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The Percentage of Stained Cells is a More Reliable Parameter in Immunohistochemical Analysis than Scoring the Intensity of Staining: Expression of 9 Molecular Markers in Progression and Liver Metastases of Colorectal Cancer

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Abstract

Aims: Research for reliable molecular markers that provide prognostic and predictive information in colorectal cancer (CRC) is based on solid evidence that the staging system on its own cannot definitively predict the tumor behavior and guide clinical management of the disease. **Methods and results:** In this study we examined the immunohistochemical expression of 9 markers, namely membrane-bound mucin 1 (MUC1), paxillin (PAX), Focal Adhesion Kinase (FAK), G-protein coupled receptor 56 (GPR56), ORAI3 and well known markers of colon carcinoma microsatellite instability (MSI): MSH2, MSH6, MLH1 and PMS2 with respect to the percentage of stained cells and intensity score in colon carcinoma cells, both in the primary tumor and liver metastasis. We have related all of the mentioned markers with clinicopathological data, including the age of patients, grading of the primary tumor and TNM system. Western blotting assay was performed to identify the expression. Our present study showed that the evaluation of different markers with respect to intensity of staining and percentage of stained cancer cells may be considered as a prognostic marker for tumor progression and later for liver metastasis. This has been found for the percentage of stained cells particularly. **Conclusions:** Our results implicate that the counting percentage of stained

colon cancer cells provides a more adequate method of immunohistochemical analysis than evaluation of the intensity of staining.

Keywords

Colon Carcinoma, Liver Metastasis, Tumor Markers, Immunohistochemistry

1. Introduction

Carcinogenesis of colorectal cancer (CRC) is a multistep process in which different pathways are disrupted, including cell proliferation, differentiation, and cell death. These pathological lesions first presented as aberrant crypt foci, then grow into adenoma, carcinoma in situ, invasive adenocarcinoma, and finally, carcinoma with metastases. It is reported that almost 60% of all CRC patients are diagnosed with liver metastases [1]. Over 80% of patients with metastatic CRC cannot undergo surgical resection. Hence, CRC has the fourth highest mortality rate accounting for 7.6% of cancer-related deaths [2]. It is well established that the staging system on its own cannot definitively predict the tumor behavior and guide clinical management of the disease. Molecular markers that provide prognostic and predictive information above that given by standard pathological staging of CRCs are of utmost importance.

The presence of a marker, usually a protein, in tumor milieu can be confirmed by means of a variety of laboratory methods including immunohistochemistry (IHC). Some markers evaluated using the IHC may also have predictive value, thus playing a vital role in improving the effects of cancer therapy. We have recently studied the expression of osteopontins in breast cancer and we have found that a high staining intensity of nuclear osteopontin-c was strongly associated with prognosis in patients with early breast carcinoma [3]. The cytosolic intensity of staining for osteopontins a and b was also predictive of poor outcomes [3]. The aim of the present study was to investigate biological molecules that are acknowledged to be linked to the process of CRC development, namely membrane-bound mucin1 (MUC1), paxillin (PAX), Focal Adhesion Kinase (FAK), G-protein coupled receptor 56 (GPR56), and ORAI1 homolog, ORAI3. We analyzed the expression of selected biomarkers and that of well known markers of colon carcinoma microsatellite instability (MSI): MSH2, MSH6, MLH1 and PMS2.

Mucins reveal a significant prognostic value in sporadic colorectal carcinoma, CRC, but not in hereditary CRC. Loss of MUC2 is an adverse prognostic factor in mismatch repair-proficient and MLH1-negative CRC, while the expression of MUC1 is correlated with tumor progression in mismatch repair-proficient CRC only [4].

MUC1 is expressed in colorectal carcinoma which has progressed to the metastatic stages, thus MUC1 may provide a useful marker for advanced colorectal carcinoma [5] while PAX protein level was significantly higher in colorectal cancer tissue than those in adjacent normal ones and the expression of PAX was significantly correlated to the tumor histological grade and size, and also to the

presence of distant metastasis [6]. The prognosis of the patients with higher PAX expression was poorer than those with low expression of PAX [6]. PAX, which is an intracellular adaptor protein, plays a basic role in the organization of the cytoskeleton, this connects integrins to FAK and plays an important role in both the assembly and disassembly of focal adhesions [7]. Overexpression of FAK as well as overexpression of CD98 and β1 and β3 integrins was found to be associated with the progression of colon carcinoma and its liver metastases [8] while GPR56 was only just recently studied by Sewda *et al*. [9] who have found that GPR56 expression was significantly correlated with the proximal tumor location and with the expression of mismatch repair genes. ORAI3 has recently gained more attention as a target for cancer therapy since its altered expression and function is supposed to contribute to the tumorigenesis and metastasis of a variety of tumors including breast, prostate, colorectal, brain and skin tumors [10].

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant cancer predisposition syndrome caused by germ-line mutations in DNA mismatch repair genes, MSH2, MLH1, PMS1, PMS2, and MSH6 [11]. Colorectal cancers with MSI have a significantly better prognosis compared with those with intact mismatch repair [12].

In this study we examined the immunohistochemical expression of 9 markers with respect to the percentage of stained cells and intensity score in colon carcinoma cells, both in the primary tumor and liver metastasis. We have related all of the mentioned markers with clinico-pathological data, including the age of patients, grading of the primary tumor and TNM system. Western blotting assay was performed to identify the protein expression.

