ORIGINAL PAPER

Immunohistochemical evaluation of the tumor neoangiogenesis as a prognostic factor for gastric cancers

DANIELA LAZĂR[1)], SORINA TĂBAN[2)], M. RAICA[3)], I. SPOREA[1)], MARIOARA CORNIANU[2)], A. GOLDIŞ[1)], CORINA VERNIC[4)]

[1)]*Department of Gastroenterology*
[2)]*Department of Pathology*
[3)]*Department of Histology*
[4)]*Department of Biostatistics*
„Victor Babeş" University of Medicine and Pharmacy, Timisoara

Abstract

Introduction. The angiogenesis, the process by which new blood vessels are formed, plays an essential role in the survival of the malignant cells, in the local expansion and tumor invasion, as well as in the appearance of distant metastases. *Material and methods*. We evaluated the relation between MVD, the VEGF expression, the clinicopathologic factors and the survival in patients with gastric cancer. A prospective study has been carried out, regarding the evolution and aggressiveness of the gastric cancer, with a duration of 5 years, 61 patients that underwent a surgery for gastric cancer being included in the study. The immunohistochemical reactions for CD34 and VEGF were performed for all gastric cancers cases included in the study group. *Results*. MVD has shown in the gastric carcinomas an average value significantly higher in comparison to the normal mucosa (38.7 *vs.* 12.5, p<0.001 ES). In the intestinal type we have noticed a much lower average MVD than the average MVD in the diffuse type of gastric carcinomas (36.8 *vs.* 41.6) (p = 0.02478 S). The anaplastic carcinoma and the signet ring cell carcinoma are detaching themselves as histological forms associated to an intense neoangiogenesis activity. The neoangiogenesis activity is correlated with: the histologic grade, the lymphovascular invasion, the level of extend, the lymph node metastasizing, the distant metastasizing and the TNM stage. The positive immunoreactions for VEGF are significantly more frequent in the gastric carcinomas, in comparison to the normal gastric mucosa (65.6% *vs.* 6.5%, p<0.001 ES). The immunoreactions to the VEGF protein were positive in 71.1% of the intestinal carcinomas, significantly more frequent in comparison to the diffuse type carcinomas (52.9%) (p = 0.018178 S). Our results show a tight correlation between the histologic grade, the level of the tumor invasion and the VEGF expression. *Conclusions*. Our results prove the major correlation between the VEGF expression and the 5-year survival rate of the patients with gastric cancer, the survival rate for the carcinomas with VEGF +~++ being significantly lower than for the VEGF negative ones (12.5% *vs.* 23.8%) (p = 0.027983 S).Our study proves a tight correlation between the VEGF expression and the MVD (p = 0.03986 S), these factors playing an important role in the tumoral biologic conduct, in the progression and the prognostic.

***Keywords*:** gastric cancer, tumor angiogenesis, MVD, VEGF, survival.

Introduction

The angiogenesis, the process by which new blood vessels are formed, plays an essential role in the survival of the malignant cells, in the local expansion and tumor invasion, as well as in the appearance of distant metastases. The intratumoral microvessel density (MVD), which can be evaluated immunohistochemically, seems to have an influence on the prognosis of various malignant tumors.

The forming of new intratumoral microvessels depends on the elaboration of the angiogenic growth factors by the malignant cells (vascular endothelial growth factor – VEGF, growth factor derived from thrombocytes and β1-TGF – β1 transforming growth factor). These factors' expression is correlated with the tumor angiogenesis, the neoplasia progression and the severe prognosis. Among the known angiogenic factors, VEGF plays a central role in the angiogenesis process control in the cancerous disease. The biologic functions of VEGF include the selective mitoses induction in the endothelial cells, the proliferation of these cells and overflow of plasmatic molecules.

Material and methods

In the present work, we intend to evaluate the relation between MVD, the VEGF expression, the clinicopathologic factors and the survival in patients with gastric cancer. A prospective study has been carried out, regarding the evolution and aggressiveness of the gastric cancer, with a duration of 5 years, 61 patients that underwent a surgery for gastric cancer in the Surgery Departments of the Emergency County Hospital No. 1 in Timisoara being included in the study.

The performed surgeries, of curative or palliative intention, were not preceded by a chemo- or radiotherapy treatment. The patients' survival rate was observed over a variable period of time, between one month and 68 months. For each case, clinical and morphological data were collected. The surgical gastric samples were morphologically analyzed, by

macroscopic and microscopic examination, by usual histological, histochemical and immunohistochemical stainings.

The gastric carcinomas were classified and interpreted according to the evaluation protocol recommended by the *American Joint Committee on Cancer* (AJCC) and the *International Union Against Cancer* (UICC) from January 2005. The survival period was calculated starting with the month when the surgery took place, until the month of death or the month of the survival confirmation, and the survival rate was represented by the survivals percent at the end of the observed period (in years and months).

For the immunohistochemical evaluation of the tumor neoangiogenesis, we have used the anti-CD34 monoclonal antibody, the clone QBEnd10 (DAKO Carpinteria, USA), using the LSAB technique. The sections were pre-treated by boiling for 20 minutes in Retrieval solution at 95–99^{0}C and then treated with the primary antibody, the secondary antibody and with Streptavidine for 30 minutes. The visualization system has included DAB and Hematoxylin counterstaining. In the immunohistochemical study of the VEGF expression, we have used the VEGF anti-human monoclonal antibody, the VG1 clone of IgG type (Novus Biologicals), using the LSAB+ technique. In order to expose the antigen, the sections were pre-treated by boiling for 15 minutes in the microwave oven (MW) in a pH 9 exposing solution (DAKO). The incubation period with the primary antibody of 1 : 25 dilution was of an hour. The used visualization system included DAB and modified Lillie Hematoxylin counterstaining.

The angiogenesis quantification was accomplished by measuring of the microvessel density (MVD). The sections were microscopically examined, initially using a small objective (×100). The areas where the most numerous CD34+ cells situated in the tumor invasion front were selected, surrounded by adenocarcinomatous pseudoglands and adjacent to the normal gastric structures. In these areas, we have counted the microvessels using a ×200 objective.

The isolated immunoreactive endothelial cells or groups of endothelial cells separated by the adjacent microvessels were considered to be quantifiable individual vessels. These CD34+ cells spots did not always show visible lumens or associated red cells. In absence of the discontinuity, the ramified structures were quantified as a sole vessel. The microvessels from the granulation tissue developed in the ulcerated tumors or the microvessels in the tumor necrosis areas' vicinity were not counted.

The immunohistochemical expression of VEGF was evaluated by a score corresponding to the sum between:

- the percentage of positive cells: 0 = 0% immunopositive cells; 1 = <25% positive cells; 2 = 26–50% positive cells; 3 = >50% positive cells;
- the staining intensity: 0 = negative immunoreaction; 1 = weak intensity; 2 = moderated intensity; 3 = strong intensity.

