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Correlations between Her2 oncoprotein, VEGF expression, MVD and clinicopathological parameters in gastric cancer

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Abstract

Introduction: Gastric carcinoma is one of the most common malignancies worldwide and is the second most frequent cause of cancer deaths. Several molecular factors are studied as prognostic and predictive factors for gastric cancer, VEGF and Her2 being currently in the spotlight. The aim of the study was to estimate the expression of Her2, VEGF and the MVD in gastric carcinoma and its relationship to clinicopathological and biological features of the tumors. *Materials and Methods*: In this study were included 28 patients with gastric carcinoma, of which 16 patients underwent total gastrectomy, which provided the TNM stage, and 12 patients with gastric biopsy. The gastric biopsies and the surgical samples were processed immunohistochemically using anti-Her2, anti-CD31, anti-CD34 and anti-VEGF antibodies. *Results*: Her2 oncoprotein was overexpressed in 85.71% of intestinal type gastric cancer cases and 14.29% in diffuse type (p=0.01), and also more in stage I an II comparatively with stage III and IV (p=0.13). Her2 positive tumors were significant low grade (G1/G2) (p<0.01). MVD is higher in Her2 positive tumors than in the negative ones but not statistically significant (p=0.29 for CD31 and p=0.52 for CD34). Positive immunoreaction of VEGF was observed in 55.5% of the intestinal type carcinomas and in 80% of diffuse type. The correlation between expression of VEGF and TNM stage showed that this angiogenic factor is more frequent positive in the first two stages comparative with the IIIrd and IVth stages. The expression of VEGF is more frequent in G1–G2 tumors (p=0.003).There was a close relationship between tumor vascularity detected with CD34 and two main histological parameters: tumor type according to Lauren's classification (diffuse type; p=0.04) and tumor grade (well and moderately differentiated tumors; p=0.01). There was also a significant correlation of mean CD34 MVD value and the TNM stage being more expressed in stage III/IV than in I/II stages (p=0.004). The mean CD34 MVD value of VEGF positive tumors was 30.8 and was a significantly higher MVD than that of VEGF negative tumors (p<0.05). *Conclusions*: Overexpression of Her2, the selecting factor of patients that benefit from a specific therapy, occurs at a significant frequency in gastric carcinomas, especially in intestinal type. The correlation between VEGF expression and CD34 MVD suggest that two molecular biomarkers play a major role in the biological tumor behavior and are able to be used as important prognostic parameters, which predict the aggressiveness of gastric carcinomas.

Keywords: gastric carcinoma, Her2/neu, VEGF, MVD.

Introduction

Gastric carcinoma is one of the most common malignancies worldwide and is the second most frequent cause of cancer deaths [1].

Most patients are diagnosed at an advanced (unresecable) stage and, despite benefits of palliative radiotherapy and chemotherapy, survival of patients with advanced tumors remains poor [2, 3].

The best promise to improve this poor survival is provided by new agents acting against specific molecular targets [4, 5]. Nowadays, several molecular factors are studied as prognostic and predictive factors for gastric cancer. Those include oncogenes, growth factors or angiogenic factors. Of these, VEGF and Her2 are currently in the spotlight [6–8].

The human epidermal growth receptor 2 gene (Her2, also know as erbB2 or Her2/neu) is now well recognized as a key in the development of certain solid human tumors, most notably in breast cancer. The c-erbB-2 proto-oncogene encodes a 185-kDa transmembrane glycoprotein with intrinsic tyrosine-kinase activity that is homologous to, but distinct from, the epidermal growth factor receptor [9].

Amplification of the Her2 gene or overexpression of Her2 protein has been observed in various solid tumors, including breast and gastric carcinomas [10]. Data reported in the literature for Her2 positively rates in gastric cancer vary from 7–54% [11, 12]. Furthermore, a Her2-positive status in gastric cancer also appears to be associated with poorer prognosis, more aggressive disease and shorter survival [13, 14].

Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in cancer cells survival, local tumor growth and development of distant metastasis. Angiogenesis is a very complex phenomena and essential for the growth of solid tumors measuring more than a few millimeters [15].

It is not easy to develop a single method capable of detecting such a complex biological function. At present, the most widely used method to asses angiogenesis in human malignancies is the quantification of microvessel density (MVD) of tumors using specific markers for endothelial cells, including factor VIII-related antigen, CD31 and CD34 [16–19].

The process of angiogenesis is the outcome of an imbalance between positive and negative angiogenic factors produced by both tumors cells and normal cells [15]. Numerous angiogenic factors have been described. Among the known angiogenic factors, vessel endothelial growth factor (VEGF) has emerged as the central regulator of the angiogenesis in cancer, including gastric adenocarcinoma. The biological function of VEGF include selective promotion of mitosis of endothelial cells, stimulation of their proliferation and angiogenesis, an increase in vessel transparency and extra-vascularization of large plasma molecules [20].

The aim of the study was to estimate the microvascularization detected with CD31 and CD34 antibodies in the primary resecable gastric carcinoma and its relationship to clinicopathological and biological features of the tumor, expression of VEGF and Her2 proteins.

