confidence interval (CI): 0.966–3.033] than intestinal tumors [RR: 0.303, 95% CI: 0.050–1.042]. In this study, patients with deficient MMR proteins did not differ in terms of age, gender, tumor location, lymph node invasion, tumor differentiation grade, tumor stage, perineural invasion, vascular invasion, peritumoral inflammatory infiltrate, invasion of nearby organs (Table 2). No significant differences were identified in overall survival according to the MSI status ($p=0.199$).

We found a positive aberrant cytoplasmatic expression for E-cadherin in 95.83% samples (Figure 5) and aberrant positive nuclear/cytoplasmatic beta-catenin immunostaining in all histological samples (Figure 6). There was no immunostaining for CDX2 in 25.80% cases (Figure 7).

IHC analysis of cell adhesion molecules (E-cadherin and beta-catenin), CDX2, clinicopathological features and survival rates is presented in Table 3.

Table 2 – *Association between MMR status and clinicopathological characteristics*

		N (%)	MSS N (%)	MSI N (%)	p-value	Total N (%)
Age [years]	<50	5 (16.12)	3 (9.67)	2 (6.45)	1.000**	5 (16.12)
	>50	26 (83.87)	20 (54. 8)	6 (29)		26 (83.9)
Gender	Female	16 (51.61)	11 (35.48)	5 (16.12)	0.809*	16 (51.6)
	Male	15 (48.39)	12 (38.70)	3 (9.67)		15 (48.39)
Tumor site[1]	Right	15 (48.39)	11 (35.48)	4 (12.90)	0.909*	15 (48.39)
	Left	16 (51.61)	12 (38.70)	4 (12.90)		16 (51.61)
Histological type[2]	Intestinal	21 (67.74)	18 (58.06)	3 (9.67)	0.03*	21 (67.74)
	Mucinous	10 (32.26)	5 (16.13)	5 (16.13)		10 (32.26)
Tumor grade[3]	G1	9 (29.03)	8 (25.80)	1 (3.2)	0.288*	9 (29.03)
	G2	15 (48.38)	11 (35.48)	4 (12.90)		15 (48.38)
	G3	7 (22.58)	4 (12.90)	3 (9.67)		7 (22.58)
pN	N0	13 (41.93)	10 (32.25)	3 (9.67)	0.455*	13 (41.93)
	N1	11 (35.48)	9 (29.03)	2 (6.45)		11 (35.48)
	N2	7 (22.58)	4 (12.90)	3 (9.67)		7 (22.58)
Venous emboli	Absent	24 (77.41)	17 (54.83)	7 (22.58)	0.664*	24 (77.41)
	Present	7 (22.58)	6 (19.35)	1 (3.22)		7 (22.58)
Perineural invasion	Absent	24 (77.41)	17 (54.83)	7 (22.58)	0.664*	24 (77.41)
	Present	7 (22.58)	6 (19.35)	1 (3.22)		7 (22.58)
Lymphocytic response	Absent	17 (54.83)	14 (45.16)	3 (9.67)	0.436*	17 (54.81)
	Present	14 (45.16)	9 (29.03)	5 (16.12)		14 (45.16)
Tumor necrosis	Absent	19 (61.3)	15 (48.38)	4 (12.90)	0.567*	19 (61.3)
	Present	12 (38.7)	8 (25.80)	4 (12.90)		12 (38.7)
Metastasis	Absent	28 (90.32)	20 (64.51)	8 (25.80)	0.535**	28 (90.32)
	Present	3 (9.67)	3 (9.67)	0 (0.0)		3 (9.67)
Invasion in nearby organs	Absent	29 (93.54)	21 (67.74)	8 (25.80)	0.527**	29 (93.5)
	Present	2 (6.45)	2 (6.45)	0 (0)		2 (6.45)
TNM staging [41]	I	2 (6.45)	2 (6.45)	0 (0)	0.597*	2 (6.45)
	II	9 (29.03)	6 (19.35)	3 (9.67)		9 (29.0)
	III	17 (54.8)	11 (35.48)	5 (16.12)		17 (54.8)
	IV	3 (9.7)	3 (9.67)	0 (0)		3 (9.7)
Survival status	Living	24 (77.41)	19 (61.29)	5 (16.12)	0.199*	24 (77.41)
	Deceased	7 (22.58)	4 (12.90)	3 (9.67)		7 (22.58)