The sum of the two parameters varied between 0 and 6. In our study, we have considered:

- a negative immunoreaction (-) for a score between 0 and 2;
- a slightly positive immunoreaction (+) for a score between 3 and 4;
- a strongly positive immunoreaction (++) for a score between 5 and 6.

The immunohistochemical reactions for CD34 and VEGF were performed for all gastric cancers cases included in the study group. We have observed the expression of the two antibodies as well at tumor level, as also in the peritumoral gastric mucosa.

Results

Anti-CD34 antibody's immunohistochemical evaluation

In the gastric mucosa of normal histological appearance, situated in the tumor's vicinity, the microvessels show an uniform spatial distribution among the glands (Figure 1), with small, even lumens, with approximately equal dimensions and an average MVD of 12.5/5 microscopic fields, observed using a ×200 objective (Figure 2).

The studied gastric carcinoma were characterized by an intense tumor neoangiogenesis, especially in the invasion front, with numerous microvessels and positive CD34 endothelial cell groups (hot spots) among the malignant structures (Figure 3). MVD in the gastric carcinomas showed variable values, between 12 and 65, with an average of 38.7, significantly higher comparatively to the normal mucosa (12.5, $p<0.001$ ES). In comparison to the normal mucosa, the vascular architecture is deeply modified, with unequal, agglomerated vessels, with irregular lumens, unevenly distributed among the malignant structures (Figure 4). In many cases, we have noticed positive CD34 endothelial cells isolated or in small groups, without forming vascular lumens with red cells.

The distribution of the average values of MVD depending on the observed clinicopathologic factors is presented in Table 1.

Our results show that the sex and age of the patients do not influence the tumor angiogenesis ($p>0.05$ NS). The tumors extended in the entire stomach and those located at the cardia show intense angiogenesis activities, with average MVD of 40.2 and 41.4.

The average MVD values have shown significant differences depending on the gastric cancers' Lauren classification (Table 1).

For the intestinal type (Figure 5), we have observed an average MVD of 36.8, much more reduced than the average MVD from the diffuse type of gastric carcinomas (41.6) ($p = 0.02478$ S) (Figure 6).

The anaplastic carcinoma and the signet ring cells carcinoma are emphasized as histological forms associated with an intense neoangiogenesis activity, with MVD averages of 42.5 and 41.7. The microvascularization quantification evidences immunohistochemically had values of 35.4 in the tubular adenocarcinoma (the lowest value), 37.8 in the papillary adenocarcinoma and of 36.4 in the mucinous adenocarcinoma.

Table 1 – *The relation between MVD and the clinicopathologic factors in the gastric cancer*

Clinicopathologic factors		Average MVD
Sex	Men	39.5
	Women	38.2
Age	≤60 years	38.3
	≥61 years	38.9
Location	Antrum	36.7
	Body	39.2
	Pangastric	40.2
	Cardia	41.4
	Gastric remnant	37.3
Lauren classification	Intestinal type	36.8
	Diffuse type	41.6
	Mixed type	38.1
Histological type	Tubular adenocarcinoma	35.4
	Papillary adenocarcinoma	37.8
	Mucinous adenocarcinoma	36.5
	Signet ring cell carcinoma	41.7
	Undifferentiated carcinoma	42.5
Histologic grade	G1	34.6
	G2	38.4
	G3	42.2
Lymphovascular invasion	Present	43.1
	Absent	34.6

We have noticed a tight correlation between the degree of histologic differentiation and the quantification of angiogenesis. As the histologic grade is dropping, an important growth of the intratumoral neovessels amount takes place.

The well differentiated tumors (G1) have shown an average value of 34.6, significantly lower in comparison to the average values registered for the moderately differentiated G2 carcinomas (38.4) and the slightly differentiated G3 carcinomas (42.2) ($p = 0.0413$ S) (Figure 7).

In our study, the angiogenesis activity is correlated with the lymphovascular invasion, being significantly higher within the carcinomas associated with the intratumoral carcinomatous emboli (43.1) ($p = 0.0328$ S).

MVD is correlated with the level of invasion, the noted values being higher in the pT3 stage (40.2) and the pT4 stage (39.8) (Table 2).

In the pTis classified case we have noticed an average MVD value of 21.4, showing a more intense angiogenesis activity in comparison with the normal gastric mucosa.

The angiogenesis activity definitely influences the pN stage. We have noted MVD values of 32.1 for the pN0 tumors, of 33.5 for the pN1 tumors, of 40.5 for the pN2 tumors and of 42.4 for the pN3 tumors. In addition, there is a tight correlation between MVD and the presence of distant metastases (pM stage).

The MVD is much lower for the pM0 tumors pM0 (33.9) in comparison with the MVD of pM1 tumors (43.2) ($p = 0.02791$ S).

We have noticed a directly proportional growth between the MVD and the TNM stage. Our results show small differences of the MVD between the IA–IB and IIIA–IIIB stages.

Table 2 – *The relation between MVD and the pTNM stage*

Clinicopathologic factors		Average MVD
pT	Tis	21.4
	T1	35.7
	T2	35.4
	T3	40.2
	T4	39.8
pN	N0	32.1
	N1	33.5
	N2	40.5
	N3	42.4
pM	M0	33.9
	M1	43.2
pTNM	0	21.4
	IA	35.6
	IB	35.2
	II	36.3
	IIIA	39.4
	IIIB	38.9
	IV	42.2

In order to evaluate the prognostic role of the neoangiogenesis activity, the tumors have been classified in two categories, depending on the average MVD value (tumors with MVD<38 and tumors with MVD>38). In our study, we have identified 34 carcinomas with MVD<38 (55.7%) and 27 carcinomas with MVD>38 (44.3%).

The obtained results show that the MVD constitutes an important prognostic factor for the gastric cancer (Figure 8). For the carcinomas with MVD<38, we have registered 15 survivals at 1 year, 11 survivals at 2 years, 10 survivals at 3 years and eight survivals at 4 years, the survival rate at 5 years being of 23.5%.

In comparison to these data, the patients with carcinomas with MVD>38 have presented the following survival rate: eight cases for 1 year, three cases for 2 years and two cases for 3, 4 and 5 years. The final 5-year survival rate was significantly lower (7.4%) ($p = 0.0179$ S).

The calculation of the survival in months for the patients with gastric cancers has shown the following values: 22.4 months for the patients with MVD<38 carcinomas and 11.1 months for the patients with MVD>38 (Figure 9).

VEFG immunohistochemical expression in the gastric cancer

The immunoreactions for the VEGF protein, performed for all the gastric carcinomas cases have shown an important staining of the tumor epithelial cells' cytoplasms (diffuse and granular pattern) and sometimes a membrane immunostaining (Figure 10). Occasionally, dispersed epithelial cells of the normal mucosa, adjacent to the proliferation, have shown a weak immunopositivity (Figure 11). For carcinomas, we have noticed the most intense positivity in the tumor invasion front.