Materials and Methods

In this study, we included 28 patients with gastric carcinoma, of which 16 patients underwent total gastrectomy that could provide the TNM stage, and 12 patients were included right after the gastric biopsy made through upper digestive endoscopy, which confirmed the diagnosis of gastric carcinoma, but that was unable to evaluate the TNM stage.

The gastric biopsies and the surgical samples were processed by paraffin embedding technique, stained initially with Hematoxylin–Eosin technique and then processed immunohistochemically using anti-Her2, anti-CD31, anti-CD34 and anti-VEGF antibodies.

The expression of Her2-neu oncoprotein was assessed using anti-Her2 polyclonal antibody (Dako, Glostrup, Denmark) diluted 1:250 in PBS and the LSAB+/HRP technique. Following heat-induced mediated antigen retrieval in citrate buffer, pH 6, the sections were incubated 30 minutes at room temperature with the prediluted primary antibody. Visualization was achieved with DAB incubation and counterstaining with Harris Hematoxylin.

Control samples included normal gastric mucosa and breast cancer tissue.

Interpretation of the HER2 membrane immunohistochemical staining was performed according to criteria modified by Hofmann M *et al.*, in 2008 (Table 1).

In this study, cases with Her2/neu score +2 or +3 were considered positive and those with score 0 or +1 were considered negative [11].

Table 1 – ***Her2 scoring system***

Intensity score of immunological marking	Pattern of immunological marker	Her2 status
0	Without membrane reactivity or reactivity in less than 10% of tumor cells.	Negative
+1 (ob. 40×)	Barely perceptible membrane reactivity in over 10% of tumor cells, or membrane reactivity is incomplete.	Negative
+2 (ob. 10×–20×)	Weak to moderate membrane reactivity, basolateral or lateral, in over 10% of tumor cells.	Equivocal
+3 (ob. 2.5×–5×)	Strong membrane reactivity, basolateral or lateral, in over 10% of tumor cells.	Positive

For the immunohistochemical evaluation of the tumor neoangiogenesis (MVD), the EnVision two-step method was performed according to the manufacturer's instructions using EnVision+/HRP polymer (Dako, Cytomation). We have used the anti-CD34 monoclonal antibody (clone QBEnd10, DAKO Cytomation, Denmark) and anti-CD31 monoclonal antibody (clone JC70A, DAKO Cytomation, Denmark) diluted 1:50 in PBS. The sections were pre-treated 20 minutes with heat-induced epitope retrieval (MW) in DakoCytomation target retrieval solution High pH and incubated 30 minutes at room temperature with primary antibodies. Visualization was achieved with DAB incubation and counterstaining with Harris Hematoxylin. Negative control was Dako Cytomation Mouse IgG, diluted in the same concentration as primary antibody and the positive slides provided by the reagent kit were used as the positive control.

MVD was assessed by using initially low-power magnification for identification of the "hot spots". After that, a high-power magnification (400×) was used for counting the vessels in three different fields and an average was calculated for each case and statistically presented as the mean ± SD. The isolated immunoreactive endothelial cells or groups of endothelial cells separated by the adjacent microvessels were considered to be quantifiable individual vessels. A visible lumens or the presence of associated red cells were not obligatory.

For the immunohistochemical study of the VEGF expression, we use the LSAB+/HRP technique and the anti-VEGF monoclonal antibody (clone VG1, Dako Cytomation, Denmark), diluted 1:50 in PBS. After 20 minutes pre-treatment of tissues with heat-induced epitope retrieval (MW) in DakoCytomation target retrieval solution, pH 9, the sections were incubated 30 minutes at room temperature with primary antibody. Visualization was achieved with DAB incubation and counterstaining with Harris Hematoxylin. Negative control was DakoCytomation Mouse IgG, diluted in the same concentration as primary antibody. External positive control was the human colon, which was positive in all immunohistochemical run.

To quantify the VEGF immunological marker, in

each case, the entire section was examined at a magnification of 200×. Signals for VEGF expression was detected as brown in the cytoplasm and/or cell membrane. Only cells labeled with undoubtedly higher than the background intensity were interpreted as positive. The expression of VEGF was assessed according to the percentage of immunoreactive cells on a total of 100 neoplastic cells. The immunoreactivity was graded as follows: positive when more than 10% of carcinoma cells were stained and negative when no detectable expression or less than 10% of carcinoma cells were stained [21].

Statistical analysis

Statistical analysis was performed using the SPSS statistical software package. Significant differences were compared with Student's *t*-test used to detect the relationship between the expression of VEGF, Her2 and MVD, and between MVD and pathological characteristics. The *chi*-square test was performed on the numeric data to dispose the expression of VEGF and Her2 and pathological features. A value of $p<0.05$ was considered statistically significant.

Results

The median age of the patients was 58.79 years with a standard deviation (SD) of 14.35 years (range 30 to 87 years). Thee cut-off point used in subsequent statistical analysis was 59 years.