MMR: Mismatch repair; MSI: Microsatellite instability; MSS: Microsatellite stable; N: No. of cases; TNM: Tumor, node, metastasis. [1]Tumor site: right colon location – cecum, ascending colon; left colon location – transverse segment to the sigmoid. [2]Mucinous type: more than 25% mucus pools. [3]Tumor grade: G1 – well differentiated; G2 – moderately differentiated; G3 – poorly differentiated. *p*-value <0.05 was considered statistically significant. **Chi*-squared test; **Fisher's exact test.
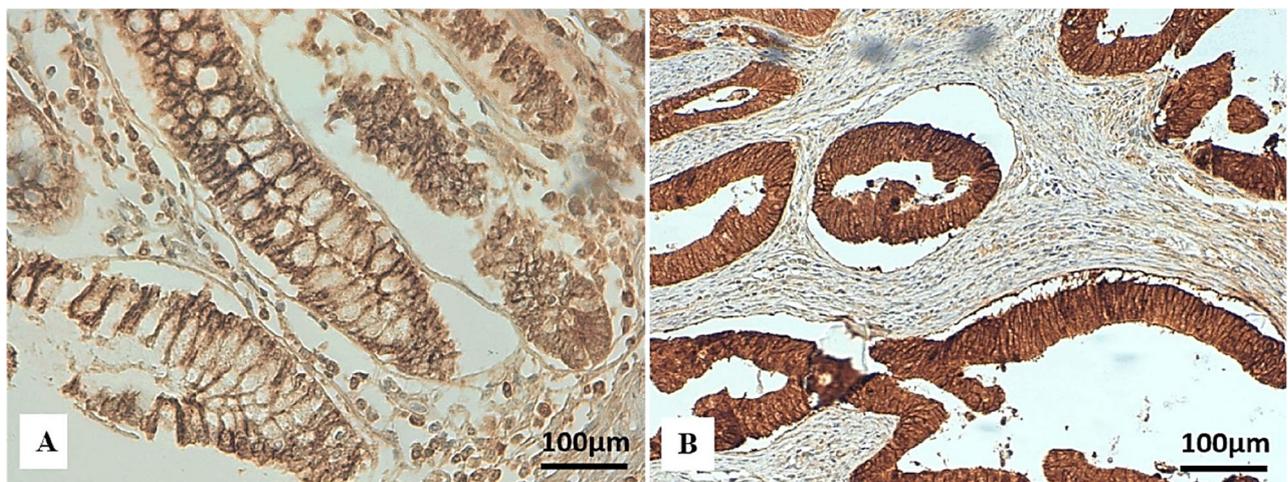

