Immunohistochemical Expression of CD8, CDX2, P53, D2-40 and KI 67 in Colorectal Adenocarcinoma, Conventional and Malignant Colo-rectal Polyps

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82 patients, who had been diagnosed with colo-rectal adenocarcinoma in our department between 2007 and 2014, were included in our study. Additionally, 31 patients with colo-rectal polyps (20 conventional adenomatous polyps and 11 malignant colo-rectal polyps) were also included in this study. The patients with colo-rectal adenocarcinoma were reevaluated in terms of gender, age, topography of recto-colic tumor, TNM stadialization, vasculo-lymphatic invasion, lymph nodes metastases and distant metastases. The study of CD8, Ki67, p53, CDX2 and D2-40 immunoexpression in colo-rectal cancer patients revealed: an elevated positivity index in patients with pT2 and pT3 stages and in patients with lymph node metastases in case of the CD8; an elevated positivity index in patients with pT2, pT3 and pT4 stage in case of Ki67; an elevated positivity index in patients with pT4 stage in in case of the p53; an increased positivity index in the pT2, pT3 stage, the absence of vasculo-lymphatic invasion, the absence of lymph node and distant metastases in case of the CDX2 and an increased positivity index in patients with the pT2, pT3 stage, the abscence of lymph node and distant metastases in case of the D2-40 Tumoral staging (pT2, pT3 and pT4) has been shown to be correlated with immunoexpression of the following markers: CD8, CDX2 and p53.

Keywords: CD8, CDX2, D2-40, distant metastases, Ki-67, lymph node metastases, p53, vasculo-lymphatic invasion

The incidence of colo-rectal cancer is higher and the International Agency for Research on Cancer reported that the most commonly diagnosed cancers in 2012 were the lung, breast and colo-rectal types [1]. Colo-rectal carcinogenesis are histologically and moleculary heterogenous, resulting from different pathways of carcinogenesis [2]. The majority sporadic of the colo-rectal cancers arise from the conventional adenoma-to-carcinoma stepwise progression [3] and a subset of carcinomas develops from serrated precursors [4]. Colo-rectal cancers arise from 3 different pathways: the adenoma to carcinoma chromosomal instability pathways in 50-70% of cases, the serrated pathways in 30-35% of cases and the mutator *Lynch syndrome* route in 3-5% of cases [5]. In the conventional pathway of colo-rectal cancers tumorigenesis, the early lesion is the conventional adenoma (tubular, tubulo-villous or villous adenoma). Progression from conventional adenoma to adenocarcinoma is associated with mutations in genes in particular in KRAS which was reported in up to 50% of villous adenoma and up to 18% of tubular adenoamas [5].

The aim of this study was to investigate immuno-expression of CD8, CDX2, Ki-67, D2-40 and p53 proteins according to some pathological parameters related to colo-rectal adenocarcinomas, conventional adenomatous and malignant colo-rectal polyps.

Experimental part

Material and methods

Patient data

82 patients, who had been diagnosed with colo-rectal adenocarcinoma in our department between 2007 and 2014, were included in our study. In this study there were also included 31 patients with colo-rectal polyps (20 conventional adenomatous polyps and 11 malignant colo-rectal polyps). The patients with colo-rectal adenocarcinoma (fig. 1) were reevaluated in terms of gender, age, topography of recto-colic tumor, TNM stadialization, vasculo-lymphatic invasion, lymph nodes metastasis and distant metastasis.

Colo-rectal polyps were examined for each case containing mucosa of conventional adenomatous polyp (adenoma, tubulo-villous adenoma, villous adenoma, tubular adenoma) and malignant polyps. There were no serrated polyps. Slides of these cases obtained from the archive were reevaluated under light microscopy (Leica,

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Tokyo Japan). We tried to select a paraffin-embedded tissue block for each case containing tumoral and preferably adjacent normal mucosa.

Patient biopsy specimen

Malignant and conventional colo-rectal polyps were visualized by colonoscopy (Pentax 290-Kp) and microscopic examination was performed after endoscopic resection (ERBE 200 S) in 2014-2016. Patients with colorectal cancer were operated in our department in the period 2007-2014. Surgical treatment of patients with colorectal adenocarcinoma was based on tumor location: right hemicolectomy, left hemicolectomy, total colectomy, segmental colectomy, resection of the rectum, rectum amputation

In patients with conventional polyps (fig. 2) of the colo-rectum, the topography of the polyps was: recto-sigmoid (3 patients), the sigmoid colon (3 patients), the descending colon (3 patients), the spleen angle of the colon (1 patient), transverse colon (4 patients), hepatic angle of the colon (1 patient), the descending colon (3 patients), cecum (1 patient), ileo-cecal valve (1 patient). For malignant polyps (fig. 3), their distribution was: sigmoid colon (5 patients), the descending colon (4 patients), the transverse colon (1 patient), cecum, and ascending colon (1 patient).

Conventional polyps were tubular (12 patients), tubulo-villous (7 patients) or villous adenomas (1 patient) and represented a range of dysplastic changes from low to severe. Adenomas with high-grade dysplasia showed higher proliferative activity, when regular adenomas shown superficial labelling only. Conventional polyps had low-grade dysplasia (5 patients), low and medium grade dysplasia (4 patients), high grade dysplasia (8 patients), hypersecretion and medium dysplasia (3 patients). High-grade dysplasia was present in 7/20 (35%) cases.

The average age of patients with colo-rectal polyps was 61 years (range 53-85), 12 patients were female and 19 patients were male.