2. Materials and Methods

2.1. Patients

The study comprised of 63 patients who have been surgically treated between 2014 and 2015. All studied cases refer to colon adenocarcinoma, grades 1, 2 and 3, without other cancer types such as neuroendocrine carcinoma. All information regarding the patients was received from the Department of General and Oncological Surgery in Wroclaw, Diagnostyka-Consilio Division of Pathology in Łódź and from the Division of Oncological Surgery, Walbrzych in Poland. The inclusion criteria were primary carcinoma of the colon with metastases to the liver and no neoadjuvant chemotherapy. In our study we did not consider the overall effects of adjuvant therapy with regard to the survival rate. Since the observations were cut off by the end of 2015, data, such as survival time, was unavailable. For all patients, who met these criteria, formalin-fixed and paraffin-embedded blocks were collected for histological and immunohistochemical studies. The clinicopathological data comprised of pathological TNM, grading (G) and age of the patients. The study was approved by the local ethics committee at Wroclaw Medical University, Poland. The patients' characteristics are shown in **Table 1**.

Table 1. Characteristics of patients enrolled in the study. Both TNM and grading were attributed relevant ranks.

Age (years)	$M \pm SD$	67.1 ± 9.1
Tumor	Rank	N (%)
T1	1	4 (6.3)
T2	2	14 (22.2)
T3	3	29 (46.0)
T3b	4	3 (4.8)
T3d	5	1 (1.6)
T4	6	6 (9.5)
T4a	7	4 (6.3)
T4b	8	2 (3.2)
Lymph node		N (%)
N0	1	19 (30.2)
N1	2	3 (4.8)
N1a	3	5 (7.9)
N1b	4	8 (12.7)
N1c	5	6 (9.5)
N2	6	6 (9.5)
N2a	7	7 (11.1)
N2b	8	9 (14.3)
Metastasis		N (%)
M0	1	7 (11.1)
M1	2	24 (38.1)
M1a	3	1 (1.6)
M1b	4	3 (4.8)
M2	5	21 (33,3)
M3	6	7 (11.1)
Grading		N (%)
1	1	5 (7.9)
2	2	47 (74.6)
3	3	11 (17.5)

2.2. Immunohistochemistry

The anti-human antibodies used in this study were MUC1 (HPA008855, Sigma, Poland; diluted 1:50), ORAI3 (HPA015022, Sigma, Poland; diluted 1:50), FAK (sc-1688, Santa Cruz, Biotechnology, USA; diluted 1:50), Paxillin, PAX (ab-32084, Abcam, UK; diluted 1:50), GPR56 (HPA046065, Sigma, Poland; diluted 1:50), and MSH2 (M3639, IR085), MSH6 (M3646, IR086), MLH1 (M3640, IR079), PMS2 (M3647, IR087) all 4 from Dako, Glostrup, Denmark, in ready-to-use concentrations. MUC1 resulted in cytoplasmic/membranous staining, ORAI3 in cytoplasmic/nuclear, FAK, PAX and GPR56 in cytoplasmic, and all 4 MSI markers revealed nuclear staining. It needs to be emphasized that the staining of GPR56 resulted in a very weak or even no staining with regard to both the percentage and intensity scores and therefore we had to exclude this protein

from further analysis.

2.3. Immunohistochemical Staining

Formalin-fixed and paraffin-embedded tissue blocks were sectioned at 5 μm, deparaffinized in two changes of xylene (9 min each) and rehydrated in alcohols (96%, 80% and 70% for 1 min each). The sections were then washed twice in distilled water and placed in 0.01 M sodium citrate (pH 6.0) in a microwave oven (350W) for 10 min for a heat-induced epitope retrieval. Following two 5-min washes in distilled water, specimens were incubated for 10 min with Peroxidase Blocking Reagent (Dako, Glostrup Denmark) and rinsed twice with phosphate-buffered saline (PBS), for 5 min each time. Incubation with Protein Block Serum Free Reagent (Dako, Glostrup, Denmark) was performed for 15 min, then the specimens were incubated with primary antibodies at indicated above dilutions for 1 h at room temperature, and then rinsed twice with PBS, each time for 5 min. Subsequently, the secondary antibody was applied for 30 minutes: when applying a mouse monoclonal antibody, Dako EnVision + System-HRP Labeled Polymer anti-mouse, K4001, was used and when applying a polyclonal rabbit antibody, Dako EnVision + System-HRP Labeled Polymer anti-rabbit, K4003, was used. Following rinsing twice with PBS for 5 min each time, 3,3'-diamino-ben-zidine (K3468, Dako, Glostrup, Denmark) was applied to the samples at the original dilution, and after the next rinsing (twice for 5 min each rinse) in distilled water, the slides were counterstained with hematoxylin for 1 minute. Following washing in tap water for 10 min, the samples were dehydrated in alcohols (70%, 80% and 96%), for 3 minutes each, and the slides were covered with cover slips. Negative controls for each antibody were created by omitting the first antibodies. All stainings have been performed in a Dako autostainer.

For each antibody, the tissues were scored for intensity (maximum intensity of the sample-3, and the lowest-1; *i.e.* 1, 2 or 3) and percent positivity (low 1 (< 10%), medium 2 (> 10% - 50%), high 3 (> 50% - 100%), (accordingly to Dabbs, 2010). In each case and for each antibody a formalin-fixed and paraffin-embedded biopsy specimen from primary cancer tissue and relevant metastatic tumor from liver was cut on a microtome in 5 m slices. All microscopic slides were independently evaluated by three pathologists and in the cases of discrepant initial scores a final score was agreed after discussion. All microphotographs of stained histological slides were taken using the Olympus BX40 light microscope at the magnification 40x, digital camera Q Imaging, Micro Publisher 3.3 RTV, and a software Q-Capture Pro 7 2010, Canada.