Among the studied gastric carcinomas, we have obtained positive immunoreactions for VEGF in 40 cases (65.6%), significantly more frequently than in

comparison to the normal gastric mucosa (four cases – 6.5%, p<0.001 ES). The positive VEGF immuno-reactions have been encountered more frequently in males (69.7%), but without a statistically significant difference regarding the VEGF immunopositivity in females (55.6%) (Table 3).

Table 3 – ***The correlation between VEGF and the patients' age, sex and tumors' location***

Clinicopathologic factors		VEGF expression		P
		- (n = 21)	*+~++ (n = 40)*	
Sex	Men	13	30 (69.7%)	0.165857 NS
	Women	8	10 (55.6%)	
Age	≤60 years	11	18 (62.1%)	0.639207 NS
	≥61 years	10	22 (68.7%)	
Topography	Antrum	9	22 (71%)	0.348443 NS
	Body	6	9 (60%)	
	Pangastric	4	6 (60%)	
	Cardia	1	1 (50%)	
	Gastric remnant	1	2 (66.7%)	
Lauren classification	Intestinal type	11	27 (71.1%)	0.018178 S
	Diffuse type	8	9 (52.9%)	
	Mixed type	2	4 (66.7%)	
Histological type	Tubular adenocarcinoma	8	20 (71.4%)	0.064672 NS
	Papillary adenocarcinoma	2	3 (60%)	
	Mucinous adenocarcinoma	2	6 (75%)	
	Signet ring cell carcinoma	8	9 (52.9%)	
	Undifferentiated carcinoma	1	2 (66.7%)	
Histologic grade	G1	1	1 (50%)	0.067889 NS
	G2	6	14 (70%)	
	G3	14	25 (64.1%)	
Lymphovascular invasion	Present	14	24 (63.1%)	0.51269 NS
	Absent	7	16 (69.5%)	

In our study, the patients' age does not influence the expression of VEGF. Even though the VEGF values obtained depending on the tumors' location have been relatively close, we notice the positive immunoreactions encountered in 71% of the antral cancers.

The immunoreactions for the VEGF protein have become positive for 71.1% of the intestinal type carcinomas (Figures 12 and 13), significantly more frequent in comparison to the diffuse type carcinomas (52.9%) (Table 3).

The histologic type of adenocarcinoma does not influence the expression of VEGF, but we have obtained immunostainings in 71.4% of the tubular adenocarcinomas and only in 52.9% cases of the signet ring cell carcinomas.

Our results show a correlation between the histologic grade and the VEGF expression, but without reaching the statistic signification threshold. For the well-differentiated carcinomas, 50% of the cases have become positive, in the moderately differentiated carcinomas we have obtained positive reactions in 70% of the cases, and for the slightly differentiated carcinomas, in 64.1% of the cases.

The lymphovascular invasion does not influence the VEGF expression.

We have remarked a significant correlation between the VEGF expression and extend of the carcinomas (Table 4).

Depending on the pT stage, we have registered the following positive VEGF immuno-reactions values: 0% for the pTis stage, 50% for the pT1 stage, 55.6% for the pT2 stage, 64.7% for the pT3 stage and 73.3% for the pT4 stage.

The VEGF quantification depending on the pN and pM stage did not identify the relation between these factors.

The results obtained show that the pTNM stage does not influence the expression of the VEGF protein in gastric cancers.

Table 4 – ***The correlations between VEGF and the pTNM stage***

Clinicopathologic factors		VEGF expression		P
		- (n = 21)	*+~++ (n = 40)*	
pT	Tis	1	0 (0%)	0.010817 S
	T1	2	2 (50%)	
	T2	4	5 (55.6%)	
	T3	6	11 (64.7%)	
	T4	8	22 (73.3%)	
pN	N0	6	12 (66.7%)	0.639207 NS
	N1	5	11 (68.7%)	
	N2	8	15 (65.2%)	
	N3	2	2 (50%)	
pM	M0	16	31 (66%)	>0.05 NS
	M1	5	9 (64.3%)	
pTNM	0	1	0 (0%)	0.174783 NS
	IA	1	2 (66.7%)	
	IB	2	3 (60%)	
	II	2	5 (71.4%)	
	IIIA	4	7 (63.6%)	
	IIIB	3	5 (62.5%)	
	IV	8	18 (69.2%)	

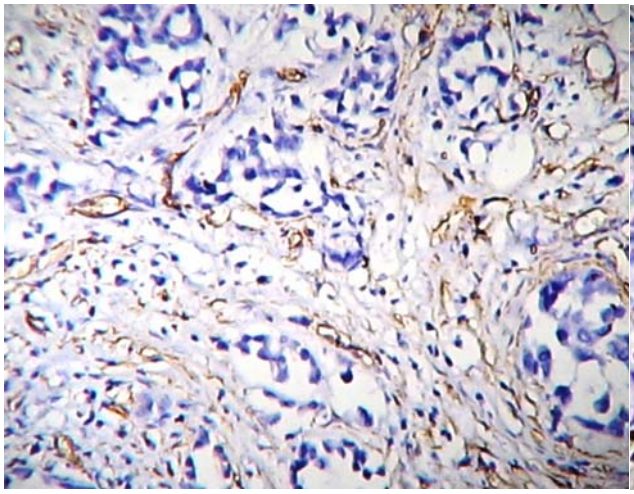


Figure 1 – ***Normal gastric mucosa. MVD average = 125 (CD34, DAB immunoreaction)***

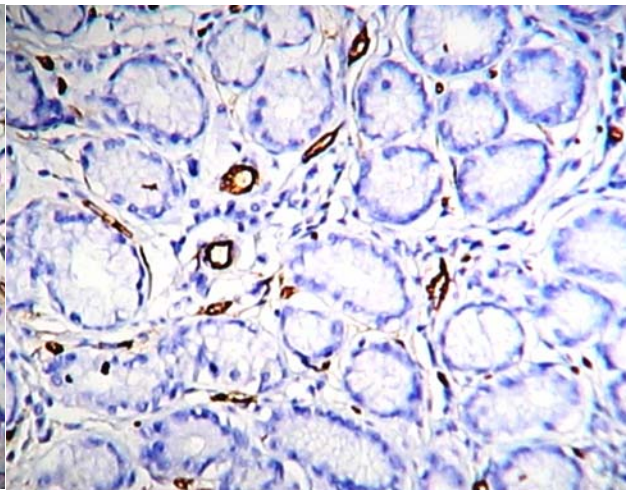


Figure 2 – ***Even lumens microvessels, distributed among the gastric glands (CD34, DAB immunoreaction)***