According to the TNM stage, which is assessed from 16 patients with surgical gastric cancer samples: only six (37.5%) cases were in the first two stages, while 10 (62.5%) cases were classified as stage III or IV.

Among the all 28-gastric cancer samples, the diffuse type of gastric carcinoma was found in 10 (35.71%) cases and the intestinal type, in 18 (64.29%) cases. Histological grading of carcinoma revealed that six (21.43%) patients had G1 grade (well differentiated), four (14.29%) patients had G2 grade (moderately differentiated) and 18 (64.29%) patients had G3 grade (poorly differentiated).

Her2 protein status in gastric carcinoma tissues samples was scored as: score 0 in four (14.29%) cases, score +1 in 10 (35.71%) cases, score +2 in 10 (35.71%) cases and score +3 in four (14.29%) cases. The positive rate was approximately 50% (14/28 cases) (Figure 1).

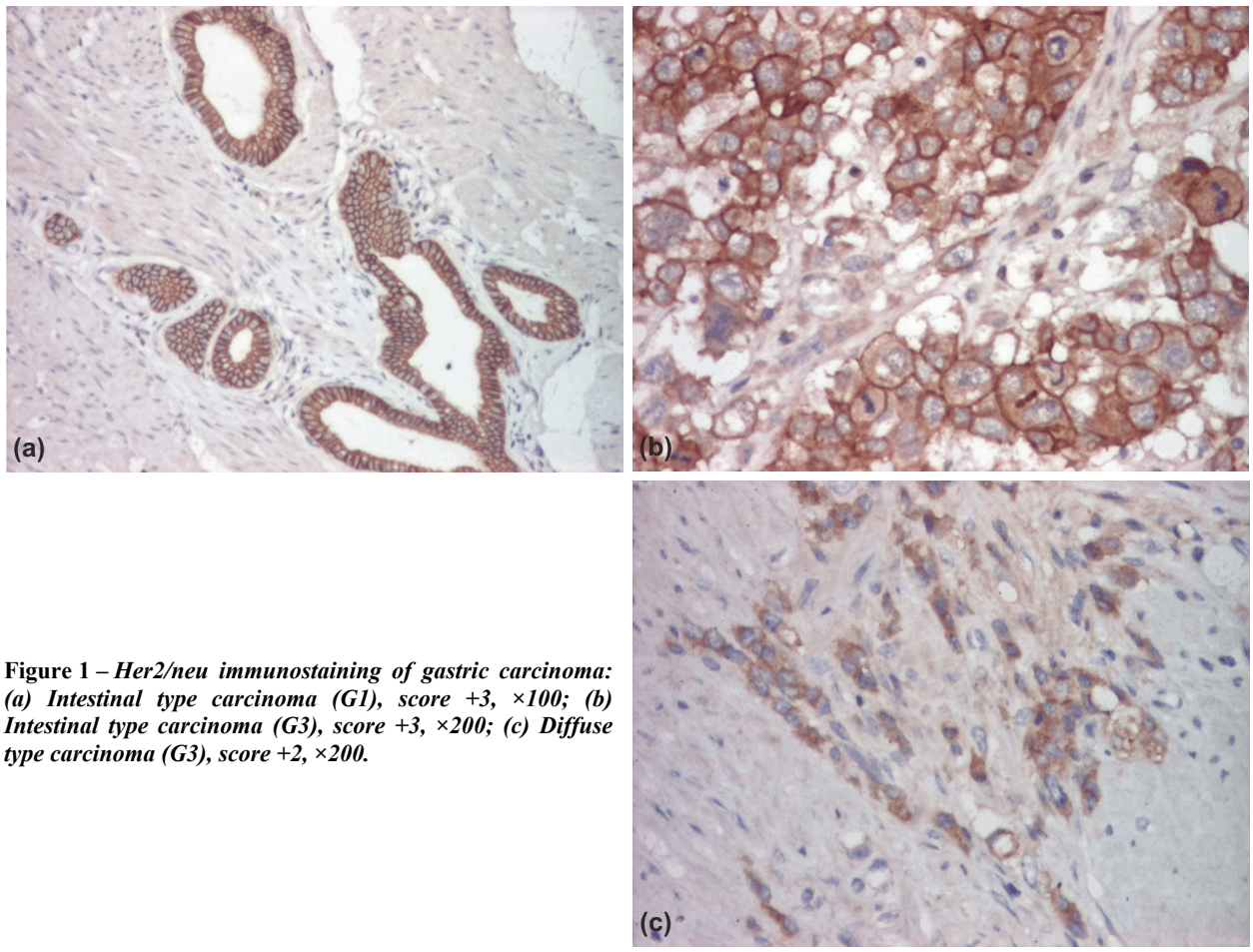

Figure 1 – ***Her2/neu immunostaining of gastric carcinoma: (a) Intestinal type carcinoma (G1), score +3, ×100; (b) Intestinal type carcinoma (G3), score +3, ×200; (c) Diffuse type carcinoma (G3), score +2, ×200.***

Clinicopathological differences were observed in gastric cancer samples with or without Her2 expression (Table 2).