Figure 5 – *Immunohistochemical staining for E-cadherin in colon cancer: (A) Normal membranous immunoexpression of E-cadherin in normal colon mucosa (×200); (B) Strong cytoplasmatic and membranous immunoexpression of E-cadherin in tumor cells (×200).*
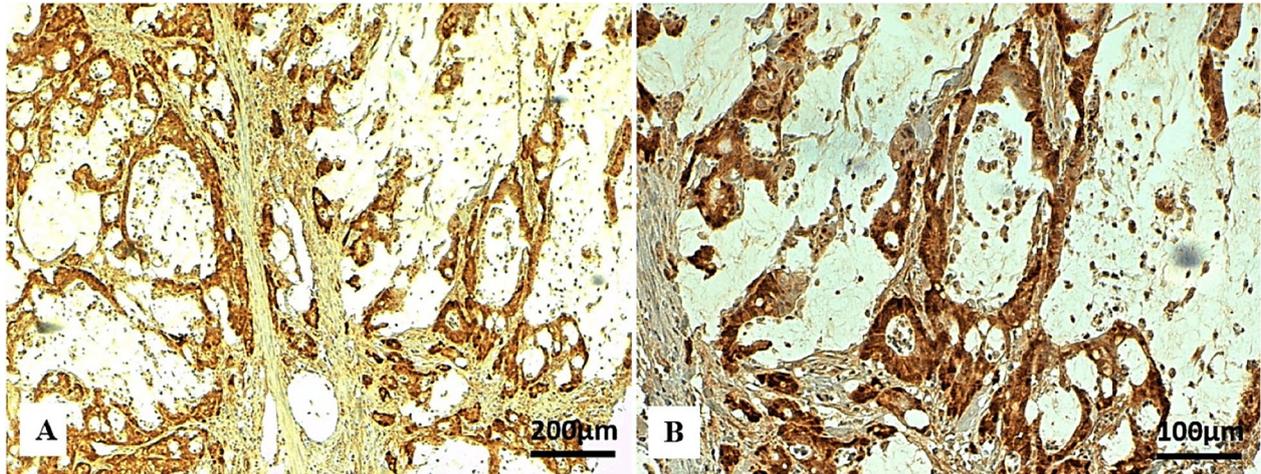
Figure 6 – *Immunohistochemical staining for beta-catenin in colon cancer: (A) Diffuse beta-catenin immunopositivity in a mucinous colon adenocarcinoma (×100); (B) Strong nuclear immunostaining for beta-catenin in tumor cells (×200).*
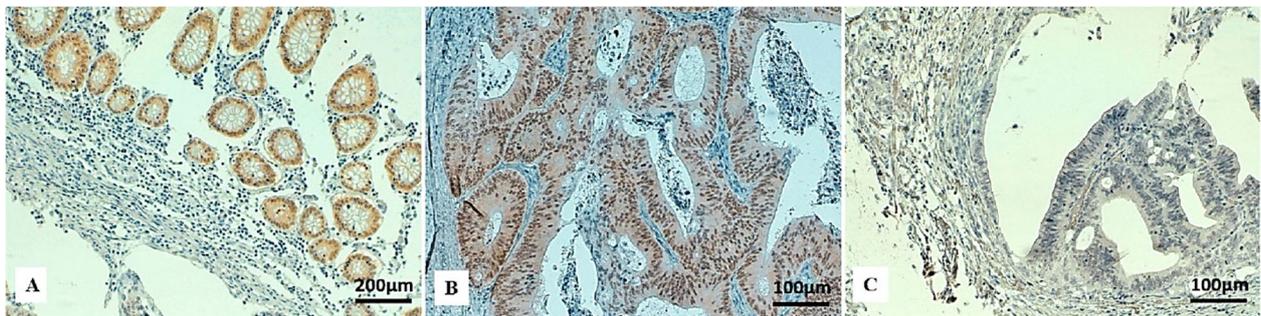

Figure 7 – *Immunohistochemical staining for CDX2 in colon cancer: (A) Positive nuclear immunostaining for CDX2 in normal intestinal mucosa (×100); (B) Strong positive nuclear immunoreactivity for CDX2 in tumor cells (×200); (C) Absence of nuclear immunostaining for CDX2 in tumor cells (×200). CDX2: Caudal-type homeobox 2.*

Table 3 – *Interrelation between beta-catenin, E-cadherin, CDX2 protein immunoexpression and clinicopathological parameters*

		N (%)	Beta-catenin Mean [%]	p-value	E-cadherin Mean [%]	p-value	CDX2 positive Mean [%]	p-value	CDX2 loss N (%)	p-value
Age [years]	<50	5 (16.12)	93.44	0.654	73.44	0.830	32.34	0.740	3 (9.67)	0.43
	>50	26 (83.87)	93.00		72.67		31.67		5 (16.12)	
Gender	Female	16 (51.6)	92.81	1.00	75.94	0.446	37.03	0.800	3 (9.67)	0.43
	Male	15 (48.4)	93.67		70.00		26.67		5 (16.12)	
Tumor site[1]	Right	15 (48.4)	95.00	0.299	76.67	0.599	40.00	0.151	2	0.20
	Left	16 (51.6)	91.56		69.69		24.53		6	
Histological type[2]	Intestinal	21 (67.74)	94.29	0.681	70.00	0.493	31.25	0.653	3 (9.67)	0.69
	Mucinous	10 (32.26)	92.35		75.59		32.65		5 (16.12)	
Tumor grade[3]	G1	9 (29.03)	92.50	0.237	83.13	0.018	35.31	0.353	3 (9.67)	0.58
	G2	15 (48.38)	91.88		68.12		25.63		4	
	G3	7 (22.58)	97.14		72.86		42.86		1	
pN	N0	13 (41.9)	94.23	0.046	73.08	0.277	34.04	0.837	4	0.24
	N1	11 (35.48)	89.09		68.64		33.64		1	
	N2	7 (22.6)	97.86		80.00		25.71		3 (9.67)	
Venous emboli	Absent	24 (77.4)	92.71	0.563	72.08	0.473	35.52	0.532	6	1.00
	Present	7 (22.6)	95.00		76.43		20.00		2	
Perineural invasion	Absent	24 (77.41)	94.79	0.068	73.54	0.661	35.31	0.235	5 (16.12)	0.33
	Present	7 (22.6)	87.86		71.43		20.71		3 (9.67)	
Lymphocytic response	Absent	17 (54.8)	93.24	0.769	70.00	0.399	35.74	0.316	3 (9.67)	0.41
	Present	14 (45.2)	93.21		76.79		27.50		5 (16.12)	
Tumor necrosis	Absent	19 (61.3)	92.11	0.389	76.05	0.389	39.61	0.220	4	0.67
	Present	12 (38.7)	95.00		68.33		20.00		4	
Metastasis	Absent	28 (90.32)	93.04	0.777	72.32	0.422	30.80	0.285	8	0.55
	Present	3 (9.78)	95.00		80.00		43.33		0	