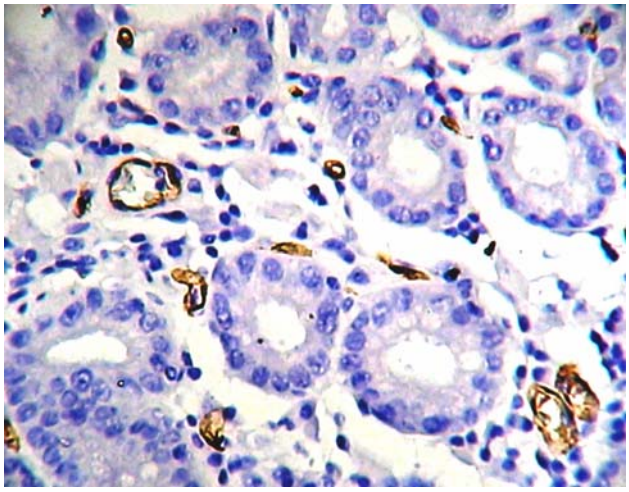


Figure 3 – ***Many microvessels and "hot spots" in the tumor invasion front (CD34, DAB immunoreaction)***

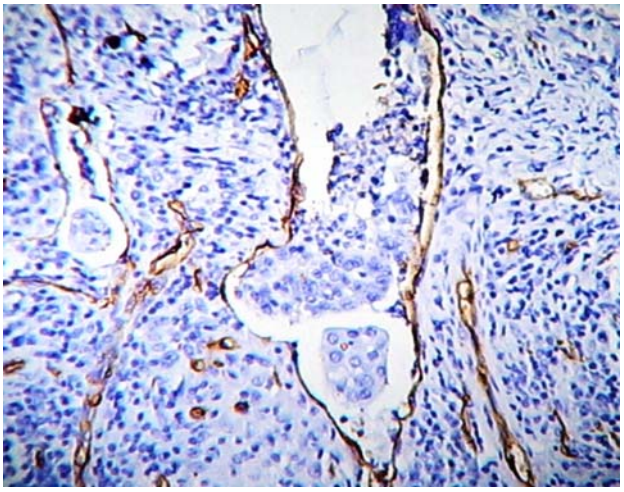


Figure 4 – ***Deeply modified vascular architecture. Carcinomatous emboli (CD34, DAB immunoreaction)***

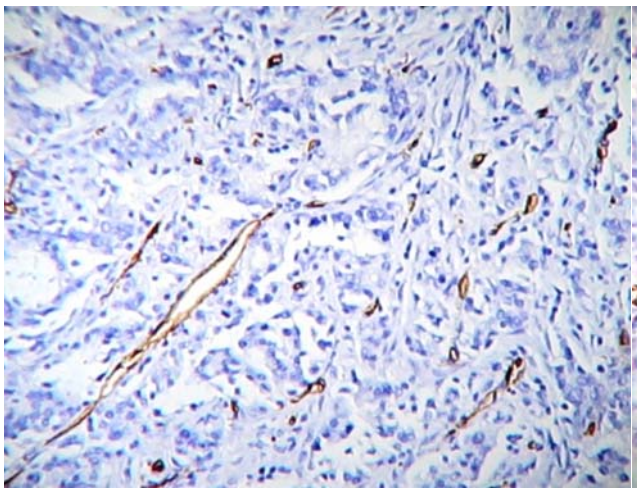


Figure 5 – ***Gastric carcinoma of intestinal type (CD34, DAB immunoreaction)***

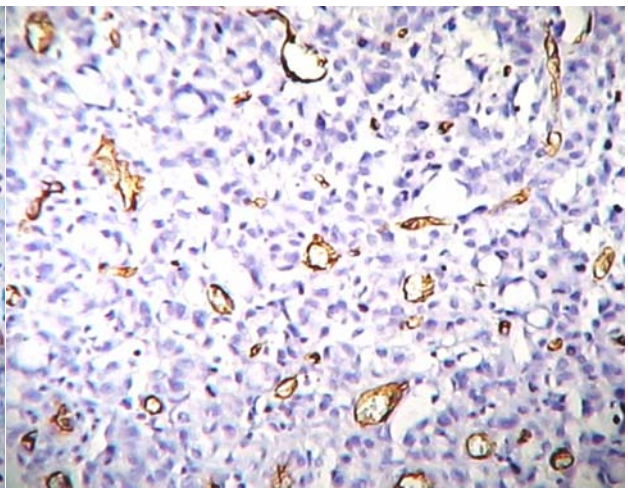


Figure 6 – ***Gastric carcinoma of diffuse type (CD34, DAB immunoreaction)***

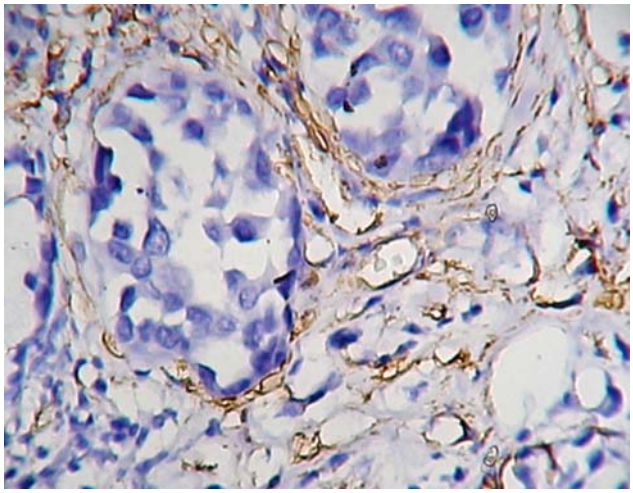
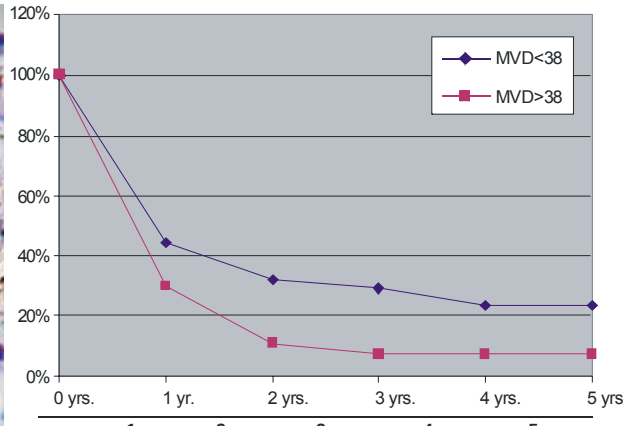


Figure 7 – ***Poorly differentiated adenocarcinoma with MVD average = 42.2 (CD34, DAB immunoreaction)***



	1 year	2 years	3 years	4 years	5 years
MVD<38	44.1%	32.3%	29.4%	23.5%	23.5%
MVD>38	29.6%	11.1%	7.4%	7.4%	7.4%