Microscopic evaluation

Sampling was conducted in concordance with the Romanian law regarding procedural norms for Ethical Code of the Romanian College Board regarding the scientific use of medical cases. Informed consent was obtained from patients included in study. Immediately after sampling from each patient conventional and malignant polyps tissue, colon cancer tissue fragments were fixed in 10% buffered formalin for 24-48h at room temperature and then processed for paraffin embedding. Sections of 3-4 μm were obtained with rotary microtome and routinely stained with Hematoxylin - Eosin.

Immunohistochemistry

Serial sections of 3 μm were dewaxed and rehydrated. Antigen retrieval was performed after microwave incubation of sections in the appropriate buffer. Endogenous peroxidase was blocked after incubation with hydrogen peroxide-methanol solution. After blocking unspecific binding, sections were incubated at 4°C with one of the mouse or rabbit monoclonal primary antibodies mentioned in table 1. Then, sections were washed and processed for amplification of the immune signal using the appropriate method: 3.3' - Diaminobenzidine tetrahidrochloride (Sigma) and hydrogen peroxide (Merck) were used for color development and Mayer's Hematoxylin for nuclear counterstaining. Slides were observed and registered with a Nikon Eclipse microscope couplet to a digital camera. Images were finally processed using Adobe Photoshop 7.0. For each antibody tested we performed a negative control in which the primary antibody was replaced by 10nM phosphate buffer saline, *p*H 7,4-7,6. Four-micron sections were taken for positive charged slides of colonic adenocarcinoma and colonic polyps by selecting appropriate formalin-fixed, paraffin-embedded tissue sections. Then CDX2, CD8, Ki67, p53 and D2-40 (podoplanin) biomarkers (table 1) were studied using a fully automated IHC staining device.

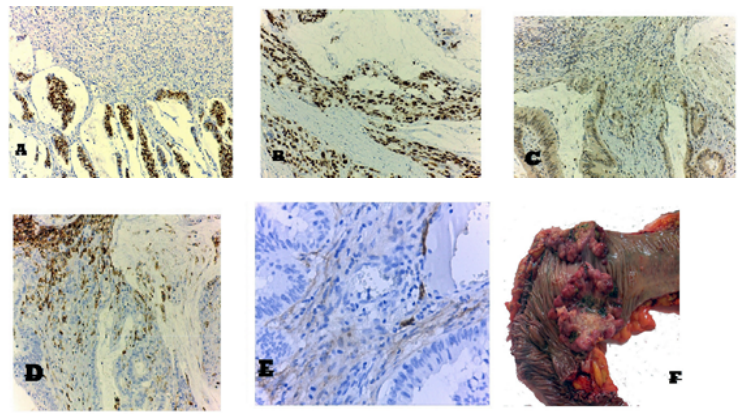

Fig. 1. CDX2, Ki67, p53, CD8 and D2-40 in colonic adenocarcinoma: (A) CDX2 immunoexpression of the sigmoid coloid adenocarcinoma G3 (x100); (B) Ki67 immunoexpression of the descending colon mucinous adenocarcinoma G2 (x100); (C) p53 immunoexpression of asecnding colon coloid adenocarcinoma G2 (x100); (D) CD8 immunoexpression of the transverse colon coloid adenocarcinoma G2 (x100); (E) D2-40 immunoexpression of sigmoid colon mucinous adenocarcinoma G2 (x100); (F) The macroscopic appearance of the hepatic angle of the colon adenocarcinoma.

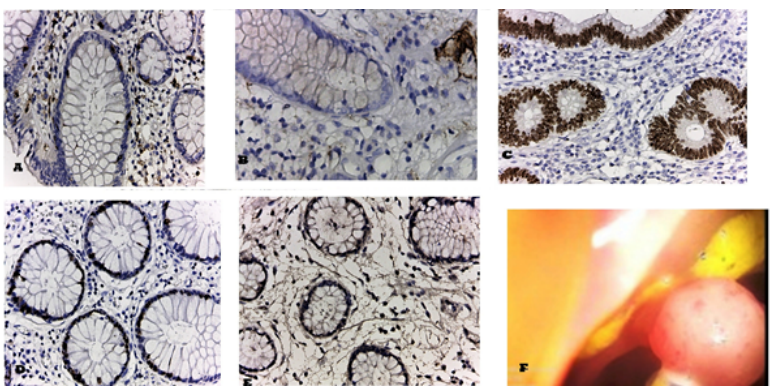

Fig. 2. CD8, D2-40, CDX2, Ki67 and p53 immunoexpression in conventional colo-rectal polyps: (A) CD8 immunoexpression of the tubular polyp from the transverse colon (x200); (B) D2-40 immunoexpression of the tubulo-villous polyp from the spleen angle of the colon (x200); (C) CDX2 immunoexpression of the tubulo-vilous polyp from the spleen angle of the colon (x200); (D) Ki67 immunoexpression of the tubulo-villous polyp fron transverse colon (x200); (E) p53 immunoexpression of the tubulo-villous polyp from the transverse colon (x200); (F) Endoscopic appearance of benign colorectal polyp.

