Overexpression of the *ITGAV* Gene Is Associated with Progression and Spread of Colorectal Cancer

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Abstract. *Background/Aim: The interaction of neoplastic cells with the extracellular matrix is a critical event for the initiation of cancer invasion and metastasis. We evaluated the relationship between the expression of SPARC, ITGAV, THBS1 and VCAM-1 genes of extracellular matrix in the progression and dissemination of colorectal cancer (CRC). Patients and Methods: Adult patients (N=114) underwent resection of CRC. Gene expression in CRC was determined by quantitative real-time polymerase chain reaction (PCR). Protein expression was analyzed by immunohistochemistry (IHC). Correlation with pathway-related molecules (p53, Bcl-2, Ki-67, EGFR and VEGF) was assessed. Results: Tumors with perineural invasion showed overexpression (p=0.028) of the ITGAV gene with regard to cancers without perineural invasion and validation of the result through IHC expression of the corresponding proteins, was significant for the expression of ITGAV protein (p=0.001). Conclusion: The overexpression of ITGAV gene was associated with higher progression and spread of CRC via perineural invasion.*

Tumor invasion and metastasis are critical steps in determining the human cancers' aggressiveness phenotype and represent the major causes of cancer-related deaths (1). Several sets of growth factors and their cognate receptors have been reported as being seriously involved in the regulation of tumor invasion and metastasis (1). Thus, the disruption of the growth factor and receptor axis is a current strategy for the development of anticancer drugs (2).

The extracellular matrix (ECM) is an association of collagen, non-collagenous glycoproteins, elastin fiber and proteoglycans synthesized and deposited in this compartment by the interstitial fibroblasts that surround the cells of the connective tissue (3). The pericellular proteolysis promotes the remodeling and degradation of the ECM and influences malignant cell transformation, tumor growth and the ability of metastatic spread, in addition to altering the mechanisms of angiogenesis and apoptosis, and impacting on the epithelial growth factors (3).

The macromolecule osteonectin (secreted protein acidic and rich in cysteine; SPARC) belongs to a family of matricellular proteins (4). The human *SPARC* gene is 26.5 kb long and located on chromosome 5q31-q33 (4). Its functions are modulating cell-to-cell and cell-to-matrix interactions, and it has also de-adhesive and growth inhibitory properties in non-transformed cells, which have led to the assessment of its role in cancer (5).

The integrins, transmembrane glycoproteins receptors used by human cells to bind cell-to-matrix, are heterodimers composed of alpha and beta subunits that act as a cell-anchoring site, as well as bi-directional-signaling molecules, controlling vital signs such as adhesion, polarization, differentiation, migration and cell division (6). Integrin alpha V (*ITGAV*) is a protein-coding gene and is located on chromosome 2q31-q32 (6, 7). The tumor expression of *ITGAV* influences angiogenesis, migration, invasion, survival of tumor cells and the initiation of metastasis (6, 7).

Thrombospondin (THBS1) is a trimeric glycoprotein stored in high concentrations in the platelet granules (8). This protein participates in platelet aggregation and clot formation and is strongly linked to the ECM (9). The gene *THBS1* is enclosed in the human chromosome 15q14 and its

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function comprises angiogenesis, apoptosis, activation of transforming growth factor-beta (TGF-beta) and immune-system regulation (8). When there is an increased expression of THBS1, tumors grow slowly, exhibit fewer angiogenesis and metastasis occurs less (8, 10). The low expression of THBS1 was inversely associated with neoplastic recurrence and a reduced overall survival in patients with colorectal cancer (CRC) (10). These features make the THBS1 an attractive target for cancer treatment (8-10).

The precursor of the vascular cell adhesion molecule 1 (VCAM-1), the 110 kDa transmembrane glycoprotein, is a sialoglycoprotein and member of the cell adhesion molecule immunoglobulin superfamily, which acts in the transit of white blood cells through the endothelial and epithelial barriers, cell-to-cell recognition, metastasis development and tumors' immune response (11, 12). It is expressed by the *VCAM-1* gene located on human chromosome 1p31-p32 (12). The interaction between VCAM-1 and its specific binding VLA-4 (complex A4b1) in the microvasculature of endothelial cells or periphery of blood cells is intricate in the mechanism that facilitates the adherence of tumor cells in the microvascular endothelium and onset of metastasis (13).