Figure 8 – ***Five-year survival rate depending on MVD***

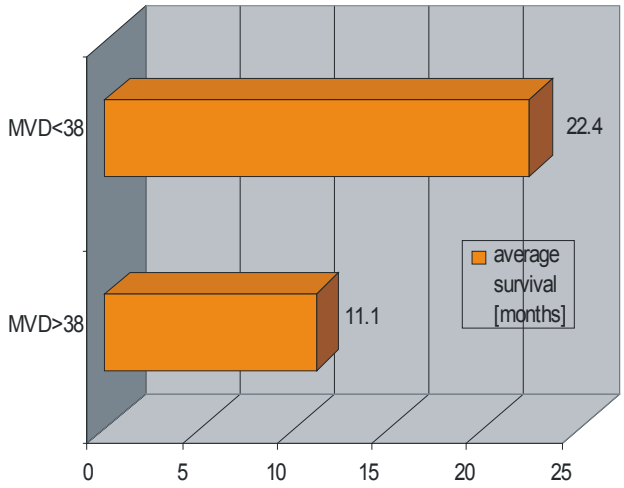


Figure 9 – ***Patients' survival (in months) reported to MVD***

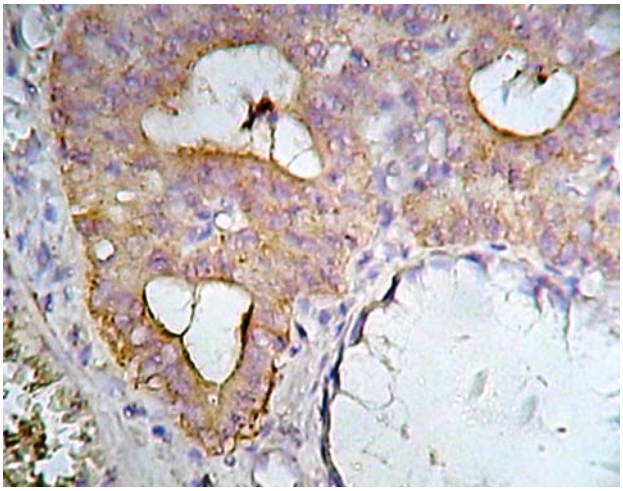


Figure 10 – ***Intensely positive VEGF immunoreaction in the tumor cells' cytoplasm (granular and diffuse pattern) (DAB)***

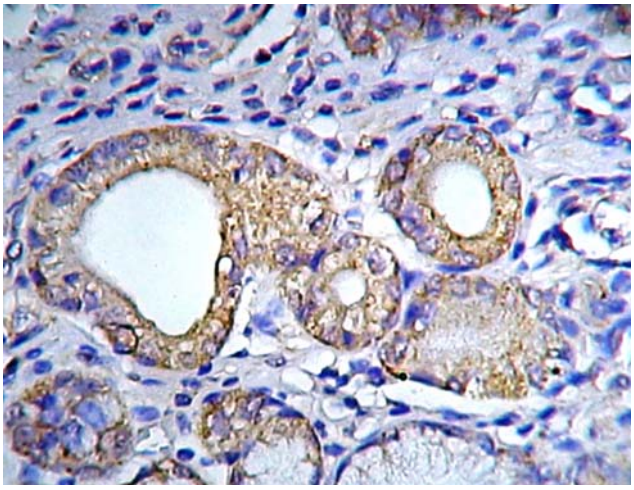


Figure 11 – ***Positive VEGF reaction in the peritumoral gastric mucosa (DAB)***

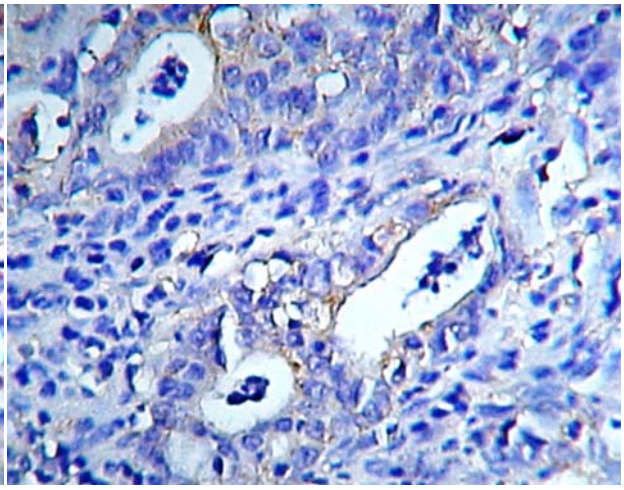


Figure 12 – ***Slightly positive VEGF immunoreaction in carcinoma of intestinal type (DAB)***

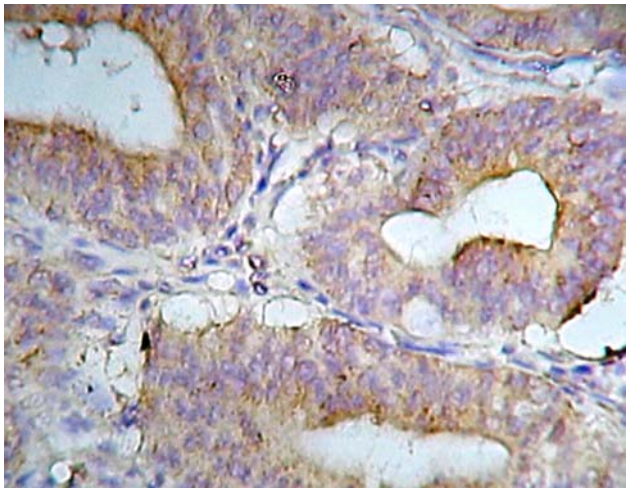
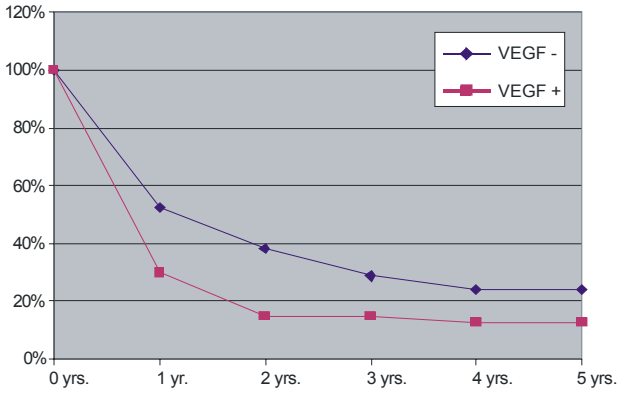


Figure 13 – ***Intestinal type of gastric carcinoma with strongly positive VEGF immunoreaction (DAB)***



	1 year	2 years	3 years	4 years	5 years
VEGF -	52.4%	38.1%	28.6%	23.8%	23.8%
VEGF +~++	30%	15%	15%	12.5%	12.5%