		N (%)	Beta-catenin Mean [%]	p-value	E-cadherin Mean [%]	p-value	CDX2 positive Mean [%]	p-value	CDX2 loss N (%)	p-value
Invasion in nearby organs	*Absent*	29 (93.5)	92.76	0.211	72.41	0.310	32.16	0.619	8	1.00
	Present	2 (6.5)	100.00		82.50		30.00		0	
TNM staging [41]	*I*	2 (6.5)	92.50	0.932	75.00	0.816	61.25	0.238	0	0.33
	II	9 (29)	94.44		70.56		23.89		4	
	III	17 (54.83)	92.35		72.94		30.88		4	
	IV	3 (9.67)	95.00		80.00		43.33		0	
Survival status	*Living*	24 (77.41)	93.26	0.924	70.87	0.288	31.41	0.924	6	1.00
	Deceased	7 (22.6)	94.29		80.71		34.29		2	

CDX2: Caudal-type homeobox 2; *N*: No. of cases; TNM: Tumor, node, metastasis. [1]Tumor site: right colon location – cecum, ascending colon; left colon location – transverse segment to the sigmoid. [2]Mucinous type: more than 25% mucus pools. [3]Tumor grade: G1 – well differentiated; G2 – moderately differentiated; G3 – poorly differentiated. *p*-value <0.05 was considered statistically significant.

Statistically significant correlation was noticed between the percentage of positive cytoplasmatic E-cadherin cells and the tumor differentiation degree ($p=0.018$; Mann–Whitney *U*-test for independent samples), tumors with low differentiation degree displaying aberrant E-cadherin expression. Moreover, there was a statistically significant correlation between the intensity of beta-catenin immunoexpression and lymph node invasion ($p=0.046$; Mann–Whitney *U*-test for independent samples), tumors with lymph node metastasis having a strong protein expression.

No significant association between CDX2 expression and tumor clinical or pathological features was found ($p>0.05$) (Table 3).

No significant differences were identified in overall survival according to E-cadherin, beta-catenin or CDX2 immunoexpression (Table 3).

Moreover, we analyzed the correlation between individual loss of MMR protein expression and CDX2 loss (Table 4). Individual MLH1 loss was strongly correlated to PMS2 loss ($p=0.006$) and to MSH2 loss ($p=0.023$); MSH2 loss was significant associated to MSH6 loss ($p=0.011$) and PMS2 loss was significantly correlated to CDX2 loss ($p=0.03$).