The aim of the present study was to evaluate the relations of the expressions of *SPARC, ITGAV, THBS1* and *VCAM-1* genes and their proteins in tumors of patients with colorectal carcinoma, correlating them with clinical and histopathology parameters of neoplastic progression and spread. The possible association among the expression of these ECM proteins and biological markers related to epithelial proliferation (epidermal growth factor; EGFR and Ki67), angiogenesis (vascular endothelial growth factor; VEGF) and apoptosis (p53 and Bcl-2) was also evaluated.

Patients and Methods

Patients and tumor samples. We studied 114 patients with CRC who underwent primary tumor resection at the Barretos Cancer Hospital (São Paulo, Brazil) between August 2006 and July 2009. All patients underwent the analysis of the expression of genes of interest in real-time PCR (RT-PCR) and immunohistochemistry (IHC) using the tissue microarray technique (TMA).

We included patients of both genders, aged ≥18 years old. Patients who had received neoadjuvant treatment (chemotherapy or radiotherapy) were excluded, as well as those without primary CRC site resection, those with previous or current diagnosis of another primary malignancy in any location of the body other than non-melanoma skin cancer or *in situ* carcinoma of the cervix and the patients with a known history of familial CRC.

Sixty-three (55.3%) patients were male and 51 (44.7%) female. Their median age was 60 years old (24-83). Both the right and left colons were affected in 41 cases (36.0%) each and the rectum was the primary tumor site in 32 cases (28.0%). Twenty-five (21.9%) patients were in tumor-node-metastasis (TNM) stage I, 39 (34.2%) were classified as TNM stage II, 34 (29.8%) as TNM stage III and 16 (14.0%) as TNM stage IV.

Table I shows the distribution of patients according to the categorization of the clinic and pathologic co-variables.

Covariates' analysis. Patients were classified according to the following clinicopathological characteristics: age group (<60 and ≥60 years old), histologic classification (adenocarcinoma not otherwise specified *vs*. mucinous adenocarcinoma), subtype tubular or villous, tumor grade [low (grades I and II) *vs*. high (grades III and IV)] and peritumoral lymphocyte infiltration (presence *vs*. absence).

Histological characteristics commonly associated with tumor dissemination and progression have been categorized as follows: venous invasion (presence *vs*. absence), lymphatic vessel invasion (presence *vs*. absence), perineural invasion (presence *vs*. absence), tumor stage (T1-2 *vs*. T3-4), lymph node metastasis (presence *vs*. absence), distant metastases (presence *vs*. absence) and TNM staging (I-II *vs*. III-IV) (14).

RNA extraction and cDNA synthesis by RT-PCR. Cryopreserved samples were embedded in a special medium for frozen tissue specimen conservation (Tissue-Tek OCT; Sakura Finetek, Torrance, CA, USA). After discarding inappropriate areas for RNA extraction, the tissue was mechanically macerated with liquid nitrogen and transferred to 1.5-ml microtubes free of RNAse and DNAse containing 1.000 μl TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was extracted according to the manufacturer's instructions and its quantification was performed using a spectrophotometer (Thermo Scientific NanoDrop 2000, Waltham, MA USA). The quality and integrity of the RNA were verified by the presence of 28S and 18S bands in agarose gel stained with 1% ethidium bromide to guarantee the absence of RNA samples degradation. RNA was purified with the RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations, eluted with 30 ml of water RNAse- and DNAse-free (Qiagen, Valencia, CA, USA), quantified in the spectrophotometer at a wavelength of 260 nm (NanoVue; GE Healthcare, Chicago, IL, USA) and stored at –80˚C until use. RT-PCR was performed using the Super-Script™ III first-strand synthesis SuperMix (Invitrogen, Carlsbad, CA, USA), as recommended by the manufacturer. The reaction was carried out in a 20-μl final volume containing 2 μg of total RNA with oligo (dT) (20) as a primer. The transcription phase was carried out in a thermal cycler (Mastercycler® ep Gradient S; Eppendorf, Hamburg, Germany) and the cDNA was stored at –20˚C for future reactions.