The Her2 protein overexpression was 85.71% (12/14) in intestinal type gastric cancer and 14.29% (2/14) in diffuse type (p=0.01). The Her2 oncoprotein overexpression was more frequent in men and in older patients, but not in a statistically significant way, and significant correlated with histological type (intestinal *vs.* diffuse type, p=0.01). The Her2 oncoprotein positively expression was 66.6% (four from six cases) in stage I an II and only 40% (four from 10 cases)

in stage III and IV (p=0.13). The expression of positive Her2 marker was 80% in well and moderated cancer and 33.3% for poor differentiated gastric carcinomas (p<0.01) (Table 2).

Table 2 – ***Correlations of clinico-morphological parameters with VEGF, Her2 expression and MVD***

	No. of cases	VEGF		Her2		MVD CD31		MVD CD34	
		- (n=10)	+ (n=18)	*0/+* (n=14)	*++/+++* (n=14)	*Low* (n=20)	*High* (n=8)	*Low* (n=16)	*High* (n=12)
Sex:			*p* NS		*p* NS		*p* NS		p=0.01
▪ *Woman*	4	2	2	2	2	2	2	0	4
▪ *Men*	24	8	16	12	12	18	6	16	8
Age:			p<0.001		*p* NS		*p* NS		*p* NS
▪ *<59 years*	10	8	2	6	4	6	4	4	6
▪ *>59 years*	18	2	16	8	10	14	4	12	6
TNM stage:			*p* NS		*p* NS		*p* NS		*p* NS
▪ *I–II*	6	0	6	2	4	4	2	6	0
▪ *III–IV*	10	4	6	6	4	8	2	6	4
Lauren's classification:			*p* NS		p=0.01		*p* NS		p=0.003
▪ *Intestinal*	18	8	10	6	12	12	6	14	4
▪ *Diffuse*	10	2	8	8	2	8	2	2	8
Grading:			p=0.003		p=0.01		*p* NS		*p* NS
▪ *G1–G2*	10	0	10	2	8	8	2	8	2
▪ *G3*	18	10	8	12	6	12	6	8	10

In terms the correlation between the Her2 expression and MVD determinate by the two markers, CD31 and CD34, we observed that the mean MVD value is higher in Her2 positive gastric cancer samples than in the negative ones, but this relationship was not statistically significant (p=0.29 for CD31 and p=0.52 for CD34).

The correlation between Her2 and VEGF expression showed that there were 10 (35.71%) cases from 28 of gastric cancer samples which presented an immunohistochemical positively expression for both markers. In more detail, two from 10 (20%) diffuse type carcinomas and eight from 18 (44.4%) intestinal type carcinomas showed positive expression for Her2 and VEGF, in the same time, without any statistical significance (p=0.269).

Among the studied gastric carcinomas, we have obtained in our study group positive immunoreactions of VEGF in 18 (64.29%) cases (Figure 2).