Table 4 – *Correlation between individual loss of MMR proteins and CDX2 loss*

			CDX2 loss	MLH1 loss	MSH2 loss	MSH6 loss	PMS2 loss
Spearman's *rho*	*CDX2 loss*	Correlation coefficient	1.000	.332	.283	.093	.391*
		p-value	.	.068	.123	.619	.030
		N	31	31	31	31	31
	MLH1 loss	Correlation coefficient	.332	1.000	.408*	.258	.479**
		p-value	.068	.	.023	.161	.006
		N	31	31	31	31	31
	MSH2 loss	Correlation coefficient	.283	.408*	1.000	.450*	.183
		p-value	.123	.023	.	.011	.324
		N	31	31	31	31	31
	MSH6 loss	Correlation coefficient	.093	.258	.450*	1.000	.134
		p-value	.619	.161	.011	.	.473
		N	31	31	31	31	31
	PMS2 loss	Correlation coefficient	.391*	.479**	.183	.134	1.000
		p-value	.030	.006	.324	.473	.
		N	31	31	31	31	31

CDX2: Caudal-type homeobox 2; MLH1: mutL homologue 1; MMR: Mismatch repair; MSH2: mutS homologue 2; MSH6: mutS homologue 6; *N*: No. of cases; PMS2: Postmeiotic segregation increased 2. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

⊟ Discussions

CRC is a heterogeneous disease with different clinical, pathological, and molecular features, even within the same TNM stage, probably because of different molecular mechanisms involved in colon tumorigenesis [4, 6]. Many existing studies have examined the relationship between different biological markers involved in colon cancer progression, but the results are controversial.

MSI or the deficiency of MMR proteins is one of the molecular pathways of colorectal tumorigenesis and characterizes approximately 15–20% of CRCs [3, 4, 43, 44].

Our study aimed to evaluate the correlation between the clinicopathological features in colon cancer and the IHC expression of different biomarkers, such as: MMR proteins (MLH1, MSH2, MSH6, PMS2), CDX2 and adhesion molecules (E-cadherin, beta-catenin). Additionally, we investigated the association between the selected biomarkers and overall survival.

We detected a dMMR proteins in 26.81% cases and the most frequently noticed protein loss was MLH1 (75%). These results are similar to those reported by other authors [10, 11, 39, 45].

In our study, most of the cases with MLH1 loss also comprised PMS2 loss (5/6 patients), which is in concordance with the results of other studies, that claimed that MLH1 and PMS2 are often lost together, and the defect is caused

by mutation in the *MLH1* gene, which is essential for stabilization of PMS2 [25, 26, 46]. The PMS2 loss was detected in 62.5% of cases, similar with the proportion reported by other authors [39].

It was interesting the fact that, in the present study, the tumors with PMS2 loss, together with tumors with MSH6 loss, covered all the dMMR tumors. Other authors reported similar results [39, 45, 47], suggesting that probably the double panel (PMS2 and MSH6) is enough to detect the dMMR status, instead of using the quadruple panel.

Our study did not find any significant relationship between individual loss of MMR proteins (MLH1, MSH2, MSH6, PMS2) and the clinicopathological features, such as: gender, tumor location, histological type, tumor stage, tumor grade, invasion, necrosis, etc. We found that tumors with loss of MLH1 were more frequent in young patients (under the age of 50), even if the difference was only approaching the significance level (p=0.06). In the current study, tumors with MSH2 loss and PMS2 loss showed high propensity for lymph node metastasis but the results were not statistically significant (p=0.161 and p=0.181, respectively).

Previous studies have shown controversial results. Some authors suggest that isolated loss of MLH1 or MSH2 is associated with right colon tumor location [41], poorly differentiated or mucinous differentiation [41, 48, 49], lymph node metastasis [49], lymphovascular invasion [41], peritumoral inflammatory infiltrate [41].

When comparing the two patient subgroups: MSI *vs.* MSS tumors and their association with clinicopathological features, the MSI status in the present study was statistically significantly correlated with mucinous histological type (p=0.003). Similar results were reported by some authors [50–52]. Our data did not reveal other particular clinicopathological features for MSI tumors. Several authors have found a positive association between MSI status and younger age [24, 50], gender [10], right location [10, 24, 50–53], poor differentiation [24, 39], lymph node invasion [39, 50]. On the other hand, some other studies did not show any significant correlation between MSI status and age [10, 53], gender [24, 39, 53], histological type [24], lymph node invasion [24, 39], tumor differentiation grade [53], tumor stage [24, 39, 53], perineural invasion, [10, 24, 39], vascular [10, 24, 39], peritumoral inflammatory infiltrate [24].