Analysis of the genes of interest. For each sample, an ECM and adhesion molecule PCR array (PAHS-013; SABiosciences, Qiagen, Valencia, CA, USA) plate was used. A mixture containing 1,275 μl of buffer with SYBR Green (2× Master Mix RT2 qPCR; SABiosciences, Qiagen, Valencia, CA, USA), 1,173 μl RNAse-free H_2O and 102 μl of cDNA sample was prepared. Afterwards, 25-μl aliquots were added to each well of a 96-well plate. Reactions were performed in a thermal cycler (ABI 7500; Applied Biosystems, Foster City, CA, USA), according to the following protocol: 95˚C for 10 min, 40 cycles at 95˚C for 15s and 60˚C for 1 min. Data analysis was performed according to http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php. Expression of each gene was classified as 'high' or 'low', considering the level of expression obtained after grouping patients by the covariates of interest after categorizing patients into the control or interest groups according to the covariates studied.

Table I. *Clinicopathological characteristics of 114 patients with colorectal carcinoma.*

Variables	N	%
Age		
<60 years	56	49.1
≥60 years	58	50.9
Gender		
Male	63	55.3
Female	51	44.7
Primary tumor site		
Right colon	41	36.0
Left colon	41	36.0
Rectum	32	28.0
Synchronic tumor		
No	112	98.2
Yes	2	1.8
Histological Classification		
Adenocarcinoma not otherwise specified	97	85.1
Mucinous adenocarcinoma	17	14.9
Histological subtype		
Tubular	99	86.8
Villous	15	13.1
Tumor grading		
Well differentiated	9	7.9
Moderately	91	79.8
Mild	14	12.3
Undifferentiated	0	0
Venous invasion		
Absent	93	81.6
Present	21	18.4
Lymphatic vessel invasion		
Absent	91	79.8
Present	23	20.2
Perineural invasion		
Absent	106	93.0
Present	8	7.0
Peritumoral lymphocyte infiltration		
Absent	21	18.4
Present	93	81.6
Resection margin status		
Positive	0	0
Negative	114	100
Lymph nodes dissected (media range)	17*	
Range	(3-67)	
Tumor stage		
T1	5	4.4
T2	27	23.7
T3	71	62.3
T4	11	9.6
Nodal stage		
N0	67	58.8
N1	25	21.9
N2	22	19.3
Distant metastasis		
Absent	98	85.9
Present	16	14.1
Distant metastasis sites		
Absent	98	85.9
Liver	9	7.9
Peritoneum	3	2.6
Lung	2	1.8
Ovary	2	1.8
Clinical stages		
I	25	21.9
II	39	34.2
III	34	29.8
IV	16	14.0

*28 patients had <12 lymph nodes dissected or analyzed.

Tissue microarray (TMA) block construction. Original paraffin blocks were sectioned at 4-μm thickness and hematoxylin and eosin (HE) stained. All sections were reviewed to confirm the diagnosis of CRC and the histopathology findings were re-evaluated.

With the aid of Beecher™ equipment (Beecher Instruments, Silver Spring, MD, USA), TMA blocks were prepared according to the manufacturer's specifications. The samples were cut to 4-μm thickness and a small roll was used to press the section on the tape. The tape with the attached histological section was then placed on a resin-coated slide (part of the adhesive system kit) and pressed with the same roll for better adherence. Afterwards, the slides were placed under UV light for 20 min and then exposed to a solvent solution (TPC) for 20 additional min. The slides were dried and the tapes removed. Subsequently, the slides were paraffin embedded and sent for storage in ideal cooling conditions.

IHC technique. Endogenous peroxidase activity was blocked by incubating the sections in a methanol bath containing 3% hydrogen peroxide for 20 min, followed by a washing in distilled water. The sections were initially submitted to heat-induced epitope retrieval using citrate buffer (pH 9.0) in an uncovered pressure cooker (Eterna®; Nigro, Araraquara, SP, Brazil).

Blocking of endogenous peroxidase was obtained with 3% H_2O_2 (10 vol.), with 3 washes of 10-min each. The slides were again washed in distilled running water and then in phosphate-buffered saline (10 mM; pH 7.4) for 5 min. Subsequently, the primary antibody was applied and the slides were incubated overnight at 8˚C.

Primary antibodies. Three primary antibodies to ECM components were imported from Abcam (Cambridge, MA, USA): anti-SPARC antibody, rabbit IgG isotype, polyclonal, 1: 400 (code: ab14174); ITGAV, mouse IgG1 isotype, clone 272-17E6, 1:400 (code: ab16821); THBS1, mouse isotype IgG1, clone A6, 1:400 (code: ab1823); and VCAM-1. One of them was imported by Santa Cruz Biotechnology (Dallas, TX, USA): mouse IgG1 isotype, clone B-N8, 1:400 (code: BMS 141).