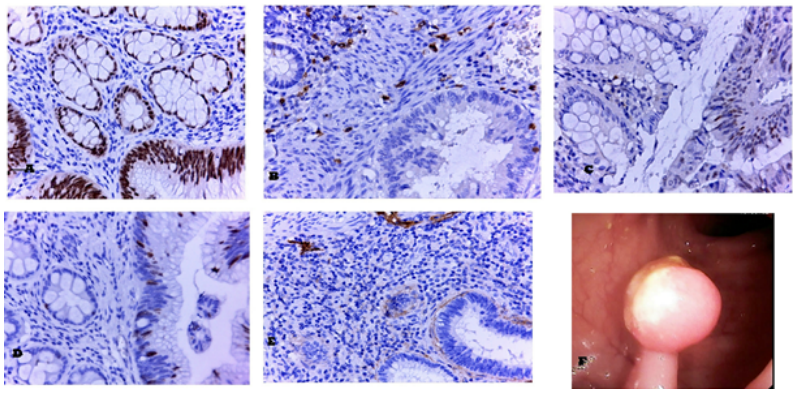

Fig. 3. CDX2, CD8, p53, Ki67 and D2-40 immunoexpression in malignant colo-rectal polyps: (A) CDX2 immunoexpression of the malignant polyp from the sigmoid colon, adenocarcinoma G2 (x200); (B) CD8 immunoexpression of the malignant polyp from the descending colon, adenocarcinoma G2 (x200); (C) p53 immunoexpression of malignant polyp from the sigmoid colon, mucinous adenocarcinoma G3 (x200); (D) Ki67 immunoexpression of the malignant polyp from the sigmoid colon, adenocarcinoma G2 (x200); (E) D2-40 immunoexpression of the malignant polyp from the descending colon, adenocarcinoma G2 (x200); (F) Endoscopic appearance of malignant colorectal polyp.

Evaluation

When evaluating immunostaining, nuclear staining of tumor cells was taken into account for CDX2, CD8, p53, Ki67 and D2-40 (podoplanin). Tumor cells that showed less than 5% for nuclear staining for p53, Ki67, CDX2, D2-40, CD8 and p53 were considered 0- (<5%), those that indicated 5-25% staining were considered as 1+, those that showed 26-75% staining were considered as 2++, and those that indicated more than 75% staining were considered as 3+++ (fig. 1-3). For all the biomarkers, staining of normal colonic glands or inflammatory cells was used as a positive control.

Statistical analysis

Figure 4 considers pairs of the five markers and searches for correlations between them using Spearman's rank correlation coefficient. Generally, there are no results strongly correlated either directly (blue) or indirectly (red). Indirectly, there is a correlation of -0.19 between CDX2 and D2.40 and of -0.17 between CD8 and CDX2. Directly, the correlations are even weaker, of only 0.15 between D2.40 and Ki67 and of 0.13 between both CDX2 and D2.40 on the one hand, and CDX2 and Ki67 on the other hand.

As the values for the five biomarkers are ordinal, the subsequent statistical tests are chosen accordingly to an ordinal distribution. There are several different populations that are statistically examined to verify if there are differences in the distributions.

For the results of each biomarker in turn, differences relative to the sexes of the patient are examined: generally, there are not significant differences between the results, with the only exception of the CD8 marker. The Kruskal-Wallis (KW) rank sum test achieves a p-value of 0.034 (< 0.05).

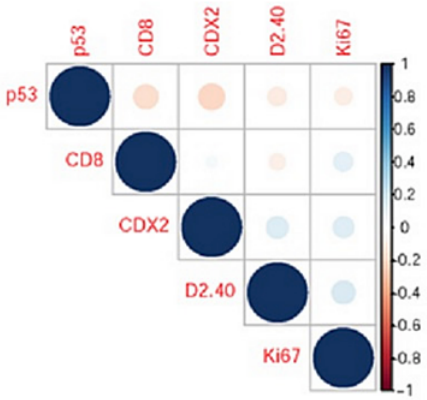

Fig.4. Spearman correlation coefficients between pairs of markers

Another considered factor that would separate the populations is the age, but the same KW statistical test shows there are no significant differences for any of the five markers.

The staging proves to be an attribute that triggers differences between the results of three out of the five markers. The p-values for CD8, CDX2 and p53, as computed via the KW test, are all below 0.05, i.e. 0.005, 0.001, 0.009.

The localization of the tumor is considered as on the left (colon_sigmoid, splenic angle of the colon, descending colon, recto-sigmoid junction, transverse colon), right (ascending colon, hepatic angle of the colon) or different (for cec and rect). When analyzing the distribution between populations based on each marker in turn, the localization proved to make no significant difference in any of the cases.

The samples that refer the polyps are compared to the cancerous ones via a Kolmogorov-Smirnov test and CDX2 and p53 proved to signal significant differences. While for the former the p-value was 8.5e-05, for the latter it was 2.6e-06.

Finally, a KW test is used to verify if the markers determine significant differences between the samples with conventional polyps and the malignant ones. CD8 conducted to a p-value of 1.1e-04 and for p53 a p-value of 0.04 was obtained, while for the rest the values were larger than 0.05.

All the above statistical observations are also validated by an analysis of variance model.

Results and discussions

CD8, Ki67, p53, CDX2 and D2-40 immunoexpression of patients with colo-rectal cancers

As shown in table 2 a total of 82 colo-rectal cancer patients aged between 31 and 93 years, predominatly male, were analyzed with histopatholgically diagnosis of mucinous colo-rectal adenocarcinoma (94.46%) and 8.54% signet ring cell colo-rectal adenocarcinoma.

Biomarker	Clone no	Company	Dilution rate
P53	Mouse DO7 7.0	Dako	predilute 453 M – 98
CDX2	Rabbit EPR 2764 7.0	Dako	predilute 235 R – 18
CD8	C8/144B 7.0	Dako	predilute 108 M – 98
Ki 67	Rabbit SP 6 7.0	Dako	predilute 275 R – 18
D2-40 (Podoplanin)	Mouse D2 – 40 7.0	Dako	predilute 322 M – 18