2.4. Western Blotting Assay

The presence of focal adhesion kinase (FAK), mucin 1 (MUC1), adhesion G protein-coupled receptor G1 (GPR56) and ORAI calcium release-activated calcium modulator 3 (ORAI3) in primary cancer tissue and relevant metastatic tumor from liver was confirmed by western blotting technique. For this study, the formalin-fixed and paraffin-embedded (FFPE) tissue blocks were used. The tis-

sue blocks were cut into 10 sections of 20 μm and put on microscope slides. Next a pathologist marked the cancerous parts of the section in accordance with the image visible in the light microscope and the appropriate tissue of each section was macro dissected (approximately 25 mg of FFPE tissue). Next, the samples were deparaffinized in three changes of xylene in a heating block with agitation, 10 min each, cleared in three changes of absolute alcohol and dried in concentrator (Eppendorf, Hamburg, Germany).

Tissue was homogenized in anlysis buffer [0.1 M Tris-HCl (pH 8.0), 0.1 M DL-dithiothreitol, 4% SDS] at 99˚C in a heating block with agitation (600 rpm) for 1 h following by the crude extracts clarification by centrifugation at 16,000 × *g* at 18˚C for 10 min. The protein lysate concentration was measured at 280 nm using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA).

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham Hybond, GE Healthcare Bio-sciences AB, Uppsala, Sweden) by NuPage System as recommended by Invitrogen (Life Technologies, Carlsbad, CA, USA). See Blue Plus 2 Prestained Protein Standard was used as a standard (Life Technologies, Carlsbad, CA, USA). Next, the membrane was washed in phosphate buffered saline with tween 20 0.1% (PBST, Sigma-Aldrich, Seelze, Germany) and blocked with 3% bovine serum albumin (Sigma-Aldrich, Seelze, Germany) in PBST for 1 h. Consequently the membrane was probed with 1:500 diluted primary polyclonal antibodies against FAK (Santa Cruz Biotechnology, Dallas, TX, USA), MUC1, GPR56 and ORAI3 (Sigma-Aldrich, Seelze, Germany) at 4˚C overnight. The following day blots were washed with PBST and incubated with the 1:1000 diluted HRP-conjugated secondary goat antibody to rabbit IgG (Abcam, Cambridge, UK) for 1 h. Finally, the bound antibodies were visualized using DAB Enhanced Liquid Substrate System for Immunohistochemistry (Sigma-Aldrich, Seelze, Germany). Documentation of blots was performed using Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA).

2.5. Statistics

Correlations between studied markers, primary and metastatic tumors, staining intensity and percent of stained cells, and clinicopathological variables were assessed with Pearson's correlation test. Correlation coefficients of > 0.1 to 0.3 were considered weak, > 0.3 - 0.5 as moderate correlation and > 0.5 as strong or very strong correlation. Sign "-" means negative correlation, otherwise if not stated, the correlation was positive. The analyses were performed using STATISTICA v.12. A p-value of 0.05 indicates statistical significance, unless stated otherwise.

For convenience, in next statistical analysis, T, N and M have been grouped in single-stage classes, e.g. M1, M1a etc. as M.

3. Results

The strongest correlation of most markers was observed in relation with distant metastases, M. Table 2 shows Spearman's coefficient of rank correlation (ρ) between selected markers, *i.e.* MUC1, PAX, ORAI3 and FAK, and intensity of

Table 2. Spearman's coefficient of rank correlation (ρ).

Marker		Age	T	N	M	Grading
MUC 1 (%)	colon					
ρ		0.096	0.049	0.165	0.029	0.095
p-value		0.452	0.698	0.193	0.822	0.453
MUC 1 (%)	liver					
ρ		0.174	0.093	0.186	**0.363**	0.112
p-value		0.171	0.462	0.143	**0.004**	0.376
MUC 1 intens	colon					
ρ		0.235	0.008	0.158	0.164	0.033
p-value		0.064	0.947	0.213	0.196	0.794
MUC 1 intens	liver					
ρ		**0.267**	0.014	0.176	0.115	0.212
p-value		**0.035**	0.911	0.165	0.364	0.095
PAX (%)	colon					
ρ		0.055	0.038	0.099	0.099	0.036
p-value		0.663	0.762	0.435	0.437	0.776
PAX (%)	liver					
ρ		0.077	0.011	0.077	**0.569**	−0.221
p-value		0.545	0.933	0.547	**< 0.001**	0.081
PAX intens	colon					
ρ		0.088	0.002	0.072	0.191	0.033
p-value		0.488	0.986	0.569	0.134	0.795
PAX intens	liver					
□		0.167	0.054	0.076	**0.409**	0.023
p-value		0.189	0.673	0.549	**0.001**	0.854
ORAI3 (%)	colon					
ρ		0.015	0.079	0.043	0.146	0.066
p-value		0.904	0.533	0.734	0.250	0.604
ORAI3 (%)	liver					
ρ		0.025	0.215	0.062	**0.366**	−0.076
p-value		0.844	0.090	0.628	**0.004**	0.548
ORAI3 intens	colon					
ρ		0.021	0.110	0.044	0.113	0.048
p-value		0.866	0.386	0.732	0.375	0.705
ORAI3 intens	liver					
ρ		0.091	0.049	0.021	0.173	0.060
p-value		0.473	0.700	0.869	0.174	0.639
FAK (%)	colon					
ρ		0.074	**0.324**	0.153	**0.368**	−0.007
p-value		0.562	**0.011**	0.230	**0.004**	0.955
FAK (%)	liver					
ρ		0.027	**0.378**	0.048	**0.642**	−0.079
p-value		0.833	**0.003**	0.708	**< 0.001**	0.536
FAK intens	colon					
ρ		0.142	0.209	0.050	**0.384**	−0.136

Continued

p-value		0.264	0.099	0.694	**0.002**	0.283
FAK intens	liver					
ρ		0.158	0.113	0.028	0.141	0.088
p-value		0.213	0.374	0.826	0.265	0.488

Table 2 shows Spearman's coefficient of rank correlation (ρ) between selected markers, *i.e.* MUC1, PAX, ORAI3 and FAK, and intensity of staining and percent of stained cells, as well as the parameters such as the age of patients, TNM and grading (G).

staining and percent of stained cells, as well as the parameters such as the age of patients, TNM and grading (G). It is evident that the percent of stained cells for all 4 markers correlated with distant metastases (M) in liver (ρ from 0.363 to 0.642). In comparison this correlation for the intensity score was found only for PAX in liver ($\rho = 0.409$) and FAK in colon ($\rho = 0.384$), and for FAK percent of stained cells which correlated also with distant metastases for primary tumors in colon ($\rho = 0.368$). The tumor grading did not correlate with any marker. The size of the tumor (T) positively correlated with some markers.