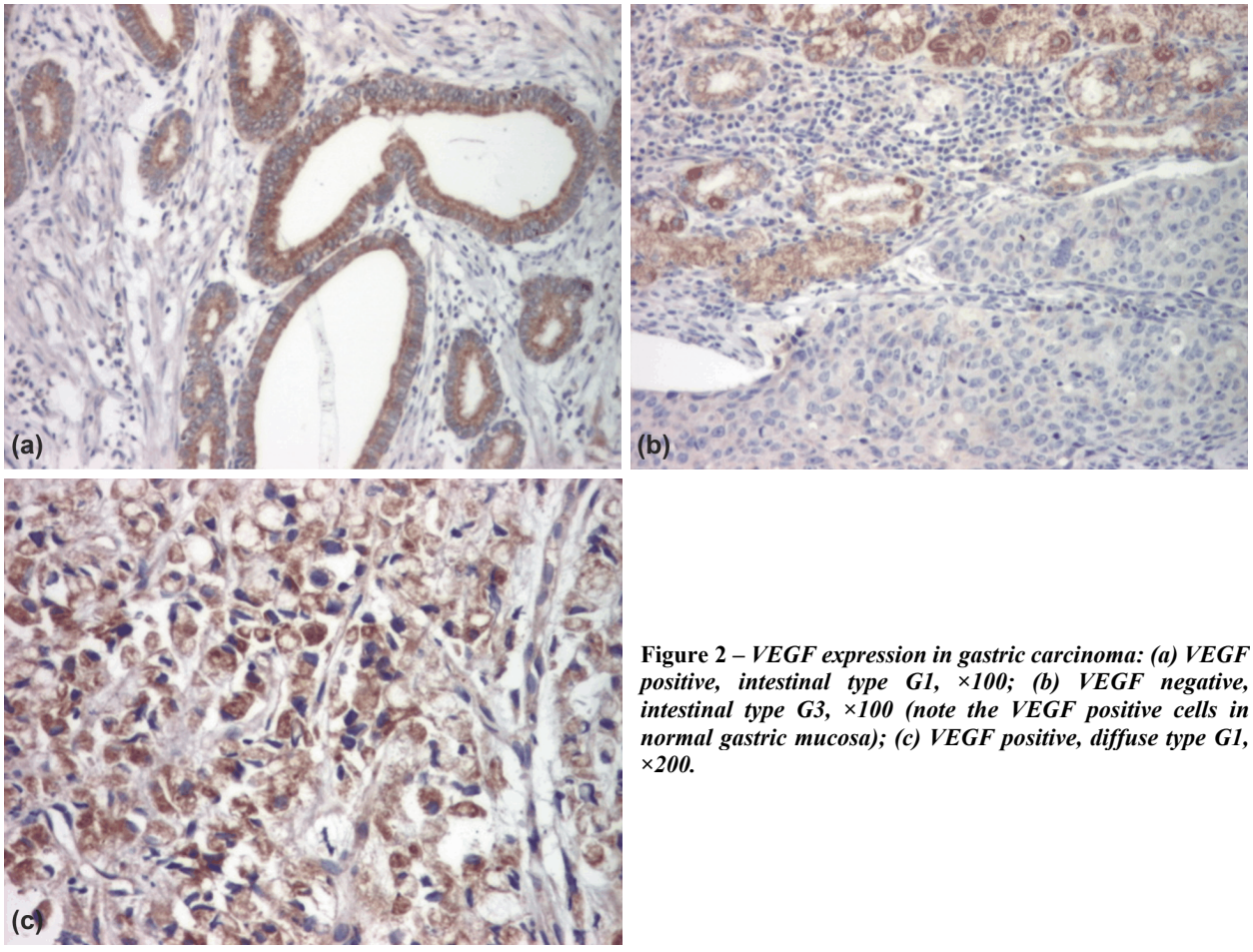

Figure 2 – ***VEGF expression in gastric carcinoma: (a) VEGF positive, intestinal type G1, ×100; (b) VEGF negative, intestinal type G3, ×100 (note the VEGF positive cells in normal gastric mucosa); (c) VEGF positive, diffuse type G1, ×200.***

The VEGF positive reaction was more frequently met in our study at the male gender (88.9% *vs.* 50% in women) and older patients (88.9% *vs.* 20% in patients under 59 years; $p<0.001$). The immunoreactions for the VEGF protein was positive in 55.5% (10/18 cases) of the intestinal type of gastric carcinoma, and in 80% (8/10 cases) of diffuse type. The correlation between expression of VEGF and TNM stage showed that this angiogenic factor is more frequent positive in the first two stages (100%; 6/6 cases) comparing with the IIIrd and IVth stages (60%; 6/10 cases). Not significant correlations were found between VEGF and these two parameters.

Comparing the well (G1) and moderately (G2) differentiated tumors with poor (G3) differentiated ones, we observed that the expression of VEGF is more frequent in G1–G2 tumors (100% *vs.* 44.4% in G3) ($p=0.003$).

The MVD value was determinate by immunohistochemical expression of CD31 and CD34 markers (Figure 3). The correlations between MVD and clinicopathological features are shown in Table 2.