Table 1
SPECIFICATIONS OF THE BIOMARKERS

Table 2

GLOBAL IMMUNOEXPRESSION OF KI67, P53, CDX2, CD8 AND D2-40 IN COLORECTAL ADENOCARCINOMA

Histological/immunoreactivity parameters		Total cases		Positivity index (N(%))			
				0 - (<5%)	+ (5-25%)	++ (26-75%)	+++ (>75%)
Global immunoexpression		82	CD8	10(12.19%)	20(24.39%)	27(32.92%)	25(30.48%)
			Ki67	0	12(14.63%)	49(59.75%)	21(25.61%)
			p53	45(54.87%)	20(24.39%)	3(3.65%)	14(17.07%)
			CDX2	13(15.85%)	2(2.43%)	37(45.12%)	30(36.58%)
			D2-40	18(21.95%)	22(26.82%)	25(30.48%)	17(20.73%)
Histopathologically type	Mucinous adenocarcinoma	75	CD8	9(12%)	19(25.33%)	23(30.66%)	24(32%)
			Ki67	0	11(14.66%)	47(62.66%)	17(22.66%)
			p53	41(54.66%)	19(25.33%)	2(2.66%)	13(17.33%)
			CDX2	13(17.33%)	2(2.66%)	37(49.33%)	23(30.66%)
			D2-40	13(17.33%)	18(24%)	23(30.66%)	16(21.33%)
	Signet ring cell adenocarcinoma	7	CD8	1(14.28%)	1(14.28%)	4(57.14%)	1(14.28%)
			Ki67	0	1(14.28%)	2(28.57%)	4(57.14%)
			p53	4(57.14%)	1(14.28%)	1(14.28%)	1(14.28%)
			CDX2	0	0	2(28.57%)	5(71.42%)
			D2-40	1(14.28%)	3(42.85%)	2(28.57%)	1(14.28%)

Table 3

KI 67, p53, CD8, CDX2 AND D2-40 IMMUNOEXPRESSION DEPENDING ON TUMOR PROGRESSION OF COLON CANCER

Histological/immunoreactivity parameters		Total cases		Positivity index (N(%))			
				0 - (<5%)	+ (5-25%)	++ (26-75%)	+++ (>75%)
Tumor progression	pT1(invades submucosa)	0		0	0	0	0
	pT2(muscularis propria)	8	CD8	0	1(12.5%)	3(37.5%)	4(50%)
			Ki67	0	2(25%)	3(37.5%)	3(37.5%)
			p53	4(50%)	3(37.5%)	0	1(12.5%)
			CDX2	0	1(12.5%)	3(37.5%)	4(50%)
			D2-40	1(12.5%)	2(25%)	2(25%)	3(37.5%)
	pT3(paracolic tissue)	65	CD8	4(6.15%)	18(27.69%)	23(35.38%)	20(30.76%)
			Ki67	0	9(13.84%)	41(63.07%)	15(23.07%)
			p53	40(61.53%)	15(23.07%)	1(1.53%)	9(13.84%)
			CDX2	8(12.30%)	3(4.61%)	28(43.07%)	26(40%)
			D2-40	8(12.30%)	14(21.53%)	21(32.30%)	23(35.38%)
	pT4(visceral peritoneum, other organs)	9	CD8	6(66.66%)	1(11.11%)	1(11.11%)	1(11.11%)
			Ki67	0	1(11.11%)	5(55.55%)	3(33.33%)
			p53	1(11.11%)	2(22.22%)	2(22.22%)	4(44.44%)
			CDX2	5(55.55%)	1(11.11%)	2(22.22%)	1(11.11%)
			D2-40	2(22.22%)	3(33.33%)	3(33.33%)	2(22.22%)

In terms of tumor progression (table 3), 8 patients were pT2 (tumor invades muscularis propria) (9.75%), 67 patients pT3 (invasion through muscularis propria into subserosa and pericolic tissue) (79.26%) and 9 patients pT4 (tumor penetrates visceral peritoneum and invasion of other organs or structures) (10.97%). In the study group there were no early-stage patients pT1 (tumor invades submucosa). Lymph node metastases were present in 29 patients (35.36%), absent in 38 patients (46.34%) and in 15 patients (18.39%) the lymph-node status could not be identified. Distant metastases (liver, lung, skeletal metastases) were present in 8 patients (9.75%), absent in 66 patients (80.48%) and in 8 patients (9.75%) the presence or absence of distant metastases could not be determined.

CD 8 immunoexpression in colo-rectal cancer patients showed an elevated positivity index (2++, 3+++) in patients with pT2 and pT3 stages and in patients with lymph node metastases. Positivity index was decreased (0-, 1+) in patients with absent distant metastases.

Ki67 immunoexpression in studied patients with colo-rectal cancer, revealed an elevated positivity index (2++, 3+++) in patients with pT2, pT3 and pT4 stage. The positivity index for Ki67 was increased (2++, 3+++) both in the presence and in the absence of vasculo-lymphatic invasion (table 4), lymph-node (table 5) and distant metastases (table 6). The positivity index was decreased (0-, 1+) in patients with the pT4 stage and when the vasculo-lymphatic invasion was absent (table 4).

The p53 immunoexpression in studied patients with colo-rectal cancer, showed an elevated positivity index (2++, 3+++) in patients with pT4 stage. The positivity index was low (0-, 1+) in patients with pT2, pT3 stage (table 3), in the absence of vasculo-lymphatic invasion (table 4), the presence of lymph-node (table 5) and distant metastases (table 6).