Table 3 shows Spearman's coefficient of rank correlation (ρ) between selected markers of microsatellite instability (MSH2, MSH6, MLH1 and PMS2) and intensity of staining and the percentage of stained cells and other parameters as shown in Table 2. It is evident that apart from PMS2 all of the other markers negatively correlated with distant metastases in the liver and this was related to both the percentage of stained cells and, in most cases, the intensity of staining. The percentage of stained cells for MSH6 and MLH1 in the liver also correlated with tumor size (T), while this correlation was not observed for MSH2 and PMS2. Interestingly enough, the intensity of staining for PMS2 in liver correlated with the size of the tumor ($\rho = -0.374$).

Table 4 shows correlations between the percentage of stained cells and intensity of staining for 8 investigated markers in primary colon carcinoma and its metastasis in liver. The strongest correlation was observed in MLH1 with respect to % of stained cells ($\rho = 0.608$) and for MSH2 in relation to intensity score ($\rho = 0.578$). Furthermore, a very strong correlation was found for FAK with regard to % of stained cells ($\rho = 0.565$), and a moderate correlation was observed for ORAI3 ($\rho = 0.360$). Moderate correlations for MUC1, PAX, MSH2 and MSH6 were also observed. P-values for all markers except for PMS2 showed significance at a level < 0.05.

Tables 5(A)-5(H) present more accurately and in a separate manner the results established for each marker. Table 5(A) shows correlation between percent of stained cells and intensity score for MUC1 in liver metastasis and primary colon carcinoma by means of Pearson Chi-square and Spearman Rank tests. It is noteworthy that the expression of MUC1 evaluated as percent of stained cells in primary colon carcinoma moderately correlated with staining in liver metastasis ($p < 0.001$), while this correlation was not significant for intensity score ($\rho = 0.186$, $p = 0.144$).

Table 3. Spearman's coefficient of rank correlation (ρ).

Marker		Age	T	N	M	Grading
MSH2 (%)	colon					
ρ		−0.122	0.233	0.084	−0.118	0.110
p-value		0.504	0.201	0.645	0.518	0.545
MSH2 (%)	liver					
ρ		−0.051	0.172	**−0.277**	**−0.323**	0.111
p-value		0.687	0.175	**0.029**	**0.011**	0.383
MSH2 intens	colon					
ρ		0.066	0.161	0.114	−0.025	0.035
p-value		0.718	0.379	0.532	0.893	0.847
MSH2 intens	liver					
ρ		−0.032	0.205	−0.213	**−0.414**	0.021
p-value		0.800	0.106	0.094	**0.001**	0.869
MSH6 (%)	colon					
ρ		−0.099	0.110	−0.022	**−0.325**	0.142
p-value		0.435	0.387	0.860	**0.010**	0.264
MSH6 (%)	liver					
ρ		−0.214	**0.322**	0.043	**−0.589**	−0.003
p-value		0.092	**0.011**	0.738	**< 0.001**	0.982
MSH6 intens	colon					
ρ		0.108	0.038	0.110	−0.174	0.029
p-value		0.396	0.762	0.387	0.170	0.821
MSH6 intens	liver					
ρ		0.020	0.193	−0.116	**−0.568**	0.069
p-value		0.876	0.128	0.361	**< 0.001**	0.586
MLH1 (%)	colon					
ρ		−0.209	**0.424**	0.027	**−0.714**	0.033
p-value		0.100	**<0.001**	0.834	**< 0.001**	0.794
MLH1 (%)	liver					
ρ		0.020	**0.319**	−0.078	**−0.456**	0.013
p-value		0.876	**0.012**	0.541	**< 0.001**	0.921
MLH1 intens	colon					
ρ		−0.115	**0.287**	−0.061	**−0.535**	−0.070
p-value		0.366	**0.024**	0.633	**< 0.001**	0.581
MLH1 intens	liver					
ρ		−0.004	0.038	−0.198	**−0.359**	0.018
p-value		0.974	0.764	0.118	**0.005**	0.888
PMS2 (%)	colon					
ρ		−0.005	0.110	−0.078	−0.184	−0.098
p-value		0.966	0.387	0.542	0.147	0.442
PMS2 (%)	liver					
ρ		0.099	−0.204	0.139	0.024	0.054
p-value		0.587	0.264	0.448	0.896	0.766
PMS2 intens	colon					
ρ		0.064	−0.094	−0.167	−0.052	−0.190

Continued

p-value		0.614	0.457	0.189	0.680	0.135
PMS2 intens	liver					
ρ		0.149	**−0.374**	−0.229	−0.040	−0.223
p-value		0.414	**0.041**	0.210	0.826	0.222

Table 3 shows Spearman's coefficient of rank correlation (ρ) between selected markers of microsatellite instability (MSH2, MSH6, MLH1 and PMS2) and intensity of staining and the percentage of stained cells and other parameters as shown in Table 2.

Table 4. Spearman's coefficient of rank correlation (ρ) between primary colon carcinoma and metastatic carcinoma in liver in relation with the percentage of stained cells and intensity score.