Figure 14 – ***Five-year survival and the VEGF expression***

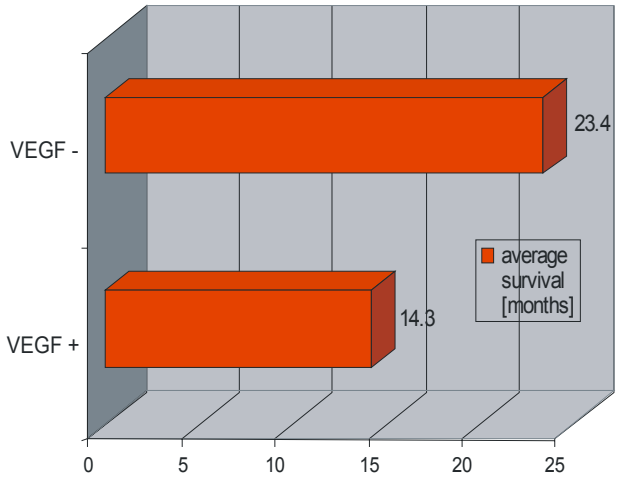


Figure 15 – ***Patients' survival (in months) depending on the VEGF expression***

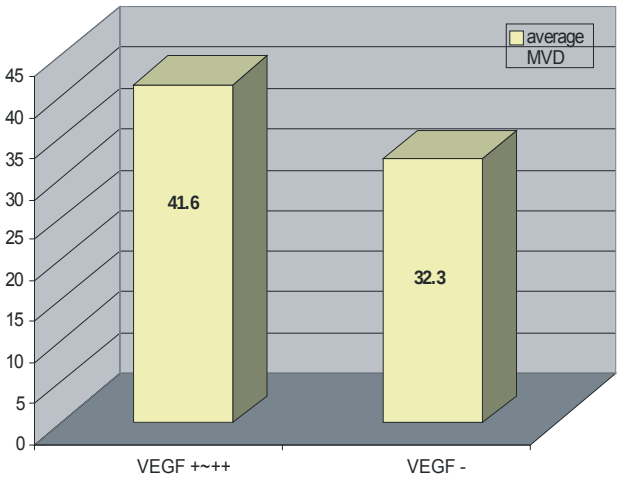


Figure 16 – ***The relation between the VEGF expression and MVD in gastric carcinomas***

The patients' survival tracking has shown the following (Figure 14):

▪ for the VEGF +~++ carcinomas were registered 12 survivals for 1 year, six survivals for 2 and 3 years, five survivals for 4 and 5 years;

▪ for the VEGF negative carcinomas we have registered 11 survivals for 1 year, eight survivals for 2 years, six survivals for 3 years, five survivals for 4 and 5 years.

Our results show the important correlation between the VEGF expression and the 5-year survival of the gastric cancer patients. At the end of the tracked period, the survival rate was of 12.5% for the VEGF +~++ carcinomas, a value significantly lower than 23.8%, meaning the 5-year survival of the patients with negative VEGF carcinomas ($p = 0.027983$ S).

By calculating the average survival in months, we have obtained clearly different values, which sustain the VEGF prognosis role in the gastric cancer: 23.4 months for the patients with negative VEGF carcinomas and 14.3 months for the patients with positive VEGF carcinomas (Figure 15).

Tracking the correlation between MVD and the VEGF expression in gastric cancers, we have noticed that the positive VEGF tumors have been characterized by an intense angiogenesis, with numerous small intratumoral vessels and with average MVD 41.6/5 microscopic fields. In the negative VEGF carcinomas, we have registered an average MVD value of 32.3, significantly lower (Table 5, Figure 16).

Table 5 – ***The relation between the VEGF expression and MVD in gastric carcinomas***

VEGF expression	No. of cases	Average MVD	P
VEGF +~++ carcinomas	40	41.6	0.03986 S
VEGF - carcinomas	21	32.3	

Discussions

The importance of the angiogenesis in the tumor growth and infiltration discovered light even since 1971, when Folkman J [1] proposed for the first time the hypothesis according to which "the solid tumors' growth and the development of metastases are dependent of the formation of new blood vessels" [2, 3].

The solid tumors' growth depends on the induction of new blood vessels [4]. In order to maintain the unlimited tumor growth, the tumor tissue depends on the constant and sustained formation of new blood vessels, this action being essential for tumors' growth over the microscopic size, ensuring the oxygenation and the nutrients' perfusion, as well as the elimination of the metabolisms products. Differently from the normal tissues, where the angiogenesis is strictly controlled, in the neoplastic tissue, the angiogenesis is uncontrolled and immature [5].

Dvorak HF has shown for the first time an association between the tumor angiogenesis and the microvascular permeability growth, fact that led to the identification of the vascular permeability factor (VPF) [6], proven subsequent by Ferrara N to be a specific angiogenesis inductor, known as the vascular endothelial growth factor (VEGF) [7].

VEGF is a mitogen and a stimulator of the endothelial cells with angiogenic effect in vivo [8–10]. Its expression is correlated with the growth of the blood vessels during the angiogenesis [11–13], with the angiogenesis in the female genital tract and with the development of the tumors [14–17]. VEGF is a homo-dimeric protein of 40–45 kDa, secreted by a large variety of cells and by most of the tumor cells, existing in five different isomorphic forms (VEGF–A, B, C, D and E) and presenting two specific receptors (Flt-1 and KDR) in the endothelial cells [18]. VEGF–C is linked to Flt-4, preferably expressed by the lymphatic endothelium [19, 20]. The VEGF expression is stimulated by the hypoxia and is frequently higher near the necrosis areas [21–23]. VEGF also induces fenestrations in the endothelium of the small venules and the capillaries, even in tissues in which the microvascularization does not present normally fenestrations, which explains partially the enhanced permeability of the tumor vessels [24, 25].

It has been suggested that *MVD* could be an independent prognostic and risk factor for metastases. The relationship between the intratumoral microvascular density and the high risk of tumor recidivations and metastases has been noticed in the carcinomas of the mammary gland and in the melanoma. More researches in the colorectal carcinomas have proven the tight correlation between MVD and the metastases in the regional lymph nodes and the distant metastases [26–29].

The establishing of the vessels density necessitates the use of some immunohistochemical techniques in order to emphasize the endothelial cells. The most used markers are the antigen correlated with the factor VIII, CD31 and CD34. Itoh *et al.* have used the con-focal microscopy in order to visualize the tridimensional angiogenesis, which is a hard and expensive technique. The density of the small vessels is estimated in areas, which contain the most capillaries and small venules (micro-vessels). These vascular hot spots can have any location, but they are most often met at the tumor edge or in the tumor invasion front [30].

The studied gastric carcinomas were characterized by an intense tumor angiogenesis, especially in the invasion front, with many micro-vessels and CD34 positive endothelial cell groups („hot spots") among the malignant structures. MVD in the gastric carcinomas had variable values between 12 and 65, with an average of 38.7, significantly higher in comparison to the normal mucosa (12.5, $p<0.001$ ES). In comparison to the normal mucosa, the vascular architecture appears deeply altered, with agglomerated vessels, unequal, with irregular lumens, unequal distributed among the malignant structures. In several cases we have noticed CD34 positive endothelial cells isolated or in small groups, but without forming vascular lumens with red blood cells.