Additionally, the following non-ECM primary antibodies were used in this study: anti-p53 antibody, IgG2b class, clone DO-7, 1:300 (reference: M7001); anti-Bcl-2 antibody, mouse IgG1 isotype, clone 124, 1:600 (reference: M0887); anti-VEGF antibody, mouse IgG1 isotype, clone VG 1, 1:100 (reference: M7273); anti Ki67 antibody, mouse IgG1 isotype, clone MIB-1, 1:500 (reference: M7240), all of them from DAKOCytomation (Glostrup, Denmark); and anti-EGFR antibody, mouse IgG1 isotype, clone EGFR-25, 1:100 (reference: NCLEGFR-384) from Novocastra (Newcastle, UK).

The IHC analysis positive controls were the following: osteosarcoma for SPARC; human tonsils for ITGAV, THBS1, VCAM-1, VEGF, Ki67, p53, and Bcl-2, and placenta for EGFR.

Immunostaining analysis. Tissue expression of SPARC, ITGAV, THBS1, VCAM-1, EGFR, VEGF, p53, Bcl-2 and Ki67 markers were categorized dichotomously as 'overexpression' or 'low expression' according to the 'quick score' method (15, 16). This score system uses a combination of the percentage of stained cells (P) and intensity of staining (I); the 'quick score' was calculated by multiplying both values.

The scores used for the percentage of stained tumor cells were as follows: 0 points (absence of stained cells), 1 point (stained cells ≥25%), 2 points (stained cells from 26 up to 50%) and 3 points (stained cells >50%). Scores used for the intensity of the cancer cell

Table II. *Comparison among the SPARC, ITGAV, THBS-1 and VCAM-1 extracellular matrix genes expression by qRT-PCR, the clinicopathological covariates and validation through the corresponding protein expression evaluated by IHC in colorectal carcinoma (n=114).*

Genes	Clinicopathologic covariate	Comparisons	Fold regulation	*p*-Value	Validation by IHC	*p*-Value
SPARC	Histological classification	Non mucinous carcinoma *vs.* mucinous carcinoma	2.25	0.007	No	0.25
SPARC	Histological subtype	Tubular *vs.* villous	–1.71	0.02	No	0.27
ITGAV	Perineural invasion	Absence *vs.* presence	1.37	0.028	Yes	**0.001**
THBS-1	Age	<60 years *vs.* ≥60 years	–1.4	0.03	No	0.853
VCAM-1	Histological subtype	Tubular *vs.* villous	1.63	0.02	No	0.23

vs.=*Versus*; Statistical significance was determined by the χ^2 or Fisher's exact tests. Significant values are bold.

staining were as follows: 1 point (mild intensity), 2 points (moderate intensity) and 3 points (intense staining). As a result, expression of a gene product in tumor cells was considered to be high (overexpressed) when the final score was >4 (P × I >4) and the markers that presented a final score ≤4 were considered to have low expression. Stromal and tumor cells were not treated separately during IHC analysis and only the level of expression of markers on tumor cells was considered for scoring.

Statistical analysis. Statistical associations between gene and protein expression levels of SPARC, ITGAV, THBS1 and VCAM-1, as well as the clinicopathological factors were determined using a non-parametric Mann-Whitney *U*-test for quantitative variables and a Chi-square (χ^2) test for qualitative variables or frequencies and proportions. When the two assumptions were not met, the Fisher's exact test was used.

To measure the association between ECM markers (SPARC, ITGAV, THBS1 and VCAM-1) and non-ECM markers (EGFR, VEGF, p53, Bcl-2 and Ki67) as ordinal variables, the Spearman correlation coefficient was used. The Shapiro-Wilk test was used to verify the data's normal distribution.

The statistical significance level was set at 5% ($p<0.05$) and the data were analysed using the SPSS software™ (Statistical Package for Social Sciences; SPSS, Chicago, IL, USA), version 15.0.