Marker	Positivity (%)		IntensityScore	
	ρ	p	ρ	P
MUC 1	**0.463**	**< 0.001**	**0.186**	**< 0.001**
PAX	**0.384**	**0.002**	0.076	0.554
FAK	**0.565**	**< 0.001**	0.242	0.057
ORAI3	**0.360**	**0.004**	0.230	0.070
MSH2	**0.401**	**0.026**	**0.578**	**< 0.001**
MSH 6	**0.408**	**< 0.001**	**0.295**	**0.019**
MLH 1	**0.608**	**< 0.001**	**0.328**	**0.009**
PMS 2	−0.207	0.263	0.284	0.121

Table 5(A). Correlation between percent of stained cells and intensity score for MUC1 in liver metastasis and primary colon carcinoma.

liver MUC 1 (%)	colon MUC 1 (%)						Total	
	1		2		3			
	n	(%)	N	(%)	n	(%)	n	(%)
1	7	70,0	9	37,5	3	10,3	19	30,2
2	2	20,0	10	41,7	12	41,4	24	38,1
3	1	10,0	5	20,8	14	48,3	20	31,7
Total	10	15,9	24	38,1	29	46,0	63	100,0
Pearson Chi-square = 15.38, df = 4, ***p* = 0.004**; Spearman Rank ρ= 0.463 (***p* < 0.001**)								
liver MUC 1 intensityscore	colon MUC 1 intensity score						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	13	59,1	5	23,8	8	40,0	26	41,3
2	7	31,8	14	66,7	8	40,0	29	46,0
3	2	9,1	2	9,5	4	20,0	8	12,7
Total	22	34,9	21	33,3	20	31,7	63	100,0
Pearson Chi-square = 7.55, df = 4, p = 0.110; Spearman Rank ρ= 0.186 (p = 0.144)								

Table 5(B) shows correlation between the percentage of stained cells and intensity score for PAX in liver metastasis and primary colon carcinoma by means of two statistic tests. A moderate, statistically significant, correlation was observed with regard to the percentage of stained cells in primary colon carcinoma and liver metastasis ($\rho = 0.384$, $p = 0.002$).

Table 5(B). Correlation between the percentage of stained cells and intensity score for PAX in liver metastasis and primary colon carcinoma.

liver PAX (%)	colon PAX (%)						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	5	50,0	6	26,1	2	6,7	13	20,6
2	2	20,0	11	47,8	10	33,3	23	36,5
3	3	30,0	6	26,1	18	60,0	27	42,9
Total	10	15,9	23	36,5	30	47,6	63	100,0
Pearson Chi-square = 12.94, df = 4, **$p = 0.012$**; Spearman Rank $\rho = 0.384$ (**$p = 0.002$**)								
liver PAX intensityscore	colon PAX intensity score						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	7	43,7	10	34,5	5	27,8	22	35,0
2	5	31,3	14	48,3	9	50,0	28	44,4
3	4	25,0	5	17,2	4	22,2	13	20,6
Total	16	25,4	29	46,0	18	28,6	63	100,0
Pearson Chi-square = 1.80, df = 4, $p = 0.772$; Spearman Rank $\rho = 0.076$ ($p = 0.554$)								

Table 5(C). Correlation between percent of stained cells and intensity score for FAK in liver metastasis and primary colon carcinoma.

liver FAK (%)	colon FAK (%)						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	12	48,0	4	33,3	1	3,8	17	27,0
2	8	32,0	7	58,3	5	19,2	20	31,7
3	5	20,0	1	8,3	20	76,9	26	41,3
Total	25	39,7	12	19,0	26	41,3	63	100,0
Pearson Chi-square = 27.29, df = 4, **$p < 0.001$**; Spearman Rank $\rho = 0.565$ (**$p < 0.001$**)								
liver FAK intensityscore	Large intestine FAK intensity score						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	24	66,7	6	35,3	5	50,0	35	55,6
2	10	27,8	9	52,9	3	30,0	22	34,9
3	2	5,6	2	11,8	2	20,0	6	9,5
Total	36	57,1	17	27,0	10	15,9	63	100,0
Pearson Chi-square = 6.13, df = 4, $p = 0.190$; Spearman Rank $\rho = 0.242$ ($p = 0.057$)								

Table 5(C) shows correlation between the percentage of stained cells and intensity score for FAK in liver metastasis and primary colon carcinoma by means of Pearson Chi-square and Spearman Rank tests. Of utmost importance is a very strong, significant correlation with regard to percent of stained cells in primary colon carcinoma and liver metastasis ($\rho = 0.565$, $p < 0.001$), while the intensity score showed no correlation ($p > 0.05$).

Table 5(D). Correlation between percent of stained cells and intensity score for ORAI3 in liver metastasis and primary colon carcinoma.

liver ORAI3 (%)	colon ORAI3 (%)						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	5	45.5	8	36.4	5	16.7	18	28.6
2	5	45.5	4	18.2	6	20.0	15	23.8
3	1	9.1	10	45.5	19	63.3	30	47.6
Total	11	17.5	22	34.9	30	47.6	63	100.0
Pearson Chi-square = 10.70, df = 4, **$p = 0.030$**; Spearman Rank $\rho = 0.360$ (**$p = 0.004$**)								
liver ORAI3 intensityscore	colon ORAI3 intensity score						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	19	59.4	12	50.0	2	28.6	33	52.4
2	13	40.6	10	41.7	3	42.9	26	41.3
3	0	0.0	2	8.3	2	28.6	4	6.3
Total	32	50.8	24	38.1	7	11.1	63	100.0
Pearson Chi-square = 8.72, df = 4, $p = 0.069$; Spearman Rank $\rho = 0.230$ ($p = 0.070$)								

Table 5(E). Correlation between percent of stained cells and intensity score for MSH2 in liver metastasis and primary colon carcinoma.