The tumors spread in the whole stomach and the ones located in the cardia show intense angiogenesis activities, with average MVDs of 40.2 and 41.4. The average values of MVD were significantly different depending on the Lauren classification of the gastric cancers. In the intestinal type, we have noticed a much lower average MVD than the average MVD in the gastric carcinomas of diffuse type (36.8 *vs.* 41.6). The anaplastic carcinoma and the signet ring cell carcinoma are standing out as histological forms associated with an intense neoangiogenesis activity, with average MVDs of 42.5 and 41.7. The quantification of the microvascularization emphasized immunohisto-chemically had values of 35.4 in the tubular adenocarcinoma (the lowest value), 37.8 in the papillary adenocarcinoma and of 36.4 in the mucinous adenocarcinoma.

We have noticed a tight correlation between the histologic grade and the quantification of the angiogenesis. As the tumor differentiation diminishes, there can be noticed an important growth of the amount of intratumoral neovessels. The well differentiated tumors (G1) had an average value of 34.6, significantly lower in comparison to the average values registered in the poor differentiated carcinomas G3 (42.2). In our study, the angiogenesis activity is correlated with the lymphovascular invasion, being significantly higher in the carcinomas associated with intratumoral carcinomatous emboli (43.1).

In the study of Zhao ZS *et al.* [31], in the neoplastic tissue and in the peritumoral stroma of the carcinomas with vascular invasion, the average MVD was much higher in comparison to the tumors without vascular invasion (44.68 *vs.* 25.69), leading to the conclusion that the tumor angiogenesis is one of the main biological aspects of the gastric carcinomas. The studies from the literature show a positive correlation between MVD and the infiltrative pattern of growth, the lymph node metastases and the distant metastases (hepatic and peritoneal) in the gastric cancer, indicating that the infiltration and the metastasizing are linked to the angiogenesis phenomenon, MVD being able to be used as prognostic marker [32].

Within the studied group, MVD is correlated with the level of extend, the registered values being higher in the stages pT3 (40.2) and pT4 (39.8). In the classified case pTis we have noticed an average MVD value of 21.4, proving a more intense angiogenesis activity in comparison to the normal gastric mucosa.

The data from literature prove a significantly higher MVD in patients with lymph node metastases, in comparison to the patients without lymph node metastases, suggesting that a high MVD and angiogenesis in the gastric cancers may lead to the pervasion of the neoplastic cells in the blood circulation, and that the lymph node metastasizing could take place when the tumor cells pervade the lymphatic vessels [33].

Our results confirm the fact that the angiogenesis activity does certainly influence the pN stage. We have noticed MVD values of 32.1 in the pN0 tumors, of 33.5 in the pN1 tumors, of 40.5 in the pN2 tumors and of 42.4 in the pN3 tumors.

There is also a tight correlation between the immunohistochemically measured MVD and the presence of the distant metastases (pM stage). MVD is much lower in the pM0 tumors (33.9) in comparison with the MVD in the pM1 tumors (43.2). We have noticed a direct proportional growth between the MVD and the TNM stage. Song ZJ *et al.* have shown that the MVD was significantly higher in patients with gastric cancers in the stages III and IV in comparison to the stages I and II, showing the fact that the MVD is tightly linked to the clinical stage of the gastric cancer, MVD and the tumor angiogenesis rising in parallel to the tumor invasion [34–36]. Hence, a high MVD may reflect the advanced stage of the gastric cancer, as well as the extension of the tumor angiogenesis and the metastasizing, being able to be used as important predictive marker in the patients with gastric carcinoma.

The studies have shown that a high MVD is significantly associated to the presence of the lymph node metastases and with a low survival rate. The MVD value and the presence of the lymph node metastases were independent prognostic factors in the patients with gastric cancer [37].

The obtained results show that the microvascular density is an important prognostic factor in the gastric cancer. In the carcinomas with MVD < 38, the survival rate at 5 years is of 23.5%, whilst the patients with a MVD>38 have a significantly lower survival rate at 5 years (7.4%).

The calculation of the survival rate in months from the patients with gastric cancers had as result the following values: 22.4 months for the patients with carcinomas with a MVD<38 and 11.1 months for the patients with MVD>38.

Li JJ *et al.* have emphasized a high expression of the VEGF in 76.7% from the gastric carcinomas, expression significantly higher in comparison to the normal gastric mucosa [33]. Among the studied gastric carcinomas, we have obtained in our study group positive immunoreactions for VEGF in 65.6% of cases, significantly more frequent in comparison to the normal gastric mucosa (6.5%, p<0.001 ES).

The studies from literature have proven a tight correlation between the VEGF expression and the invasion depth, the presence of the lymph node metastases and the survival rate in 5 years, VEGF representing an independent prognostic factor [38, 39].

The VEGF positive immunoreactions were more frequently met in our study at the male gender (69.7%), but without a statistically significant difference towards the VEGF immunopositivity in women (55.6%). The patients' age did not influence the VEGF expression.

Although the VEGF values, obtained depending on the location of the tumors, were relative close, we notice the positive immunoreactions met in 71% in the antral cancers.

Some studies have not registered a significant correlation between the VEGF expression and the histological types, respectively the macroscopic aspect of gastric cancer. The immunoreactions for the VEGF protein were positive in 71.1% of the intestinal type of gastric carcinomas, significantly more frequent in comparison to the diffuse type carcinomas (52.9%). The histological type of adenocarcinoma does not influence the VEGF expression, but we have obtained immunostaining in 71.4% cases of tubular adeno-carcinomas and only in 52.9% of the cases with signet ring cells carcinomas.

Our results emphasize o tight correlation between the histologic grade and the VEGF expression. Among the well-differentiated carcinomas, 50% of the cases had positive reactions, and in the poor differentiated carcinomas, 64.1% of the cases.

The lymphovascular invasion does not influence the VEGF expression in the gastric carcinomas.

The studies from literature show a tight correlation between the VEGF expression and the invasion depth, it being higher in the gastric carcinoma with invasion of the serosa, indicating the fact that the VEGF does contribute to the tumor invasion enhancing, relevant for the presence of the lymph node metastases [33]. Within the studied group, we have noticed a significant correlation between the VEGF expression and the level of extend. Depending on the pT stage, we have registered the following values of the VEGF positive immunoreactions: 0% in the stage pTis, 50% in the stage pT1, 55.6% in the stage, 64.7% in the stage and 73.3% in the stage pT4.

Song ZJ *et al.* have shown an association between the VEGF expression and the presence of the lymph node metastases, respectively the TNM stage. A high expression of the VEGF was noticed in patients with gastric cancer presenting lymph node metastases and at those in the IIIrd and IVth disease stage [34, 39].

Kakeji Y *et al.* [40] have proven that the VEGF represents an independent prognostic factor and an independent risk factor for the hepatic metastasizing.

Karayiannakis AJ *et al.* [41] have shown a significant association between the serum VEGF level disease stage, the depth of the tumor invasion and the presence of the distant metastases. VEGF was correlated with the invasion and the metastasizing of the gastric cancer, being so able to represent a predictive factor for the status and the prognostic of the tumor in advanced gastric cancer and being able to offer important prognostic information over the conventional clinicopathologic prognostic factors [42–45].