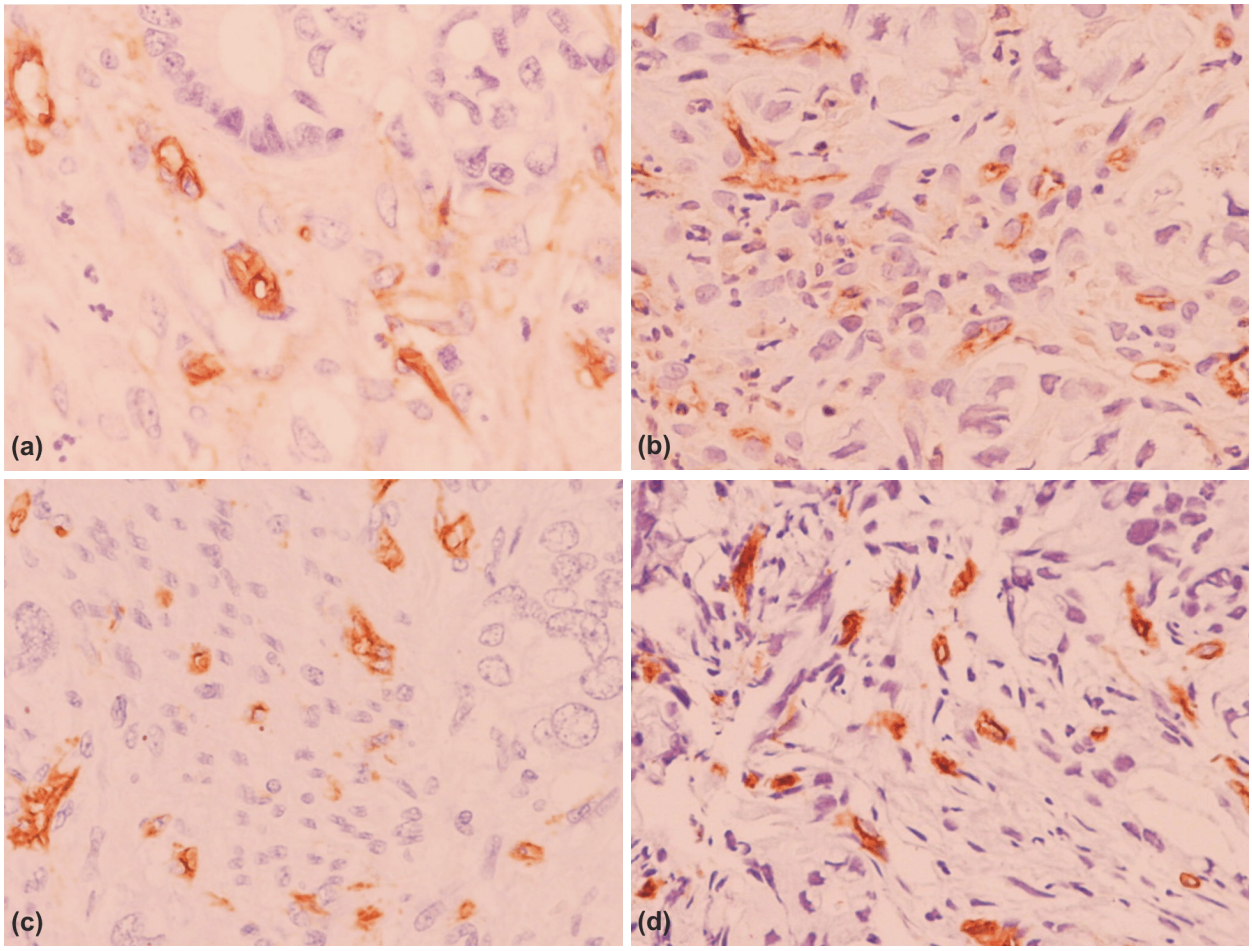

Figure 3 – ***(a) CD31 low MVD, intestinal type G1 carcinoma, ×400; (b) CD31 high MVD, diffuse type carcinoma, ×400; (c) CD34 low MVD, intestinal type G2 carcinoma, ×400; (d) CD34 high MVD, intestinal type carcinoma G3, ×400.***

The MVD for 28 tumors specimens expressed by CD31 ranged from 12 to 27 with a mean MVD value of 19.14±4.25 SD. When a mean MVD value of 19 for CD31 was chosen as the cut-off point for discrimination of 28 patients, 20 patients were categorized as low MVD CD31 and eight as high MVD CD31.

In addition, the MVD expressed by CD34 ranged from 16 to 45, and the mean MVD value was 28.64± 8.64 SD. When a mean MVD value of 29 for CD34 was chosen as the cut-off, 16 patients were categorized as low MVD CD34 and 12 as high MVD CD34.

This study revealed the close relationship between tumor vascularity detected with CD34 and two main histological parameters: tumor type according to Lauren's classification and tumor grade. MVD value was higher in the diffuse type of gastric cancer in comparison to the intestinal type (33.4 *vs.* 26) ($p=0.04$). In well and moderately differentiated tumors, MVD was significantly lower in comparison to the group of poorly differentiated cancers samples (23.8 *vs.* 31.3) ($p=0.01$).

There was also a significant correlation of mean CD34 MVD value and the TNM stage and gender, being more expressed in stage III/IV than in I/II stages (28.2 *vs.* 21.66; $p=0.004$), in women than in men (37 *vs.* 27.25; $p=0.01$). The mean CD34 MVD value of VEGF positive tumors was 31.8 and was a significantly higher MVD than that of VEGF negative tumors ($p<0.05$). We found a strong association between VEGF expression and CD34 MVD. However, the relationship between the status of Her2 expression and MVD was not statistically significant ($p=0.52$) (Table 3).

All data we observed in the MVD value expressed

by CD34 marker, whereas the CD31 expression was less associated with any of the clinicopathological parameters.

Table 3 – ***Correlations of VEGF and Her2 expression with MVD***

VEGF	MVD CD31	*p*-value	MVD CD34	*p*-value
Negative (*n*=10)	18.11	0.083	27.44	<0.05
Positive (*n*=18)	21		31.8	
Her2				
0/+ (*n*=14)	18.29	0.296	27.57	0.522
++/++++ (*n*=14)	20		29.71	

Discussion

In this study, we evaluated the Her2 and VEGF expression in 28 samples tissues of gastric cancer, and the correlations of these two markers with MVD value (detected by using the CD31 and CD34 antibody to visualize the endothelial cells) and some clinico-pathological parameters.

Many studies have examined human gastric cancer at the genetic level, and several genetic alterations, including amplification of the c-erbB-2 gene or overexpression of its protein. The putative prognostic significance of overexpression of Her2 protein in gastric cancer is controversial and the results published are contradictory. Her2 overexpression could be regarded as an independent prognostic factor for patients with gastric carcinoma [13, 14], whereas other studies did not reveal the prognostic value of Her2 expression in gastric carcinoma [22–24].