liver MSH 2 (%)	colon MSH 2(%)						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	3	75,0	0	0,0	2	14,3	5	16,1
2	1	25,0	6	46,2	2	14,3	9	29,0
3	0	0,0	7	53,8	10	71,4	17	54,8
Total	4	12,9	13	41,9	14	45,2	31	100,0
Pearson Chi-square = 16.00, df = 4, **$p = 0.003$**; Spearman Rank $\rho = 0.401$ (**$p = 0.026$**)								
liver MSH 2 intensityscore	colon MSH 2 intensity score						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	4	40,0	2	14,3	0	0,0	6	19,4
2	5	50,0	8	57,1	1	14,3	14	45,2
3	1	10,0	4	28,6	6	85,7	11	35,5
Total	10	32,3	14	45,2	7	22,6	31	100,0
Pearson Chi-square = 12.71, df = 4, **$p = 0.013$**; Spearman Rank $\rho = 0.578$ (**$p < 0.001$**)								

Table 5(D) shows in turn correlations for ORAI3 in liver metastasis and primary colon carcinoma by means of two statistic tests. A moderate, statistically significant correlation was observed with regard to percent of stained cells in primary colon carcinoma and liver metastasis ($\rho = 0.360$, $p = 0.004$).

Table 5(F). Correlation between percent of stained cells and intensity score for MSH6 in liver metastasis and primary colon carcinoma.

liver MSH 6 (%)	colon MSH 6 (%)						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	6	75,0	12	70,6	11	28,9	29	46,0
2	1	12,5	3	17,6	12	31,6	16	25,4
3	1	12,5	2	11,8	15	39,5	18	28,6
Total	8	12,7	17	27,0	38	60,3	63	100,0
Pearson Chi-square = 11.58, df = 4, **$p = 0.021$**; Spearman Rank $\rho = 0.408$ (**$p < 0.001$**)								
liver MSH 6 intensityscore	colon MSH 6 intensity score						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	9	81,8	13	54,2	11	39,3	33	52,4
2	1	9,1	5	20,8	6	21,4	12	19,0
3	1	9,1	6	25,0	11	39,3	18	28,6
Total	11	17,5	24	38,1	28	44,4	63	100,0
Pearson Chi-square = 6.14, df = 4, $p = 0.189$; Spearman Rank $\rho = 0.295$ (**$p = 0.019$**)								

Table 5(G). Correlation between percent of stained cells and intensity score for MLH1 in liver metastasis and primary colon carcinoma.

liver MLH1 positivity (%)	colon MLH1 (%)						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	20	64,5	2	12,5	2	12,5	24	38,1
2	9	29,0	8	50,0	3	18,7	20	31,7
3	2	6,5	6	37,5	11	68,8	19	30,2
Total	31	49,2	16	25,4	16	25,4	63	100,0
Pearson Chi-square = 27.8, df = 4, **$p < 0.001$**; Spearman Rank $\rho = 0.608$ (**$p < 0.001$**)								
liver MLH1 intensityscore	colon MLH1 intensity score						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	22	66,7	8	34,8	2	28,6	32	50,8
2	9	27,3	11	47,8	4	57,1	24	38,1
3	2	6,0	4	17,4	1	14,3	7	11,1
Total	33	52,4	23	36,5	7	11,1	63	100,0
Pearson Chi-square = 7.37, df = 4, $p = 0.118$; Spearman Rank $\rho = 0.328$ (**$p = 0.009$**)								

Table 5(H). Correlation between percent of stained cells and intensity score for PMS2 in liver metastasis and primary colon carcinoma.

liver PMS2 (%)	colon PMS2 (%)						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	2	25,0	1	11,1	5	35,7	8	25,8
2	0	0,0	5	55,6	3	21,4	8	25,8
3	6	75,0	3	33,3	6	42,9	15	48,4
Total	8	25,8	9	29,0	14	45,2	31	100,0
Pearson Chi-square = 8.22, df = 4, $p = 0.089$; Spearman Rank $\rho = -0.207$ ($p = 0.263$)								
liver PMS 2 intensity score	colon PMS2 intensity score						Total	
	1		2		3			
	n	(%)	n	(%)	n	(%)	n	(%)
1	7	53,8	6	35,3	0	0,0	13	41,9
2	5	38,5	8	47,1	0	0,0	13	41,9
3	1	7,7	3	17,6	1	100,0	5	16,2
Total	13	41,9	17	54,8	1	3,2	31	100,0
Pearson Chi-square = 6.56, df = 4, $p = 0.161$; Spearman Rank $\rho = 0.284$ ($p = 0.121$)								

Tables 5(E)-5(H) present more accurately and in a separate manner the results obtained for the microsatellite instability (MSI) markers. The strong correlation was observed with regard to the percentage of stained cells and intensity of staining for MSH2 in primary colon carcinoma and liver metastasis ($\rho = 0.401$, $p = 0.026$ and $\rho = 0.578$, $p < 0.001$, respectively), **Table 5(E)**.

Table 5(F) shows correlations for MSH6 in liver metastasis and primary colon carcinoma. A statistically significant correlation was found with regard to percent of stained cells and intensity of staining for MSH6 ($\rho = 0.408$, $p < 0.001$ and $\rho = 0.295$, $p = 0.019$, respectively).

Table 5(G) shows correlations for MLH1 in liver metastasis and primary colon carcinoma. A statistically significant correlation was found with regard to the percentage of stained cells and intensity of staining for MLH1 ($\rho = 0.608$, $p < 0.001$ and $\rho = 0.328$, $p = 0.009$, respectively).

Table 5(H) presents correlations for PMS2 in liver metastasis and primary colon carcinoma. No statistically significant correlation was found with regard to percent of stained cells and intensity of staining for this marker ($\rho = -0.207$, $p = 0.263$ and $\rho = 0.284$, $p = 0.121$, respectively).

