Assessment of CD105, α-SMA and VEGF expression in gastric carcinomas

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Abstract
In this study, we analyzed the microvessel density (MVD) for CD105+ and α-SMA+ vessels and the VEGF immunoexpression in 38 gastric carcinomas. CD105+ MVD had superior values at the advancing edge compared with the intratumoral area, no matter of the analyzed clinico-pathological parameters, the difference being significant only in intestinal type, moderate differentiated carcinomas as well as in T2–T3 carcinomas, without lymph node metastases (p<0.05). Intratumoral expression of CD105+ MVD indicated significant differences related to histological type (p=0.006), depth of invasion (p=0.027) and lymph node metastases (p=0.009), but without statistical association in case of the advancing edge or metastases. The assesses of α-SMA+ MVD indicated no differences between intratumoral and advancing edge areas, no matter of the analyzed parameters, excepting intestinal type carcinomas, which presented significant high values (p=0.003) at the advancing edge. VEGF score revealed significant differences related to histological type (p=0.020), differentiation degree of the intestinal type carcinomas (p=0.036) and depth of invasion (p=0.049). In case of metastases, the levels of VEGF expression were higher in the primary tumor, without statistically significant differences (p>0.05). It were significant differences of intratumoral VEGF expression depending on CD105+ MVD values (p=0.019), but not with α-SMA+ MVD (p>0.05). Angiogenesis evaluated through the VEGF and MVD (CD105+ and α-SMA+) expression is correlated with the progression and metastasis of gastric cancer and could be considered a prognostic marker of these tumors.

Keywords: angiogenesis, VEGF, CD105, α-SMA, gastric carcinomas.

Introduction
Angiogenesis process is essential for tumor growth and metastasis, because increased vascularity enhance the growth of primary tumors and hematogenous metastasis by supplying nutrients and oxygen [1, 2].

Previous studies indicated a significant correlation between the incidence of metastasis and the number and density of blood vessels [3, 4]. Thus, the microvessel density (MVD) is a surrogate marker of gastric carcinomas angiogenesis used for the appreciation of tumor prognosis [5, 6]. Moreover, VEGF expression was associated with vascular invasion, hepatic metastases [7], and lymph node metastases [8].

It was found that inhibition of VEGF pathway using monoclonal antibodies has strong antitumor effects in animal models [9], the aspect that could constitute a new approach for the treatment of these tumors. Thus, although the therapy directed at tumoral angiogenesis has been speculated almost 40 years ago, antiangiogenic therapy had been used only in the last decade [10].

This study evaluated tumor angiogenesis in gastric cancer using quantification of MVD assessed by CD105 (CD105+ MVD) and α-SMA (α-SMA+ MVD), VEGF expression and possible correlations with clinical and morphological parameters.

Materials and Methods
This study evaluated 38 gastric adenocarcinomas diagnosed in the Laboratory of Pathology, Emergency County Hospital of Craiova, Romania, between 2009 and 2012. Biologic material was represented by gastric resection pieces fixed in 10% buffered formalin, processed by the usual histological paraffin embedding technique and Hematoxylin–Eosin staining.

The clinico-morphological parameters represented by age, sex, histological type, differentiation degree, depth of invasion and presence of lymph node metastases were analyzed, lesions being classified in accordance to the latest WHO criteria [11].

The study was approved by the local Ethical Committee, and written informed consent was obtained from all the patients.

For the immunohistochemical analysis, we used the following antibodies panel (Table 1).
The immunohistochemical analysis was performed by simple reactions (anti-human CD105, α-SMA and VEGF) and double reactions (anti-human CD105/α-SMA). For simple reactions, antigenic retrieval was followed by endogenous peroxidase blocking and non-specific site blocking, the sections being incubated over the night with monoclonal antibodies anti-human CD105, anti-human α-SMA and respectively anti-human VEGF. The next day, sections were incubated with biotinylated secondary antibodies; reactions were subsequent amplified by using LSAB2-HRP system (code K0675, Dako) and developing was accomplished with DAB chromogen (code 3467, Dako).

To determine the maturity degree of neof ormation vessels, we used double reactions CD105/α-SMA. In these cases, we followed sequential protocols, the LSAB2-HRP system (code K0675, Dako) and LSAB2-AP System (code K0674, Dako) were used for the reactions amplification and DAB (code 3467, Dako), respectively Vulcan Fast Red chromogen (code FR805S, Biocare Medical) were used to see the reactions. The Avidin–Biotin blocking system was used between the two reactions. For the positive reactions validity, there were used positive external controls (data not shown) and negative external controls, by omitting the primary antibody.