We investigated Her2 expression in gastric cancer and found Her2 overexpression in 50% of primary tumor specimen. The Her2 status was correlated with sex, being more frequent in men, with age at diagnosis (>59 years), more frequent in older patients and with histological classification (intestinal *vs*. diffuse type, p=0.01), which was similar to findings of previous studies [23, 25].

The Her2 oncoprotein positively expression was 66.6% (four from six cases) in stage I an II and only 40% (four from 10 cases) in stage III and IV (p=0.13). These findings suggest that overexpression of Her2 oncoprotein is correlated, but not in a statistically way, with firs two TNM stages of gastric carcinomas. Comparatively with our results, several studies showed a correlation of Her2 expression and the advance stages of the gastric tumors [26]. Zhang XL *et al*. results indicated that Her2 overexpression was significantly associated with advanced TNM stage and strongly associated with tumor progression and poor prognosis of patients with gastric cancers [14].

The majority of the studies revealed a strong correlation between Her2 overexpression and well and moderately differentiated gastric tumors [14, 25, 27], results which was seen also in our study. The expression of positive Her2 marker was 80% in well and moderated cancer and 33.3% for poor differentiated gastric adeno-carcinoma (p=0.01). The fact that positive staining was also found in poorly differentiated indicates that Her2 is not uniquely linked to a specific differentiated type. The Her2 protein overexpression was 85.71% (12/14) in intestinal type gastric cancer and 14.29% (2/14) in diffuse type (p=0.01).

In our study, the overexpression of Her2 was more frequent in intestinal type than diffuse (66.67% *vs*. 20%), with a statistical significance difference between the two histological types (p=0.01). The gastric cancer specimens who present an amplification of c-erbB-2 gene are more associated with the intestinal type than diffuse type in Lauren's classification and with a poorer prognosis [24, 28].

These two histological types of gastric cancer differ in their epidemiology, pathogenesis, clinical outcome and even genetic changes. A high correlation between Her2 expression and intestinal type gastric cancer was reported by several research studies [13, 14, 25]. The reasons for the selective overexpression of Her2 in the intestinal type of gastric cancer are though to be a complex and unclear of the present time. The association of this oncogene with a particular tumor type indicates that certain characteristics may be expressed together preferentially. However, since not all tumors of the intestinal type overexpress Her2, this cannot be the only factor involved [13].

We observed that the mean MVD value is higher in Her2 positive gastric cancer samples than in the negative ones, but this relationship was not statistically significant (p=0.29 for CD31 and p=0.52 for CD34).

The correlation between Her2 and VEGF expression showed that there were 10 cases (35.71%) from 28 of gastric cancer samples which presented an immuno-histochemical positively expression for both markers. 20% from diffuse type carcinomas and 44.4% from intestinal type carcinomas showed positive expression for Her2 and VEGF, in the same time, without any statistical significance (p=0.269).

The assessment of Her2 status is a critical issue in selecting gastric cancer patients that might benefit from trastuzumab therapy. Only patients with tumors overexpressing Her2, as defined by score IHC +3 or IHC +2 and confirmatory FISH+ results are eligible for trastuzumab therapy [5]. Trastuzumab, a humanized monoclonal antibody (mAb) directed against the extra-cellular domain of Her2/neu receptor improves survival rates in Her2 positive breast cancer patients. In practical models, trastuzumab demonstrated ability to inhibit the growth of Her2 positive but not of Her2 negative gastric cell lines [29–31]. As results of these preclinical data, several clinical trials are exploring the potential of anti-Her2 therapies in gastric cancer patients [8, 32, 33]. In their work, Grávalos C *et al*. [32], conducting a phase II trial, tried to evaluate the efficacy and tolerance of trastuzumab in combination with cisplatin in Her2 positive advanced or metastatic gastric carcinoma patients. They observed that this association of therapies is an active regime with a good toxicity profile. A good response to the trastuzumab therapy of a metastatic gastric cancer was also observed in combination with proton beam therapy [34] or oxaliplatin [35].

Trastuzumab improves the survival in gastric carcinoma and in combination with chemotherapy, represent a new gold standard for the treatment of patients with Her2 positive gastric cancers.

One of the essential factors on which malignant tumor progression depends, is the induction of a micro-circulation from the surrounding environment. The

process of angiogenesis is the outcome of an imbalance between positive and negative regulators of neovascularization. Since Folkman's initial discovery that tumors are angiogenesis dependent, a variety of positive and negative regulators of angiogenesis have been discovered [36].

Dvorak HF *et al.* 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) [37], proven further by Ferrara N *et al.* to be a specific angiogenesis inductor, known as the vascular endothelial growth factor (VEGF) [20].