Figures 1(A)-1(H) show the results of immunohistochemical stainings for 4 markers (with exception of MSI markers and GPR56). **Figures 1(A)-1(D)** show staining from primary colon adenocarcinoma, whereas **Figure 1(E)-1(H)** from metastatic adenocarcinoma in the liver. **Figure 1(A)** shows the results of staining against PAX in colon. It is clearly seen that the diffuse cytoplasmic PAX expression has been observed in all cancer cells, whereas MUC1 in colon cancer cells has been found mainly in membranes, and to lesser extent in cytoplasm (**Figure 1(B)**). **Figure 1(C)** shows staining for ORAI3 in colon cancer cells

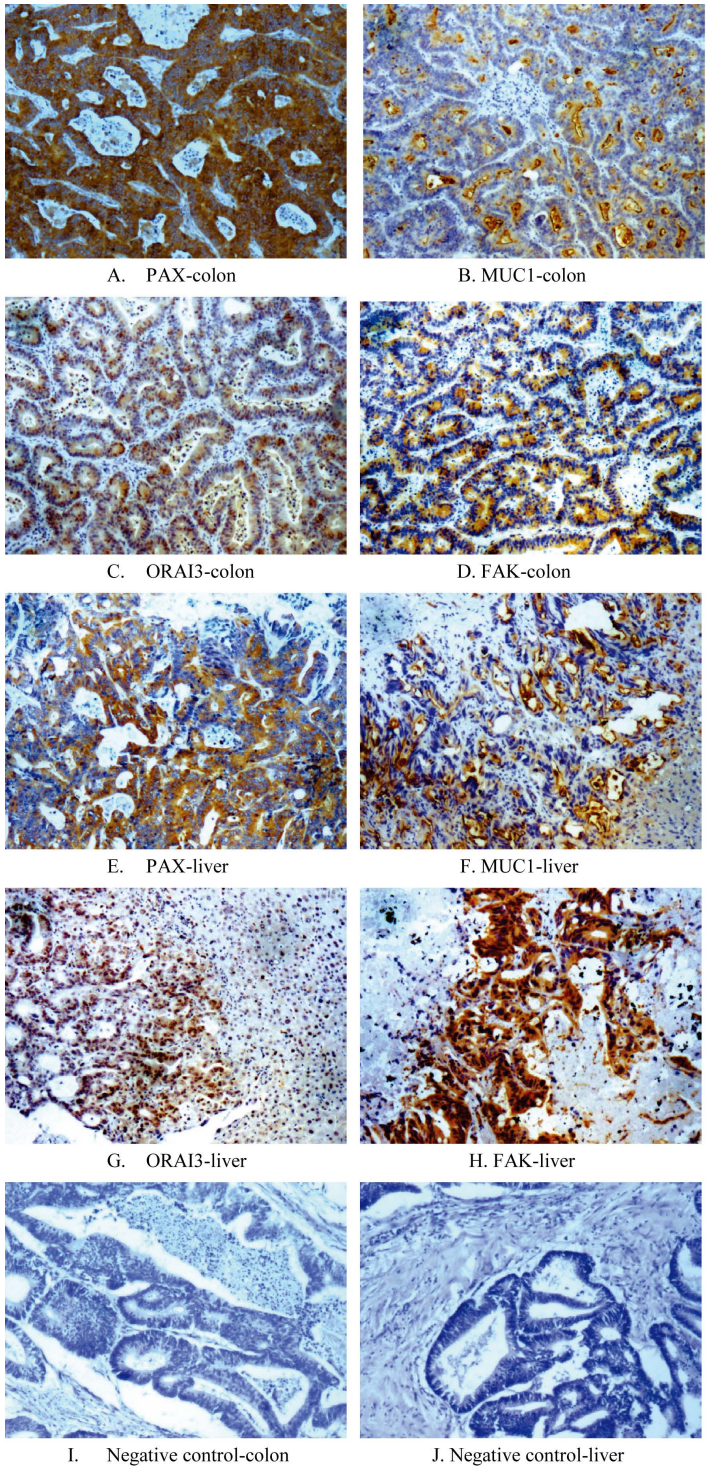

Figure 1. Representative immunohistochemical images of PAX, MUC1, ORAI3 and FAK expression patterns in colon carcinoma (A)-(D) and in liver metastases (E)-(H) (hematoxylin counterstained, 40x). (A) shows the results of staining against PAX in cytoplasm of colon cancer cells. (B) shows expression of MUC1 in membranes of colon cancer cells, and to lesser extent in cytoplasm. (C) shows staining for ORAI3 in nuclei of colon cancer cells, which was in part cytoplasmic, whereas (D) staining against FAK in cytoplasm of cancer cells. Identically, in metastatic colon cancer cells in liver (E)-(H) the pattern of staining was the same. (I)-(J) shows the negative control of colon carcinoma and liver metastasis; the first antibody was omitted here (hematoxylin counterstained, 40x).

which has turned out to be rather nuclear, and in part-cytoplasmic, whereas staining against FAK in cancer cells has been cytoplasmic (**Figure 1(D)**). Identically, in metastatic colon cancer cells in liver the staining pattern has been found to be the same (**Figures 1(E)-1(H)**). **Figures 1(I)-1(J)** show negative controls in primary colon cancer and in metastatic tumor where the first antibody was omitted.

Figure 2 shows results from western blotting assay. FAK presented a band at 125 kDa, MUC1 at 122 kDa, and less intense bands were found for GPR56 at 65kDa, and ORAI3 at 31 kDa.