The present study revealed that individual MLH1 loss was strongly correlated to PMS2 loss (p=0.006) and MSH2 loss (p=0.023). These findings are similar to those reported by other authors [25, 46], who also revealed that PMS2 loss was often associated with MLH1 loss. Moreover, MSH2 loss was significant associated to MSH6 loss (p=0.011). This can be explained by the fact that MMR proteins form dimers and work in pairs, MLH1/PMS2 and MSH2/MSH6 [12, 24, 46], therefore loss of MLH1 or MLH2 induces loss of PMS2, respectively MSH6. However, loss of PMS2, respectively MSH6, is not always associated with MLH1 respectively MLH2 loss, maybe because of other MMR proteins (*e.g.*, MSH3) which bind and stabilize MLH1 or MSH2 [46].

Our data showed better survival for patients with MSI tumors, but the difference was not statistically significant (p=0.199). We also found that tumors with individual loss

of MSH6 showed tendency towards a better survival (p=0.131). Since in our study mucinous histological type has been associated with MSI status, and mucinous tumors usually tend to be associated with poor prognosis [52], we could suggest that there is a subgroup of mucinous colon tumors with MSI status that may have better outcomes [52].

There is a considerable body of literature on the relationship between the overall survival and MSI status; several systematic reviews and meta-analysis have shown an improved overall survival in patients with MMR deficiency [3, 54, 55]. The meta-analysis conducted by Guastadisegni *et al.* [54] involving 12782 patients revealed a significantly improved prognosis for MSI tumors [odds ratio (OR): 0.58, 95% CI: 0.47–0.72, p<0.0001]. Even so, other authors did not find any significant correlation between MMR deficient status and overall survival [3, 13, 49].

E-cadherin is a transmembrane glycoprotein normally expressed in the basolateral membrane of epithelial cells (adherens junctions), being involved in growth and development in the early human organogenesis, as well as in cell-to-cell interactions [30, 56, 57]. In pathological conditions, it has been associated with loss of epithelial differentiation, EMT, tumor progression, invasion, metastasis, because of loss of cell adhesiveness [30, 31].

Beta-catenin is a structural component of cadherin-based adherens junctions and an important component of the Wnt signal cascade [33, 58, 59], playing a role in the control of cell proliferation or cell death [60]. Beta-catenin has been reported to be implicated in carcino-genesis, activation of this pathway causing an aberrant accumulation of nuclear/cytoplasmatic beta-catenin and reduced membranous expression [33, 58, 59].

Immunohistochemistry is an effective technique to identify reduced membranous expression levels of adhesion molecules, aberrant E-cadherin, and beta-catenin expression and Wnt pathway activation.

In the current study, we detected a high percentage of aberrant E-cadherin and beta-catenin immunoexpression in the tumor cells (95–100%). Other authors have also reported high proportion (80–100%) of aberrant E-cadherin or beta-catenin expression in colon cancer cells [61–64].

We found a statistically significant correlation between the percentage of aberrant cytoplasmatic E-cadherin cells and the tumor differentiation degree (p=0.018), which is consistent with the results of other studies [28, 31, 39, 56, 61, 62], where aberrant or decreased membranous E-cadherin immunoexpression was associated with a low degree of tumor differentiation. These findings could be explained in part by the fact that during the EMT, the dissolution of the E-cadherin-mediated adherens junction causes loss of E-cadherin normal immunoexpression [31, 65].

Furthermore, in our study, a statistically significant correlation was detected between the intensity of nuclear beta-catenin immunoexpression and lymph node invasion (p=0.046), tumors with lymph node metastasis having a strong beta-catenin nuclear expression. Our data are in accordance with the results published by other authors which indicate that high nuclear beta-catenin expression has a high incidence of lymph node metastasis and shorter overall survival [66, 67].

In our group of patients, there was no significant correlation between E-cadherin or beta-catenin aberrant expression and other different clinicopathological parameters (gender, age, tumor location, histological type, tumor stage, perineural invasion, vascular invasion, peritumoral inflammatory infiltrate) our results being similar to other published studies [61, 62]. The present study did not find any statistically significant correlation between aberrant adhesion molecules expression and local invasion or metastasis. This was a surprising result, considering the role of these molecules in cell-to-cell interactions, and can be explained by the fact that in cancer progression, alteration of adhesion proteins can be associated with alterations of other molecules [68]. Ahmad & Hahn-Stromberg [69] also reported no statistical association between E-cadherin and beta-catenin immunoexpression and metastasis in colon cancer, while other authors [61, 62, 64] proved that E-cadherin or beta-catenin aberrant expression led to a risk of aggressive biological behavior.