Results

RT-PCR-based analysis of the expression of the genes of interest. Table II shows the results concerning the expression of the relevant genes studied by RT-PCR and IHC of the corresponding proteins. Overexpression was observed ($p=0.007$) for the *SPARC* gene in colorectal adenocarcinomas of the mucinous type compared to non-mucinous adenocarcinomas, as well as for comparison between tubular and villous subtypes ($p=0.002$). Tumors with perineural invasion showed overexpression ($p=0.028$) of the *ITGAV* gene with regard to cancers without perineural invasion and the validation of these result through IHC expression of the corresponding proteins was significant for the expression of the ITGAV protein ($p=0.001$). There was low expression ($p=0.03$) of the *THBS1* gene in patients <60 years old and overexpression ($p=0.02$) of the VCAM-1 gene compared to the tubular villous CRC.

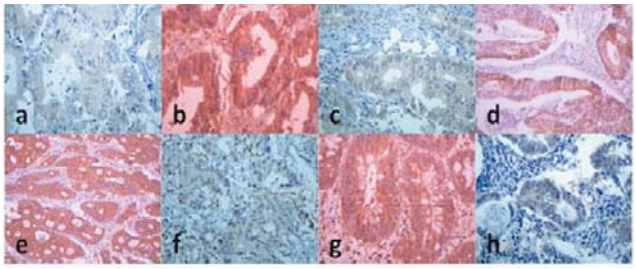

Figure 1. *Photomicrograph of immunohistochemical expression of SPARC, ITGAV, THBS1 and VCAM-1 extracellular matrix proteins in colorectal carcinoma. The expression is represented by the stained cell cytoplasm. a, SPARC low expressed (×200); b, SPARC overexpressed (×400); c, ITGAV low expressed (×200); d, ITGAV (×100); e, VCAM-1 overexpression (×100); f, VCAM-1 low expression (×200); g, THBS-1 overexpression (×400); h, THBS-1 low expression (×200).*

Analysis of the expression of the relevant biological markers according to the IHC technique. The IHC expression of SPARC, ITGAV, THBS1 and VCAM-1 proteins are shown in Figure 1. Table III displays the analysis of the results for proteins' IHC expression in operated patients with CRC, according to the studied covariate.

Patients with no metastatic colorectal tumors showed an overexpression of SPARC protein ($p=0.027$) compared to those with distant metastasis.

The ITGAV protein was significantly overexpressed in tumors without venous invasion ($p<0.001$), lymphatic invasion ($p<0.001$), neural invasion ($p=0.001$), lymph node metastasis ($p<0.001$) and no systemic metastasis ($p<0.001$), and less advanced (I+II) clinical stages ($p<0.001$) but with deeper invasion (T3+T4) of the intestinal wall ($p=0.022$).

There was a significant lower expression ($p=0.003$) of the *THBS1* gene in tumors with deeper degree of neoplastic invasion (T3+T4) in the intestinal wall (Table III).

Tabla III. *Analysis of immunohistochemistry expression of extracellular matrix proteins SPARC, ITGAV, THBS-1 and VCAM-1 according to covariates in patients with colorectal carcinoma (n=114).*

Covariates	Categorization	SPARC		ITGAV		THBS-1		VCAM-1	
		–/+ n	*p**	–/+ n	*p**	–/+ n	*p**	–/+ n	*p**
Age	<60 years	5/51	0.486	27/29	0.259	31/25	0.853	22/34	0.107
	≥60 years	3/55		35/23		31/27		14/44	
Histological type	Adenocarcinoma non mucinous	6/91	0.341	54/43	0.601	50/47	0.19	29/68	0.356
	Adenocarcinoma mucinous	2/15		8/9		12/5		7/10	
Histological subtype	Tubular	4/77	0.257	44/37	0.594	39/42	0.174	22/59	0.234
	Villous	2/14		10/6		11/5		7/9	
Tumor grading	Low grade	7/93	1.000	56/44	0.401	52/48	0.253	28/72	**0.028**
	High grade	1/13		6/8		10/4		8/6	
Peritumoral lymphocyte infiltration	Absent	0/21	0.348	10/11	0.629	13/8	0.478	7/14	0.848
	Present	8/85		52/41		49/44		29/64	
Venous invasion	Absent	5/88	0.163	58/35	<0.001	48/45	0.235	25/68	**0.023**
	Present	3/18		4/17		14/7		11/10	
Lymphatic vessel invasion	Absent	7/84	1.000	59/32	**<0.001**	50/41	0.812	29/62	0.895
	Present	1/22		3/20		12/11		7/16	
Perineural invasion	Absent	8/98	1.000	62/44	**0.001**	56/50	0.287	30/76	**0.012**
	Present	0/8		0/8		6/2		6/2	
Tumor stage	T1+T2	2/30	1.000	23/9	**0.022**	10/22	0.003	5/27	**0.025**
	T3+T4	6/76		39/43		52/30		31/51	
Lymph node metastasis	Absent	4/63	0.715	59/8	**<0.001**	33/34	0.252	17/50	0.089
	Present	4/43		3/44		24/18		19/28	
Distant metastasis	Absent	5/93	**0.027**	62/36	**<0.001**	52/46	0.592	27/71	**0.022**
	Present	3/13		0/16		10/6		9/7	
Clinical stage	I+II	4/60	0.728	59/5	**<0.001**	32/32	0.345	17/47	0.192
	III+IV	4/46		3/47		30/20		19/31	