The study of CDX2 immunoexpression in colo-rectal cancer patients revealed an increased positivity index (2++, 3+++) in the pT2, pT3 stage, the absence of

Table 4

KI 67, p53, CD8, CDX2 AND D2-40 IMMUNOEXPRESSION DEPENDING ON VASCULO-LYMPHATIC INVASION OF COLON CANCER

Histological/immunoreactivity parameters		Total cases		Positivity index (N(%))			
				0 - (<5%)	+ (5-25%)	++ (26-75%)	+++ (>75%)
Vasculo-lymphatic invasion	Presence	9	CD8	3(33.33%)	2(22.22%)	4(44.44%)	0
			Ki67	0	2(22.22%)	2(22.22%)	5(55.55%)
			p53	4(44.44%)	1(11.11%)	1(11.11%)	3(33.33%)
			CDX2	6(66.66%)	3(33.33%)	0	0
			D2-40	1(11.11%)	2(22.22%)	4(44.44%)	2(22.22%)
	Absence	73	CD8	17(23.28%)	15(20.54%)	28(38.35%)	13(17.80%)
			Ki67	0	8(10.95%)	53(72.60%)	12(16.43%)
			p53	26(35.61%)	24(32.87%)	10(13.69%)	13(17.80%)
			CDX2	13(17.80%)	14(19.17%)	26(35.61%)	20(27.39%)
			D2-40	14(19.17%)	20(27.39%)	33(45.20%)	16(21.91%)

Table 5

Ki 67, p53, CD8, CDX2 AND D2-40 IMMUNOEXPRESSION DEPENDING ON LYMPH-NODE METASTASES OF COLON CANCER

Histological/immunoreactivity parameters		Total cases		Positivity index (N(%))			
				0 - (<5%)	+ (5-25%)	++ (26-75%)	+++ (>75%)
Lymph node metastases	Presence	29	CD8	10(34.48%)	9(31.03%)	8(27.58%)	2(6.89%)
			Ki67	0	9(31.03%)	7(24.13%)	13(44.82%)
			p53	12(41.37%)	7(24.13%)	3(10.34%)	7(24.13%)
			CDX2	16(55.17%)	12(41.37%)	1(3.44%)	0
			D2-40	4(13.79%)	8(27.58%)	10(34.48%)	7(24.13%)
	Absence	38	CD8	5(13.15%)	11(28.94%)	15(39.47%)	7(18.42%)
			Ki67	0	17(44.73%)	14(36.84%)	7(18.42%)
			p53	17(44.73%)	4(10.52%)	6(15.78%)	11(28.94%)
			CDX2	0	1(2.63%)	18(47.36%)	19(50%)
			D2-40	5(13.15%)	1(2.63%)	14(36.84%)	18(47.36%)
	Not identify	15		0 all markers	0	0	0

Table 6

Ki 67, p53, CD8, CDX2 AND D2-40 IMMUNOEXPRESSION DEPENDING ON DISTANT METASTASES OF COLON CANCER

Histological/immunoreactivity parameters		Total cases		Positivity index (N(%))			
				0 - (<5%)	+ (5-25%)	++ (26-75%)	+++ (>75%)
Distant metastases	Presence	8	CD8	2(25%)	2(25%)	3(37.5%)	1(12.5%)
			Ki67	0	2(25%)	4(50%%)	2(25%)
			p53	4(50%)	1(12.5%)	1(12.5%)	2(25%)
			CDX2	5(62.5%)	3(37.5%)	0	0
			D2-40	1(12.5%)	2(25%)	3(37.5%)	2(25%)
	Abscence	66	CD8	17(25.75%)	34(51.51%)	9(13.63%)	6(9.09%)
			Ki67	0	16(24.24%)	31(46.96%)	19(28.78%)
			p53	37(56.06%)	13(19.69%)	7(10.60%)	9(13.63%)
			CDX2	0	2(3.03%)	39(59.09%)	25(37.87%)
			D2-40	16(24.24%)	12(18.18%)	25(37.87%)	23(34.84%)
	Not identify	8		0 all markers	0	0	0

vasculo-lymphatic invasion, the absence of lymph node and distant metastases. The positivity index was decreased (0-. 1+) in patients with pT4 stage, when vasculo-lymphatic invasion was present and in the presence of lymph node and distant metastases.

The study of D2-40 (podoplanin) immunoexpression revealed an increased positivity index (2++, 3+++) in patients with colo-rectal cancers in the pT2, pT3 stage, in patients with the abscence of lymph node and distant metastases. The positivity index was decreased in patients with vasculo-lymphatic invasion.

CD8, p53, D2-40, CDX2 and Ki67 immunoexpression of the patients with conventional and malignant colo-rectal polyps

As shown in table 7 in patients with conventional colo-rectal polyps, CD8 immunoassay indicated an increased positivity index (2++, 3+++) and, in the case of malignant polyps a low positivity index (0-, 1+) was found.

Ki67 immunoexpression in patients with colo-rectal polyps revealed an increased positivity index (2++, 3+++) for conventional polyps and an approximately uniform distribution of the positivity index for malignant polyps.

Table 7

CD8, p53, D2-40, CDX2 AND KI67 IMMUNOEXPRESSION OF THE PATIENTS WITH CONVENTIONAL AND MALIGNANT COLO-RECTAL POLYPS

Histological/immunoreactivity parameters		Total cases	Positivity index (N(%))			
			0 (<5%)	+ (5-25%)	++ (26-75%)	+++ (>75%)
Cp	CD8	20	0	7(35%)	7(35%)	6(30%)
Mp	CD8	11	5(45.45%)	6(54.54%)	0	0
Cp	p53	20	0	6(30%)	7(35%)	7(35%)
Mp	P53	11	0	2(18.18%)	0	9(81.81%)
Cp	D2-40	20	7(35%)	8(40%)	3(15%)	2(10%)
Mp	D2-40	11	4(36.36%)	1(9.09%)	4(36.36%)	2(18.18%)
Cp	CDX2	20	0	0	3(15%)	17(85%)
Mp	CDX2	11	2(18.18%)	0	0	9(81.81%)
Cp	Ki67	20	2(10%)	0	9(45%)	9(45%)
Mp	Ki67	11	0	4(36.36%)	2(18.18%)	5(45.45%)

Cp=conventional polyp
Mp=malignant polyp

Immunoexpression of p53 in patients with conventional colo-rectal polyps indicated an almost uniform distribution of the positivity index and in case of malignant polyps an increased positivity index (3+++).