VEGF quantification score results by multiplying the number of labeled cells (P) with the stain intensity (I) [12]. Thus, according to the number of the labeled tumor cells, cases were divided into one of the following categories: 1 (below 25% labeled cells), 2 (26–49% labeled cells), 3 (50–74% labeled cells) and 4 (75% labeled cells). The immunostain intensity categories were: 1 (poor), 2 (moderate), 3 (strong). For the statistical analysis, VEGF immuno-expression was considered low for 1–4 score, medium for 6–8 score and high if the score was 8–12.

The microvessels density (MDV) of the stained vessels was performed using the “hot spot” method introduced by Weidner N et al., whereby, the 10× microscope objective identifies the most vascularized areas, three fields are chosen to count vessels at 20× microscope objective and an average is computed [13]. Vascular microdensity was analyzed both intratumoral and in the advancing edge. In α-SMA+ MVD, only vessels with double stain CD105+ and α-SMA+ were counted.

For the statistical analysis, there were used Student’s t-test, one-way ANOVA, chi-square and Pearson’s correlation index, using SPSS 10 software. Average values are reported ±standard deviation (SD). Image acquisition was performed using Nikon Eclipse E600 microscope and Lucia 5 software.

**Results**

The clinico-pathological data analysis indicated the predominance of gastric carcinomas in patients over 50 years (81.6%), in males patients (57.9%), most lesions being intestinal type gastric carcinomas (73.7%), poorly differentiated (52.7%) with lymph node metastases (47.3%) (Table 2).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone/Source</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>External positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Polyclonal/ Santa Cruz Biotechnology</td>
<td>1:200</td>
<td>Citrate, pH 6</td>
<td>Colonic adenocarcinoma</td>
</tr>
<tr>
<td>CD105 (endoglin)</td>
<td>Polyclonal/ Thermo Scientific</td>
<td>1:50</td>
<td>Citrate, pH 6</td>
<td>Kidney</td>
</tr>
<tr>
<td>α-SMA (smooth muscle actin)</td>
<td>Clone 1A4/ Dako</td>
<td>1:50</td>
<td>Citrate, pH 6</td>
<td>Colon</td>
</tr>
</tbody>
</table>

Table 1 – Antibodies panel

Table 2 – Statistical analysis of CD105+ MVD, α-SMA+ MVD and VEGF immunoexpression related with the investigated clinico-pathological parameters