+/– = Low/high expression. Statistical significance was determined by the χ^2 or Fisher's exact tests. Significant values are bold.

Table IV. *Logistic regression for the immunoexpression of extracellular matrix proteins SPARC, ITGAV, THBS-1 and VCAM-1 in patients with colorectal carcinoma (n=114).*

Covariate	Protein	Coefficient	SE*	OR**	CI*** (95%)	*p*-Value
Lymph node metastasis present	ITGAV	4.684	0.706	108.167	27.127 a 431.305	**<0.001**
Degree of infiltration (T1+T2 *vs*. T3+T4)	THBS1-1	–1.471	0.469	0.230	0.092 a 0.575	**0.002**
Perineural invasion present	VCAM-1	–2.028	0.844	0.132	0.025 a 0.689	**0.016**

*SE=Standard error, **OR=Odds ratio, ***CI=Confidence interval. Significant values in bold.

The VCAM-1 protein was significantly overexpressed in tumors with low degrees of cell differentiation (p=0.028) and without venous (p<0.023) or perineural (p=0.012) invasion, which had a more profound (T3+T4) invasion of the bowel wall (p=0.025) and no systemic metastases (p=0.022).

Table IV shows the logistic regression results for immune staining to SPARC, ITGAV, THBS1 and VCAM-1 proteins. Independent variables were ITGAV protein overexpression for lymph node metastasis (OR=108.1, 95% CI=27.1-431.3, p<0.001), THBS1 protein overexpression (OR=0.230 95% CI=0.092-0.575, p=0.002) in the presence of a deeper level (T3+T4) intestinal wall tumor infiltration and the overexpression of VCAM-1 protein (OR=0.132, 95% CI=0.25-0.689, p=0.016) for the presence of perineural invasion.

Correlation of SPARC, ITGAV, THBS1, VCAM-1, EGFR, VEGF, p53, Bcl-2 and Ki67 expression. Table V describes the analysis results for the comparative expression of genes *SPARC, ITGAV, THBS1* and *VCAM-1* with the respective proteins and epithelial biological markers related to

proliferation (epidermal growth factor; EGFR) and Ki67 and angiogenesis (vascular endothelial growth factor; VEGF) and apoptosis (p53 and Bcl-2). The respective values of the Spearman correlation coefficients (r) are also displayed.

The SPARC protein had a significant weak and inverse correlation with VEGF expression (r=-0.197, p=0.036). The ITGAV marker showed a strong and direct correlation with EGFR score (r=0.774, p<0.001), an average direct correlation with the Ki-67 marker (r=0.262, p=0.005) and a weak and direct correlation with the p53 marker (r=0.215, p=0.022). The VCAM-1 marker showed a weak inverse correlation toward Ki-67 (r=-0.247, p=0.008) and p53 (r=-213, p=0.023). The THBS1 protein had no significant correlation with any epithelial markers.

Table V. *The Spearman coefficient correlation (r, two-tailed model) for associations among immunohistochemistry expression of extracellular matrix proteins SPARC, ITGAV, THBS-1 and VCAM-1 and immunohistochemistry expression of cell proliferation markers (EGFR and Ki67), angiogenesis (VEGF) and apoptosis (p53 and Bcl-2) in patients with colorectal carcinoma (n=114).*

	EGFR	VEGF	Ki67	p53	Bcl-2
SPARC					
r	–0.059	–0.197	–0.030	–0.016	–0.069
p-Value	0.532	**0.036**	0.748	0.866	0.468
ITGAV					
r	0.774	–0.103	0.262	0.215	–0.106
p-Value	**0.000**	0.277	**0.005**	**0.022**	0.263
THBS1					
r	–0.127	0.109	–0.155	–0.180	0.070
p-Value	0.178	0.249	0.079	0.055	0.456
VCAM1					
r	–0.108	–0.052	–0.247	–0.213	0.075
p-Value	0.254	0.585	**0.008**	**0.023**	0.425

0=No correlation; ≤0.25=weak correlation; 0.25-0.50=regular correlation; 0.50-0.75=moderate correlation; >0.75=strong correlation; 1.0=perfect correlation. Significant values are bold.