4. Discussion

For decades now, researchers have resorted to tumor biomarkers in order to gain more information and a better understanding of neoplasms. It started with alpha-fetoprotein (AFP), discovered nearly 60 years ago by Bergstrand and Czar [13], and considered the major serum fetal protein in mammals ever since. Over the recent decades a large number of newly described markers were applied in immunohistochemistry, and both their prognostic and predictive values were widely accepted. In this study we selected two well known and characterized markers, MUC1 and PAX, and three markers with a more poorly identified role in colon carcinoma, FAK, GPR56 and ORAI3. Accordingly to literature [5] [6] [8] [10] these markers, MUC1, PAX, FAK and ORAI3, are correlated with a prognosis in CRC and metastases. GPR56 has been considered as a protein strongly correlated with mismatch repair genes and thus with MSI [9]. However, in our present study we could not relate expression of GPR56 with neither metastasis nor other parameters (T, N) due to the lack of or a very low expression of this protein in examined tissues.

In mismatch repair-proficient colorectal carcinoma, MUC1 protein expression was more often observed in tumors at a higher stage and higher grade [4], but we have not observed this kind of correlation with stage or grade in our

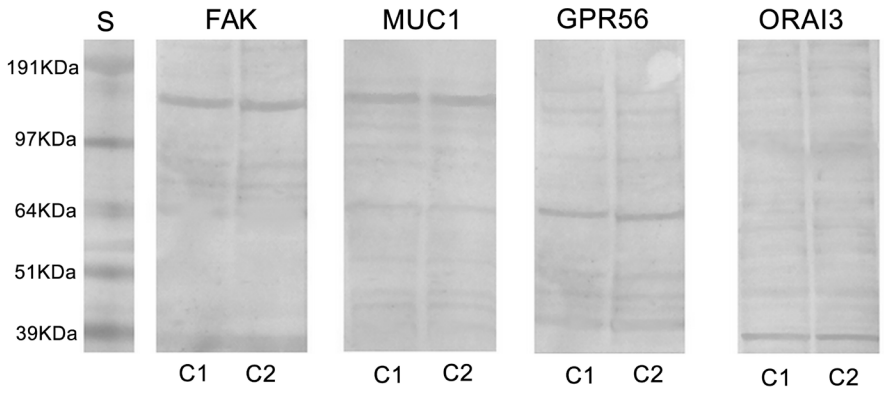

Figure 2. Western blot analysis of focal adhesion kinase (FAK, 125 kDa), mucin 1 (MUC1, 122 kDa), adhesion G protein-coupled receptor G1 (GPR56, 65 kDa) and ORAI calcium release-activated calcium modulator 3 (ORAI3, 31 kDa) expression in colorectal cancer tissues. C1, C2: primary colon cancer and metastatic tumor, respectively. S-Standard.

study. In MLH1-negative colorectal carcinoma there was no association between MUC1 expression and clinicopathological features, as well as it was presumed in HNPCC [4]. Survival analysis proved that the prognosis of the patients with a high expression of PAX was poorer than those with its low expression [6]. Cox proportional hazards model with stepwise selection showed that age; PAX expression and clinical TNM were independent prognostic factors influencing survival [6]. In our study we have not observed any correlation of PAX expression with clinicopathological parameters except for metastasis. However, it is agreed upon that PAX was expressed at significantly higher levels in colorectal cancer tissues and therefore it may serve as a potential prognostic indicator in patients with this cancer [6].

Real-time PCR analysis of colorectal carcinoma and liver metastases demonstrated increased FAK mRNA and protein levels in tumor and metastatic tissues versus normal tissues [14]. We have also found an increased expression of FAK by means of immunohistochemistry at both sites. It is well known, that ORAI3 may form Ca^{2+}-permeable channels in breast cancer [15] and in non-small cell lung adenocarcinoma [16]. Dubois *et al.* [17] showed the overexpression of ORAI3 in cancer tissues leading to the appearance of Ca^{2+} signaling dependent on the endogenous levels and production of arachidonic acid (AA). They have established an oncogenic role for the ORAI3 channel and a mechanism via which AA metabolism is involved in prostate oncogenesis [17]. We have observed increased expression of ORAI3 in both primary colon and metastatic tumors, which indicates that ORAI3 might also be involved in similar AA metabolism in colon cancer. Our findings were confirmed by western blotting assay.

Our present study showed that the evaluation of different markers with respect to intensity of staining and percentage of stained cancer cells may be considered as a prognostic marker for tumor progression and later for liver metastasis. This has been found for the percentage of stained cells particularly. Evaluation of effects of staining with regard to this parameter only, shows that for all examined markers, including these for MSI, with the exception of PMS2, correlation coefficients (ρ) exceeded 0.3 and showed moderate correlation. Moreover, some of them, including FAK and MLH1 were strongly correlated. Based on our data the high percentage of colon cancer cells stained for FAK correlates with a relevant high percentage of stained cells in liver. Since the FAK is a well-known mediator of tumorigenesis [18] and metastasis [19] it seems that the high percentage of stained cells in primary colon cancer should correlate more significantly with changes in the liver than the intensity of staining alone does. A similar observation has been made with regard to ORAI3, a protein which is also a well-known marker [20] and two out of four MSI markers, namely MSH6 and MLH1. The percentage of stained cells for FAK moderately correlated with the size of the tumor, $\rho = 0.324$ for colon, and for liver. It is noteworthy that the percentage of stained cells for MSH6 and MLH1 is also moderately correlated with tumor size. We did not observe any correlation between staining intensity or percent of stained cells and grading. This finding is in contrast with data shown by Ay and Benzerdjeb *et al.* [16] claiming that ORAI3 was not only over-

expressed in cancer tissues in comparison to normal ones, but it was also stronger in high grade tumors.

In summary, we would like to suggest that the counting percentage of stained colon cancer cells provides a more adequate method of immunohistochemical analysis than evaluation of the intensity of staining.

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