The VEGF quantification depending on the pN and pM stage has not shown in our group the relationship between these factors. The obtained results show that

the pTNM stage does not influence the VEGF protein expression in the gastric cancers.

Our results prove an important correlation between the VEGF expression and the 5-year survival rate of the patients with gastric cancer. At the end of the pursued period, the survival rate was of 12.5 for the carcinomas with VEGF +~++, value significantly lower than 23.8%, which indicates the survival rate at 5 years of the patients with negative VEGF carcinomas. Calculating the average survival rate in months, we obtained clearly different values, which support the role of VEGF of prognostic factor in the gastric cancer: 23.4 months for the patients with VEGF negative carcinomas and 14.3 months for the patients with VEGF positive carcinomas.

The studies have shown a tight correlation between the VEGF expression and a high density of the tumor microvascularization, the malignancy degree and the metastasizing in the gastric cancer. These results indicate the fact that the VEGF and the angiogenesis induced by it do play an important role in the growth, the infiltration and the tumor metastasizing in the gastric carcinoma. Therefore, the VEGF and MVD expression have prognostic significance. There has been noticed a heterogeneity of the VEGF expression and of the forming of new vessels in the tumoral tissue, lacking in the basal membrane. This aspect proves the hyperpermeability of the neovascularization, fact that facilitates the penetrating of the tumor cells in the blood vessels and the metastasizing [31]. The VEGF expression shown by positive cells located in the center of the tumor or at the peripheries of the necrosis areas can be explained by the presence of the hypoxia, which may stimulate the expression and the biological activity of VEGF.

Du JR *et al.* [46], Maeda K *et al.* [47] have shown that the VEGF expression is correlated with MVD, MVD in the VEGF (+) gastric cancers being higher in comparison to the VEGF (-) gastric tumors, the density being higher especially in the areas in which the VEGF expression was positive.

Pursuing the correlation between MVD and the VEGF expression in the gastric cancers, we have noticed that the positive VEGF tumors were characterized by an intense angiogenesis, with several small intratumoral vessels and with an average MVD of 41.6/5 microscopical field. In the VEGF negative carcinomas, we have registered an average MVD value of 32.3, significantly lower. Our study proves a tight correlation between the VEGF expression and the MVD, fact that shows the ability of VEGF to induce the forming of new blood vessels. These data suggest that VEGF and MVD do play a major role in the biological tumor behavior, in the progression and in the prognostic.

The VEGF overexpression and the active angiogenesis are present in the gastric cancer, VEGF and MVD representing two precious prognostic figures in this kind of neoplasm.

The anti-angiogenic therapy is the only therapeutic possibility which acts upon the tumoral vascularization and not directly on the tumor cells, reason wherefore it holds a large applicability in case of most solid tumors. Despite the fact the many growth factors are implied in the angiogenesis process, VEGF is considered to play a major role in the tumor angiogenesis [48–50].

The use of the VEGF as ideal target for the blocking of the tumor angiogenesis was successfully confirmed by laboratory experiments (Kim YB *et al.*, 1993; Warren RS *et al.*, 1995; Borgström P *et al.*, 1996; Wang G *et al.*, 1998) [51–54].

In the cases where there were administrated anti-VEGF antibodies in doses of 200 μg/day, for 14 days in a row, there has been noticed a higher suppression rate of the tumor growth (76.2%) at the mice inoculated with gastric human tumor cells. In these experiments, the suppression continued from 3–5 days after the interruption of the treatment [54].

On experimental models, using the xenograftings of human gastric tumors or of cultures of neoplastic gastric cells inoculated at mice, the therapy with anti-VEGF antibody in combination either with chemo-therapeutic agents, or with other anti-angiogenic agents (for example: anti-EGFR antibody) have proven to be an efficient inhibitory therapy of the tumor growth [55–57].

Bevacizumab (Avastin) represents an anti-VEGF monoclonal antibody, investigated as therapeutic agent in a large variety of tumors, among which the pulmonary, the mammary, the prostatic, the renal, the colorectal and the gastric carcinomas, with promising results in many clinical studies in progress [56].

Conclusions

The immunoreactions with the CD34 antibody are an optimal emphasizing and quantification method for the neovascularization in the gastric cancer. MVD has shown in the gastric carcinomas an average value significantly higher in comparison to the normal mucosa (38.7 *vs.* 12.5, $p<0.001$ ES).

The average MVD values had shown significant differences, depending on the Lauren classification of the gastric cancers. In the intestinal type we have noticed a much lower average MVD than the average MVD in the diffuse type of gastric carcinomas (36.8 *vs.* 41.6) ($p = 0.02478$ S). In comparison to the intestinal carcinoma, the intense angiogenesis in the diffuse type of gastric carcinoma is a determinant factor of the high metastasizing potential. The anaplastic carcinoma and the signet ring cell carcinoma are detaching themselves as histological forms associated to an intense neoangiogenesis activity.

The neoangiogenesis activity (expressed by the average MVD) is correlated with: the histologic grade (as the degree of tumor differentiation diminishes, there is observed a major enhancing of the amount of intratumoral neo-vessels), the lymphovascular invasion, the level of extend (the values are higher in the stages pT3 and pT4), the lymph node metastasizing, the distant metastasizing and the TNM stage (MVD and the tumor angiogenesis grow in parallel with the tumor invasion).

The obtained results show that the microvascular density is an important prognostic factor in the gastric

cancer. Thus, a high MVD may reflect an advanced stage of the gastric cancer, as well as the extension of the tumor angiogenesis and the metastasizing, and may use as important predictive prognostic marker at patients with gastric carcinoma.

The positive immunoreactions for VEGF are significantly more frequent in the gastric carcinomas, in comparison to the normal gastric mucosa (65.6% *vs.* 6.5%, p<0.001 ES).

The immunoreactions to the VEGF protein were positive in 71.1% of the intestinal carcinomas, significantly more frequent in comparison to the diffuse type carcinomas (52.9%) (p = 0.018178 S).

Our results show a tight correlation between the histologic grade, the level of the tumor invasion and the VEGF expression.

Our results prove the major correlation between the VEGF expression and the 5-year survival rate of the patients with gastric cancer, the survival rate for the carcinomas with VEGF +~++ being significantly lower than for the VEGF negative ones (12.5% *vs.* 23.8%) (p = 0.027983 S).

The per-surgery establishing of the VEGF by immunohistochemistry applied on endobiopsy fragments is useful in the therapeutic decision regarding the extending of the surgical resection of the lymph nodes and the post-surgical chemotherapy.

Our study proves a tight correlation between the VEGF expression and the MVD (p = 0.03986 S), these factors playing an important role in the tumoral biologic conduct, in the progression and the prognostic.

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Corresponding author
Marius Raica, Professor, MD, PhD, Department of Histology & Cytology, "Victor Babeş" University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300 041 Timişoara, Romania; Phone +40256–204 476, E-mail: raica@umft.ro

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