Discussion

The role of ECM components in tumor phenotype has not been studied much in CRC (1, 3). The interaction of neoplastic cells with the ECM is a critical event for the initiation of cancer invasion and metastasis. As an integral component of the microenvironment in CRC, stromal cells can influence tumor progression (17).

High SPARC immune expression is associated with better disease outcome in stage II CRC and may be of prognostic value for cancer survival (18). SPARC is found in the ECM of CRC and expressed in stromal and CRC cells (4, 18). Though SPARC's influence on CRC is not clear, epigenetically-regulated SPARC expression in the microenvironment of CRC stromal cells can affect primary CRC progression and is influenced by lymph vascular invasion (4).

Mucinous CRC accounts for 10-15% of all colorectal carcinomas (19). It has already been reported that SPARC is expressed in greater amounts in mucinous CRC than non-mucinous CRC (20) and that mucinous CRC are an independent prognostic factor for poorer outcomes (21).

Liang *et al*. (22) used human colon adenocarcinoma tissues and a corresponding non-diseased colon from 114 patients' biopsies, investigated using IHC staining the expression of SPARC and VEGF and evaluated the relationship between SPARC and VEGF, as well as their prognostic significance for patients. The results showed that SPARC expression was mainly found in the stromal cells surrounding the colon cancer and there was significant difference among those tissues, lymph node metastasis and differentiation degree of tumor. Patients with low or absent SPARC expressions had significantly worse overall survival and disease-free survival in a single factor analysis. Using the Cox regression analysis, SPARC emerged as an overall survival and disease-free survival independent prognostic factor for colon cancer. The authors concluded that the low expression or absence of stromal SPARC was an independent prognostic factor for poor prognosis of colon cancer.

SPARC may function as a modulator of chemotherapy sensitivity by enhancing apoptosis rate. Chan *et al*. (23) examined the effects of SPARC on cellular senescence in the presence of chemotherapy. They found that CPT-11-resistant cells exposed to endogenous or exogenous SPARC could be triggered into cellular senescence. This induction is associated with higher levels of p16 (*INK4A*) and phosphorylated p53. The knock down of p16 (*INK4A*) reduces drug-induced senescence in all cells but the knock down and overexpression of p53 modulates senescence only in cells exposed to SPARC. Furthermore, treatment of mice with SPARC and CPT-11 leads to significantly increased cellular senescence and tumor regression. The authors highlighted that the chemo-sensitizing effects of SPARC in CRCs are in part mediated by activating cellular senescence.

Yang *et al*. (20) made a survival analysis of 292 validation set of CRC patients that revealed a poorer prognosis for patients lacking *SPARC* expression than for patients with normal *SPARC* expression (56.8% *vs*. 75.8% 5-year survival rate, p=0.0014).

In our series, there was a significant *SPARC* gene overexpression (p=0.007) assessed by qRT-PCR in non-mucinous compared to mucinous CRC and in villous concerning tubular CRC (p=0.02) but no validation was observed by the immunohistochemical study of the corresponding protein in relation to these variables. In the present study, a significant relationship between low expression marker VEGF and overexpression of SPARC protein in the tumor was also observed. This SPARC effect was similar to those published by other authors (24).

It is significant that one of the microscopic criteria by which infiltrating growth can be recognized is perineural invasion (25). This pathologic feature is not routinely assessed or specifically reported in CRCs, yet perineural invasion itself has been an independent indicator of poor prognosis shown in a number of studies (24-26). Some authors (27-29) have shown increased risk of regional and systemic spread in tumors with perineural invasion. Thus, it is considered that the capacity of tumor cell spread through the perineural space of nerves is related to the progression and spread of CRC.