Among the studied gastric carcinomas, we have obtained, in our study group, positive immunoreactions for VEGF in 18 cases (64.29%), eight cases for diffuse type and 10 cases, intestinal type. The VEGF positive immunoreactions were more frequently met in our study at the male gender (p=0.51) and older patients (p<0.001). The immunoreactions for the VEGF protein were positive in 55.5% of the intestinal type of gastric carcinomas and in 80% of diffuse type. In our study, the correlation between expression of VEGF and TNM stage showed that this angiogenic factor is more frequent positive in the first two stages (100%; 6/6 cases) comparative with the IIIrd and IVth stages (60%; 6/10 cases). Not significant correlations were found between VEGF and these two parameters.

Comparing the well (G1) and moderately (G2) differentiated tumors with poor (G3) differentiated ones, we observed that the expression of VEGF is more frequent in G1–G2 tumors (100% *vs.* 44.4% in G3) (p=0.003).

The studies from literature have proven a tight correlation between the VEGF expression and the invasion depth [38, 39], the presence of the lymph node metastases, the distant metastases [40, 41] and the survival rate in five years. Du JR *et al.* have shown an association between the VEGF expression and the presence of the lymph node metastases, respectively the TNM stage, in the IIIrd and IVth disease stage [42, 43]. All these studies have proven that the VEGF represents an independent prognostic factor and an independent risk factor for the hepatic metastasizing.

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 [44, 45].

The majority of the studies made to evaluate the relation between the MVD and expression of VEGF demonstrated a strong correlation of these parameters. [39, 41–43, 46] In our study, we have noticed that the VEGF positive tumors were characterized by an intense angiogenesis and with an average CD34 MVD value of 31.8. In the VEGF negative tumors, the average CD34 MVD value was 27.44, significantly lower (p<0.05). 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. This data suggest that VEGF and MVD play a major role in the biological tumor behavior, in the progression and in the prognostic.

This results were observed in the immunoexpression of CD34 antigen for angiogenesis comparatively with the CD31 marker, which have not showed a significant correlation with positive expression of VEGF (p=0.08). This data conclude that the CD34 antigen mark more microvessels than CD31, being more useful in determination of tumor angiogenesis. Some studies compared the different ways of assessment of angiogenesis. It was found that MVD measured by CD31 expression might not be very useful, the authors recommending the use of CD34 [17, 47].

In our study, we observed strong positive correlation between angiogenesis in gastric carcinomas tumors measured by CD34 antigen expression and Lauren's classification (p=0.04), TNM stage and histological grade (p=0.01). There was not a significant correlation between CD34 MVD value and expression of Her2 oncoprotein (p=0.52).

In this study, the average values of CD34 MVD were different depending on the Lauren's classification of the gastric cancers. In the intestinal type, we have noticed a lower average CD34 MVD than the average CD34 MVD in the gastric carcinomas of diffuse type (26 *vs.* 33.4). There is a significant correlation between the histological type and CD34 MVD value (p=0.04).

The diffuse carcinoma is a histological form associated with an intense neoangiogenesis activity. It is well know that patients with gastric cancer of diffuse type is characterized by much worse prognosis, and the analysis of angiogenesis may be helpful to better estimation of individual survival and selection the group of patients with high risk of recurrence [16, 47]. Our results suggest that more intense angiogenesis in diffuse type of gastric adenocarcinoma could be important factor for higher metastatic potential of this type of tumors in comparison to intestinal type gastric adenocarcinoma.

In his work, Takahashi Y *et al.* observed that the expression of VEGF and the mean MVD value is more frequent found in the intestinal type of gastric carcinoma than in diffuse type, suggesting that VEGF may be one of the more important angiogenic factors studied in inducing neovascularization in intestinal type gastric tumors and, that the process of growth and metastasis in intestinal type tumors are more angiogenesis dependent than they are in diffuse type tumors [48].

We have noticed a direct proportional growth between the CD34 MVD and the TNM stage. The mean CD34 MVD value was 28.2 for the advanced gastric cancers, the III and IV stages comparatively with the first two stages of the gastric carcinomas. In this group of patient, the mean of CD34 MVD was 21.66, showing a statistically significance difference (p=0.004). Zhou YJ *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 [46]. 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 prognostic marker in the patients with gastric carcinoma.

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 [39, 41].

We also have noticed a tight correlation between the histologic grade and the quantification of the angiogenesis. As the tumor differentiation diminishes and is dedifferentiated, there can be noticed an important growth of the amount of intratumoral neovessels. The well and moderately differentiated tumors (G1–G2) had an average value of 23.8 significantly lower in comparison to the average values registered in the poor differentiated carcinomas G3 (31.3, p=0.01). Our results are in concordance with the results of other authors [41, 42, 47].

Conclusions

Overexpression of Her2 may occur at a significant frequency in gastric carcinomas, especially in intestinal type, and remains the single factor in selecting gastric cancer patients that benefit from a specific therapy.

Although an immunochistochemical evaluation of MVD with CD34 antibodies in gastric cancer does not assess the mechanism of angiogenesis, it may help in estimating of probability of hematogenous metastasis.

We believe that the tight correlation between VEGF expression and CD34 MVD suggest that two molecular biomarkers play a major role in the biological tumor behavior and are able to be used as important prognostic parameters which predict the aggressiveness of gastric carcinomas. 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.

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