Immunoexpression of CDX2 in patients with colo-rectal polyps revealed an increased positivity index (2++, 3+++) for patients with conventional colo-rectal polyps and for patients with malignant colo-rectal polyps, there was an increased positivity index (3+++).

D2-40 (podoplanin) immunoexpression in patients with conventional colo-rectal polyps revealed a low positivity index (0-, 1+) and an almost uniform distribution of the positivity index for malignant colo-rectal polyps.

Colorectal cancer is the result of sequential genetic alterations that can induce triggering anti-tumor, immunological mechanism in which cytotoxic T lymphocytes are heavily involved.

On the one hand, the activation of cytotoxic lymphocytes can cause a specific lytic effect with the destruction of the tumor cell and on the other hand, there is a change in the ratio of the lymphocyte subtypes normally present in the lamina propria at the level of the tumoral stroma with an increase in the cytotoxic T lymphocyte population [6].

In our study, CD 8 immunoexpression in colo-rectal cancer patients showed an elevated positivity index in patients with pT2 and pT3 stages and in patients with lymph node metastases. Positivity index was decreased in patients with absent distant metastases. In patients with conventional colo-rectal polyps, CD8 immunoassay indicated an increased positivity index and in case of malignant polyps a low positivity index was found. Other similar studies [7] reported at the tumoral edges percentages of CD8-positive lymphocyte infiltrated in similar proportions to those in our study, of 28% for cases with poor CD8 immunoexpression, 44% of cases with moderate immunoexpression and 28% of colorectal carcinomas with intense CD8 immunoexpression.

Although most of the studies [8] have highlighted the lack of correlation between cytotoxic T lymphocytes and stages of tumor progression, underlying the existence of a more prominent lymphocytic infiltrate, CD8 positive, especially intraepithelial in the stages of advanced tumor progression. In agreement with similar studies, in our research, we noticed a maximum intensity of CD8 immunoexpression predominant in the pT2 stage of tumor progression.

The prognostic parameters analyzed in terms of CD8 immunoexpresion revealed, in the case of lympho-vascular invasion present in colorectal mucinous adenocarcinoma, a predominance of cases with moderate positive immunoexpression. Lymph node metastases and distant metastases were manifested by lower or absent CD8 immunoexpression. Similar studies [9] indicates the involvement of CD8 positive lymphocytes in tumor immunomodulation and colorectal cancer prognosis in that the absence of CD8 positive lymphocytes increases the risk of lymph node metastases, while emphasizing the role of these lymphocytes in the immunologic mechanism to prevent the onset of systemic micrometastases.

The Ki-67 antigen, considered an important marker for cell proliferation, is expressed in all phases of cell cycle except for the G0 phase.

The cell growth fraction, directly correlated with tumor aggression, was evaluated by means of immunostaining with monoclonal antibody ki-67 with a variable immunoexpression level in all 82 studied cases of colorectal carcinomas.

Ki67 is found in the lower third of normal human colonic crypts. We confirm the results of previous studies [10] that described flattened Ki67 negative cells at the invading edge of colorectal carcinomas and such change in cell shape could be indicative of an epithelial-to-mesenchymal transition.

Ki67 staining did not correlate with age, gender, limph node metastases or tumor location, but a high Ki67 index correlated with stadialization (TNM stage). Patient with high Ki67 tended to have a poorer prognosis.

Ki-67 was extensively expressed in 25,6% of the studied carcinomas, the highest represented percentage (59,75%) being cases of medium intensity of immunoexpression and no cases of negative expression.In accordance with the results of our study, many other similar studies report medium intensity of immunoexpression [11].

Mucinous adenocarcinama showed medium intensity of immunoexpression of the Ki-67 antigen (62, 66%) and signet ring cell adenocarcinoma presented high grade immunoexpression in 57,44% of the cases. There are studies that have not identified any significant difference in Ki-67 immunoexpression of mucinous adenocarcinoma and the signet ring cell adenocarcinoma [12].

In our study, overexpression of the Ki-67 with high grade immunoexpression was found in both cases where the tumor invasion was limited to the level of muscularis propria (pT2) and to advanced cancers (pT4) that invades visceral peritoneum with values 37.5% and 33.33% respectively, expressing the lack of correlation between the proliferative activity and the infiltrative activity of the researched carcinomas.

In accordance with the results of our study, other similar studies [13] did not reveal any correlation between the tumor stage and the intensity proliferative activity of colorectal carcinomas. In contrast, the results of other studies [14] show a significantly lower rate of proliferative activity in the deeper invasion of the wall than in cancers with limited invasion of the submucosa or muscularis propria.

In mucinous adenocarcinoma whose prognostic parameters of vasculo-lymphatic invasion and lymph node metastases were identified, the Ki-67 immunoassay was expressed in significant percentages (55.55% and 44.82%) of high intensity. Medium intensity immunoassay was highlighted in 50% of colorectal cancers studied with metastases.

In similar studies, there is no consensus on the possible correlation of the Ki-67 immunoregulatory intensity and the prognostic parameters, so some specialty papers assert the absence of this correlation [12], this result being also expressed in our study. Other papers [14] indicate a poor proliferative activity of mucinous carcinomas associated with lymph node metastases and peritoneal dissemination.

The p53 acts a tumor suppresor in many tumor types: induces growth arrest or apoptosis depending on the physiological circumstances and cell type. Activated p53 by various stimuli from intrinsec and extrinsec environment controls cell cycle arrest, senescence and apoptosis Tp53 mutations, but not p53 positive immuno-histochemistry, have been consistently associated with poor prognosis in colo-rectal cancer [15].