In this series, the *ITGAV* gene was significantly (p=0.028) amplified in the presence of tumors without neural invasion, which was validated by immunohistochemical overexpression of the corresponding protein (p=0.001), despite the fact that *ITGAV* gene overexpressions have already been correlated to a greater likelihood of perineural invasion in prostate cancer (30), carcinoma of the head and neck (31) and also in CRC (32, 33). Moreover, the presence of the *ITGAV* gene overexpression is associated with an increased risk of invasion and tumor spread in laryngeal and hypopharyngeal squamous cell carcinomas (34).

In this study, ITGAV protein immune expression was also significantly overexpressed regarding the absence of venous invasion, lymphatic invasion, lymph node involvement, distant metastasis, in situ invasion of the intestinal wall and less advanced stages of CRC. The absence of lymph node involvement was an independent variable in the multiple logistic regression analysis. However, no significant alteration of the *ITGAV* gene expression, as determined by qRT-PCR in relation to this variable, was observed. The strong correlation of IHC expression observed among EGFR and ITGAV proteins suggests interaction between these two signaling pathways, as also observed by Viana *et al*. (32).

The loss of *THBS1* was observed in early colonic adenomas and it became undetectable in invasive colon cancers (35). The expression and role of *THBS1* remain controversial and the regulation of *THBS1* expression in colon cancer is poorly understood (35). Miyanaga *et al*. (36) performed IHC staining for THBS1 on 132 CRC specimens. They found that THBS1 expression significantly correlated with independent prognostic factors. This study furnishes evidence that THBS1 is expressed in tumor stroma, inhibits tumor angiogenesis and suppresses tumor growth by activating TGF beta-1. A 5-year survival rate for patients with THBS1-negative lesions at the deepest invasive tumor site was significantly poorer than that for patients with THBS1-positive lesions.

This study also suggested that the expression of the THBS1 molecule might exert a protective role for local disease progression. There was THBS1 protein overexpression in tumors with a light degree of invasion (T1+T2) in the intestinal wall, compared to tumors with deeper invasion (T3+T4). In multivariate analysis, the overexpression of the THBS1 protein was significant (p=0.002) indicating that patients with increased THBS1 protein immune staining were less likely to have an advanced tumor invasion of the intestinal wall. However, the expression of *THBS1* gene determined by qRT-PCR showed a relation only with age of the CRC operated patients.

Dymicka-Piekarska *et al*. (13) demonstrated an increased level of soluble adhesion molecule (VCAM-1) and angiogenic factor (VEGF) in CRC patients compared to the control group. The dynamics of these molecules showed a tendency for rising along tumor size and metastasis formation. Soluble VCAM-1 stimulates endothelial cells, chemotaxis and angiogenesis (13).

In the present study, the qRT-PCR technique allowed to observe that the *VCAM-1* gene low expression was significantly (p=0.016) related to the absence of perineural invasion, as validated by immune staining with significantly increased protein VCAM-1 in CRC operated patients (p=0.012). The IHC expression of VCAM-1 protein was significant (p=0.16) by multivariate analysis regarding the absence of perineural invasion for immune staining for VCAM-1 protein. The results suggest that the *VCAM-1* gene has a protective effect on perineural invasion in CRC.

It was observed that the VCAM-1 protein immune reactivity was significantly increased in low tumor grading CRC, venous invasion absence, shallow intestinal wall invasion and lack of distant metastases. However, no significant increase in expression evaluated by the qRT-PCR technique of the *VCAM-1* gene was noticed.

It is noticeable that overexpressed genes do not necessarily mean overexpressed proteins. Hence, the reason to perform the validation of the immune expression of the respective proteins is encoded by *SPARC, ITGAV, TSP-1* and *VCAM-1* genes.

Future studies should be directed to the analysis of the methylation or mutation and sequencing of the genes, which may explain the variability of the progression and spread of CRC, in accordance with the expression of genes related to the ECM inside these tumors.

Conclusion

The genetic and protein overexpression of ITGAV is associated with a higher possibility of progression and spread of CCR *via* perineural invasion. The strong correlation of IHC expression observed among EGFR and ITGAV proteins suggests interactions between these two signaling pathways. The *SPARC* gene expression seems to be associated with the anti-angiogenic effect by means of VEGF down-regulation.

Ethical Standards

The Institutional ethics committee approved the use of the human tissue for research. This study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

Conflicts of Interest

All Authors declare that they have no conflicts of interest.

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