Previous research has reported divergent results concerning the prognostic value of E-cadherin and beta-catenin in colon tumors [56, 61, 69, 70]. In the current study, we did not identify any significant effect of E-cadherin or beta-catenin on the survival rate. Some groups have reported similar results [56, 71], while others have found that loss of normal immunoreactivity of these adhesion proteins may be a predictor of poor survival in colon cancer [62, 64, 72], as well as in other tumors: breast cancer [73], hepatocellular carcinoma [74], bladder cancer [75], prostate cancer [76], cervical cancer [77].

CDX2 is an intestinal transcription factor, normally expressed in the nucleus of enterocytes [35, 36], that plays an essential role in intestinal development and differentiation during embryonic life [35, 78, 79]. In pathological conditions, CDX2 is an important marker for intestinal adenocarcinomas [36, 37, 39]. Immunohistochemistry identifies nuclear CDX2 expression in both normal and neoplastic colonic mucosa.

In the current study, loss of nuclear CDX2 expression was identified in 25.80% cases, similar to other previous reports [36, 80, 81]. No significant correlation between the loss of CDX2 immunoexpression and any of the clinicopathological features was found in the present study ($p>0.05$). Our findings are similar to those reported by Altintas *et al.* [82], but the literature review shows divergent results concerning the relationship between the CDX2 loss and clinicopathological features. Some authors reported a significant correlation between CDX2 loss and female gender [80, 81], right-sided tumor location [79, 80, 83], high-grade tumors [36, 39, 79, 81, 83], advanced tumor stage [36, 39, 79, 81, 83], increased number of tumor-infiltrating lymphocytes [79], mucin production [79].

In our study, PMS2 loss was significantly correlated to CDX2 loss ($p=0.03$). Similar results were reported by Sayar *et al.* [39]. Although there are studies that suggested a relationship between CDX2 loss and MMR protein deficiency [79, 83], other authors [84] showed no association between MSI status and CDX2 loss.

Our data did not reveal any significant impact of loss of CDX2 expression on overall survival, which is in accordance with the results of other studies [78, 81, 82, 85]. In contrast, several studies suggested that CDX2 loss was independently associated with worse overall survival [79] and progression-free survival [79, 86, 87]. In a recently published study, Ryan *et al.* [88] proved that CDX2 loss expression had a poor prognosis only in patients with MSS CRC, and patients with MSI CDX2 loss had a low risk to develop distant metastases. Therefore, maybe CDX2 loss is a worse prognostic factor only in the subgroup of patients with MSS CRC [87, 88].

Despite the numerous studies, the role of CDX2 in colorectal carcinogenesis is poorly understood and it might differ in relation with other proteins involved in tumor progression.

Study limitations

Limitations of our study can be related to some reasons: the small number of patients enrolled, the tumor heterogeneity, different types of antibodies used, various scoring systems that identify the aberrant antigen immunoexpression, etc.

⊟ Conclusions

Despite decades of research, the molecular analysis of biological markers for colon cancer continues to be a very interesting debate topic. The results of this study indicate that MSI status was statistically significantly correlated with mucinous histological type, but not with other pathological features or with survival rate. Our data indicate that MLH1 and PMS2 are often lost together. Moreover, tumors with PMS2 loss, together with tumors with MSH6 loss, covered all the dMMR tumors, suggesting that the double panel (PMS2 and MSH6) might be enough for detecting the MSI status. Our study demonstrated that the adhesion molecules (E-cadherin, beta-catenin), hallmarks of EMT, were positively associated with tumor differentiation degree and lymph node invasion. Furthermore, this research highlights the positive significant association between PMS2 loss and CDX2 loss, suggesting that CDX2 may be involved in molecular pathways that lead to loss of the MMR proteins. Despite the many studies performed, there are conflicting opinions related to the prognostic significance and the role of these molecular biomarkers in tumor progression. Future research is needed, in large representative series, to identify the most powerful prognostic panel of biomarkers in colon cancer in order to select high-risk patients and to find the most appropriate treatment algorithms.

Conflict of interests

The authors declare that they have no conflict of interests.

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