Speciality studies reveal fairly wide limits of p53 immunoexpression in colorectal carcinomas, with 86.36% [16] and 57.14% [17] as well as the relatively low level of p53 immunoexpression values in mucinous adenocarcinoma with percentage values of approximatively 44% [18].

The p53 immunoassay applied to both varieties of carcinomas studied with excess mucin production (mucinous adenocarcinoma and signet ring cell adenocarcinoma) revealed the absence of p53 immunoexpression in significant percentages, 54.66% for colloid carcinomas and 57.14% in signet ring cell adenocarcinoma. Overexpression of p53 immunoexpression was present in small number of cases of both mucinous adenocarcinoma (17.33%) and signet ring cell adenocarcinoma (14.28%).

Speciality studies [18] mention similar low values of p53 immunoexpression in colloidal carcinomas, as well as an absence of p53 immunoexpression in signet ring cell adenocarcinoma. The study of tumor progression of mucinous adenocarcinomas by the application of p53 immunoassay revealed an increase in the percentage of cases with intense immunoexpression as the tumor invasion increased, with 12.5% of these cases in the pT2 stage and 44.44% of p53 overexpression cancers in the pT4 stage.

The existence of a higher percentage of cases in deeper invasive forms of mucinous or non-mucinous colorectal carcinomas was reported in some similar papers [16], an aspect also identified in our study. In addition, there is a lack of correlation between the intensity of the p53 immunoassay and the stage of the tumor invasion [19]. There is also evidence that the p53 immunoexpression is relatively diminished to pT progression [18].

The prognostic factors of colorectal mucinous carcinomas represented by vascular and lymphatic invasion, lymph node metastases and peritoneal dissemination were investigated through p53 immunoexpression, showing in our study the absence of the p53 immunoexpression in significant percentages of 44.44%, 41.37% and respectively 50% of cases in which the predicted parameters were identified.

The highly of p53 immunoexpression was identified in 33.33% of colorectal carcinomas with vasculo-lymphatic invasion, 24.13% of those with lymph node metastases and 25% of carcinomas with distant metastases.There is no consensus in similar studies on the correlation between p53 immunoexpression and prognosis of mucinous or non-mucinous colorectal carcinomas.

In this sense, some authors assert the lack of any concordance [18,19], with an obvious correlation between these parameters not present even in our study. In other studies [16] a predominant immunoexpression of p53 was observed in colorectal adenocarcinomas with lymph node metastases.

CDX2 is known to reglate gut specific genes and processes determining differentiation of gut epithelium . In this study, CDX2 was identified with elevated mean expression levels in adenomatous polyp and carcinoma compared to normal tissue. Immunohistochemistry estabilished elevated expression of CDX2 protein in adenomatous polyps. The results of this study support the complex relationship between CDX2 and colon carcinogenesis reported in previous studies [20].

CDX2 is a sensitive and specific marker for adenocarcinoma of colon and rectum and its expression is decreased among higher grade and tumor stage.

CDX2, a nuclear transcription factor, is involved in the processes of differentiation, intestinal cell proliferation, adhesion and apoptosis, having organ-specific expression. CDX2 was expressed in our study in a majority (84.70%) and variable intensity. The existence of a CDX2 positive expression and in very close proportions to those obtained in our study was also reported in other specialized studies [21] with values over 70% CDX2 positive colorectal mucinous carcinomas. In another study [22] a 97% CDX2 immunoexpression of colorectal carcinomas was reported, of which 60% were those with increased positivity index. In our study, a majority (45.12%) of tumors with high grade positivity index of CDX2 were identified.

In terms of the histologically studied type, the immunofixation of CDX2 in colloid carcinomas had a variable appearance but with a predominance of positive cases (82.66%), among them, those with a medium intensity positivity index being the most numerous (49.33%), followed by intensive positivity index (30.66%). Signet ring cell adenocarcinoma were entirely represented by CDX2 positive cases (100%), most (71.42%) expressing with maximum positivity index CDX2.

Tumor progression of mucinous colorectal carcinomas was analyzed in terms of CDX2 immunoassay, resulting in a 100% immunoexpression of limited cases of invasion of muscularis mucosae, the percentage value decreasing for cases diagnosed in pT3 disease status to 87.69%, reaching 44.44% for tumors in pT4 stage.

CDX2 expression was analyzed in some studies [22] compared to the clinical stage of tumor progression (pT), claiming no correlation between the two parameters

analyzed, other studies [23] showing a decrease in CDX2 immunoexpression as progression goes to the tumor stage, this being the one reported in our study.

The prognostic parameters represented by the vasculo-lymphatic invasion, lymph node and distant metastases were analyzed with the CDX2 immunoexpression, revealing in our study a predominantly negative immun-expression. Thus, 66.66% of CDX2 immun-expression was missing from vascular-lymphatic invasion, 55.17% of mucosal colorectal cancers with lymph node metastases and 62.5% of tumors diagnosed with distant metastases.

The reduction of CDX2 expression in tumor types whose prognostic parameters of vasculo-lymphatic invasion and distant metastases were present was the result of specialized studies [23], consistent with the our own.

Other studies suggest that there is no correlation between CDX2 expression and vasculo-lymphatic invasion [22], or even an increase in CDX2 immunoreactivity expression in metastasis of colorectal mucinous carcinomas compared to positive immune markers obtained in metastatic cancers.

In colorectal carcinoma lymph node metastasis is an important prognostic factor .Most studies using enzyme histochemical assay specific for 5'-nucleotide alkaline phospahatase have confirmed the hypoyesis that lymphatics are absent in the lamina propria of colonic mucosa [24].

The lymphatic–specific monoclonal antibody D2-40 is used for immunohistochemical staining of lymphatic channels and has been developed to study both lymphatic vessel density and lymphatic invasion in colon carcinoma

Fogt et al., utilised D2-40 to identify lymphatics in normal colonic mucosa, adenoma and invasive carcinomas. These authors found that lymphatic were absent in normal colonic mucosa but were present whitin the lamina propria of invasive carcinoma [25].

In our study D2-40 immunoexpression had a variable expression, the maximum intensity of immunoexpression was noted in 20.37% of cases, moderately positive immunoexpression representing 30.48% of cases and low immnunoexpression of D2-40 were in 26.82% of cases.

Tumor progression of colo-rectal carcinomas revealed a high percentage of D2-40 immunoexpression (62.5%) in tumors limited to invasion of muscularis mucosae pT2 and tumors with invasion of pericolic tissue pT3 (67.88%). Advanced colo-rectal cancers pT4 showed an immunoexpression of D2-40 with almost equal distribution of cases.

The study of tumor progression of mucinous adenocarcinomas by the application of D2-40 immuno-assay revealed an increase in D2-40 immunoexpression as the tumor progression increased in pT2 and pT3 stages, 37.5% and 35.38% respectively.

In mucinous colo-rectal adenocarcinomas whose prognostic parameters represented by vasculo-lymphatic invasion, lymph node metastases and distant metastases were identified, moderately positive immunoexpression was expressed as a significant percentage of 44.44% , 34.48% and 37.5% but also in absence of identification of these parameters: 45.20, 36.84 and 37.8% respectively.

The World Health Organisation has classified serrated polyps into three types of lesions: traditional serrated adenomas (TSA), sessile serrated adenomas polyps (SSA/P) and hyperplastic polyps (HP). Conventional polyps were classified intro three types: tubular polyps, tubulovillous polyps and villlous adenoma.

The TSA has predilection of the left colon, eosinophilic cytoplasm, tubulovillous archictecture and BRAF mutation. At a molecular level, traditional serrated adenoma can be divided into 2 groups based on their BRAF or KRAS mutation status. TSA have three histological features: cytoplasmic eosinophilia, characteristic serration (luminal serration) and ectopic crypt foci. Also is present elongated, penicillated nuclei with evenly dispersated coarse chromatin and small inconspicuous nucleoli. Morphological variants of the traditional serrated adenoma are: flat TSA, filiform TSA (is less aggresive) and mucin-rich or goblet cell-rich cells.

Serrated dysplasia was graded as mild, moderate and severe on the basis of cytological and architectural criteria [26]. The cytological features were gauged along the length of the crypt starting from the base, ascending to the mild-crypt and then to the surface. Nuclear features of the serrated dysplasia suggested by Lazarus et al included: variation in size and shape, enlargement and loss of polarity. Goldstein refined these criteria [27], collapsing mild and moderate dysplasia into low-grade serrated dysplasia and added the following features: lining cells were cuboidal to short columnar, the nuclei were round to oval, chromatin pattern was vesicular and open, there was a proeminent large macronucleoli and decrease amonts of eosinophilic cytoplsm.

Bettington et al. have described a so-called serrated tubulovillous adenoma [28, 29]. This polyp resembles a conventional tubulovillous adenoma cytologeically in terms of dysplasia but is typified by projections that are probably best described as festonated rather than the usual serrated pattern seen in traditional serrated adenoma.

Ki67 and p53 staining was weak in the epithelium of pedunculated polyps whereas invasive colon carcinomas showed increased staining for one or both markers [30-36]. Ki67 is found in the lower third of normal human colonic crypts. We confirm the results of previous studies [10].

Statistically in our study, there was a slight direct correlation between D2-40 and Ki-67 ($p = 0.15$), between CDX2 and D2-40 on the one hand and between CDX2 and Ki-67 on the other hand ($p = 0.13$). There was an indirect correlation between CDX2 and D2-40 ($p = 0.19$) and between CD8 and CDX2 ($p = 0.17$).

Between CD8 and the sex of the examined patients there was a statistically significant correlation ($p = 0.034$). Staging (pT2, pT3, pT4) has been shown to be strongly correlated with immune expression of the following markers: CD8, CDX2, p53 values of p being: 0.005, 0.001 and 0.009, respectively. In the comparison between colonic polyps and colorectal cancers, there were statistical differences in CDX2 and p53 immunoexpression. In comparison between conventional and malignant polyps, the CD8 and p53 immunoexpression was statistically significant ($p = 0.04$).

Conclusions

CD8 immunoexpression in colo-rectal cancer patients revealed an elevated positivity index in patients with lymph node metastases and in patients with pT2 and pT3 stages.

High grade immunoexpression of Ki67 was found in both cases of pT2 and pT4 stages and did not reveal any correlation between the tumor stage and intensity proliferative activity of colo-rectal carcinomas or the prognostic parameters.

The absence of p53 immunoexpression in significant percentages was found in colo-rectal cancer patients with pT2, pT3 stages, with the absence of vasculo-lymphatic invasion, the presence of lymph node and distant metastases.

CDX2 immunoexpression showed a decrease positivity index in patients with colo-rectal cancers in pT4 stag,.

when vasculo-lymphatic invasion, lymph node and distant metastases were present. CDX2 immunoexpression revealed an increased positivity index for patients with conventional and malignant colo-rectal polyps.

D2-40 immunoexpression revealed a high positivity index in colo-rectal tumors of pT2 and pT3 stages.

Tumoral staging (pT2, pT3 and pT4) has been shown to be correlated with immunoexpression of the following markers: CD8, CDX2 and p53.

CD8 and p53 immunoexpression was statistically significant in comparison between conventional and malignant polyps.

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