<table>
<thead>
<tr>
<th>Average values (±SD)</th>
<th>Variable (No.)</th>
<th>IT</th>
<th>CD105</th>
<th>α-SMA</th>
<th>VEGF (score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [years]</td>
<td></td>
<td>IT</td>
<td>p*</td>
<td>IT</td>
<td>p*</td>
</tr>
<tr>
<td>&lt;50 = 7</td>
<td>10.7±1.3</td>
<td>0.019</td>
<td>1±0.5</td>
<td>0.200</td>
<td>3.8</td>
</tr>
<tr>
<td>&gt;50 = 31</td>
<td>13.8±3.2</td>
<td>0.002</td>
<td>1.6±1.5</td>
<td>0.239</td>
<td>7.0</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.054</td>
<td>0.305</td>
<td>0.293</td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>IT</td>
<td>p*</td>
<td>IT</td>
<td>p*</td>
</tr>
<tr>
<td>Male</td>
<td>13.4±3.7</td>
<td>0.001</td>
<td>1.6±1.4</td>
<td>0.900</td>
<td>5.8</td>
</tr>
<tr>
<td>Female</td>
<td>14.5±3.5</td>
<td>0.036</td>
<td>1.3±1.3</td>
<td>0.852</td>
<td>7.4</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.366</td>
<td>0.495</td>
<td>0.315</td>
<td>0.190</td>
<td></td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td>IT</td>
<td>p*</td>
<td>IT</td>
<td>p*</td>
</tr>
<tr>
<td>Intestinal = 28</td>
<td>12.9±3.5</td>
<td>0.000</td>
<td>1.6±1.5</td>
<td>0.819</td>
<td>5.6</td>
</tr>
<tr>
<td>Diffuse = 10</td>
<td>16.5±3.8</td>
<td>0.749</td>
<td>0.7±0.4</td>
<td>0.105</td>
<td>8.6</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.006</td>
<td>0.195</td>
<td>0.003</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>Differentiation degree (intestinal type)</td>
<td></td>
<td>IT</td>
<td>p*</td>
<td>IT</td>
<td>p*</td>
</tr>
<tr>
<td>WD = 4</td>
<td>10±1.6</td>
<td>0.070</td>
<td>0.7±0.5</td>
<td>0.444</td>
<td>3.2</td>
</tr>
<tr>
<td>MD = 14</td>
<td>12.8±2.9</td>
<td>0.001</td>
<td>2.2±1.7</td>
<td>0.179</td>
<td>4.8</td>
</tr>
<tr>
<td>PD = 10</td>
<td>14.2±3.1</td>
<td>0.055</td>
<td>1.2±1.3</td>
<td>0.191</td>
<td>7.3</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.064</td>
<td>0.113</td>
<td>0.854</td>
<td>0.036</td>
<td></td>
</tr>
<tr>
<td>Depth of invasion (T)</td>
<td></td>
<td>IT</td>
<td>p*</td>
<td>IT</td>
<td>p*</td>
</tr>
<tr>
<td>T1 = 3</td>
<td>13.3±3.6</td>
<td>0.204</td>
<td>1±0</td>
<td>0.423</td>
<td>2.3</td>
</tr>
<tr>
<td>T2 = 11</td>
<td>11.4±3</td>
<td>0.003</td>
<td>1.1±0.8</td>
<td>0.258</td>
<td>6.8</td>
</tr>
<tr>
<td>T3 = 16</td>
<td>14.3±3.5</td>
<td>0.020</td>
<td>1.7±1.5</td>
<td>0.463</td>
<td>6.6</td>
</tr>
<tr>
<td>T4 = 7</td>
<td>16.1±2.9</td>
<td>0.818</td>
<td>1.7±1.9</td>
<td>0.289</td>
<td>7.8</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.027</td>
<td>0.1±0.6</td>
<td>0.591</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastases (N)</td>
<td></td>
<td>IT</td>
<td>p*</td>
<td>IT</td>
<td>p*</td>
</tr>
<tr>
<td>N0 = 20</td>
<td>12.4±2.9</td>
<td>0.000</td>
<td>1.4±1.2</td>
<td>0.881</td>
<td>5.5</td>
</tr>
<tr>
<td>N1–3 = 18</td>
<td>15.4±3.6</td>
<td>0.180</td>
<td>1.6±1.5</td>
<td>0.521</td>
<td>7.5</td>
</tr>
<tr>
<td>p**/p***</td>
<td>0.009</td>
<td>0.651</td>
<td>0.878</td>
<td>0.059</td>
<td></td>
</tr>
</tbody>
</table>

p* – Student’s t-test; p** – ANOVA correlation level (CD105 and α-SMA); p*** – Chi-square correlation level (VEGF); SD – Standard deviation; IT – Intratumoral; AE – Advancing edge; WD – Well-differentiated; MD – Moderate differentiated; PD – Poor differentiated.

Immunoreaction for CD105 was identified at cytoplasmic level in all studied cases. CD105+ vessels morphology was variable, the vessels having irregular diameter, tortuous and sometimes with unicellular aspect. CD105+ MVD had superior values at the advancing edge comparing with intratumoral area, no matter of clinico-pathological analyzed parameters, but the differences were statistically significant only for moderate differentiated, intestinal type carcinomas, as well as for T2–T3 carcinomas without lymph node metastases (p<0.05, Student’s t-test) (Table 2; Figure 1, A–F).
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Figure 1 – CD105/α-SMA immunostaining, gastric adenocarcinoma, ×100: (A) Well-differentiated, intestinal type, intratumoral; (B) Well-differentiated, intestinal type, advancing edge; (C) Moderately differentiated, intestinal type, intratumoral; (D) Moderately differentiated, intestinal type, advancing edge; (E) Poorly differentiated, intestinal type, intratumoral; (F) Poorly differentiated, intestinal type, advancing edge; (G) Diffuse type, intratumoral; (H) Diffuse type, advancing edge.
Intratumoral CD105+ MVD analysis indicated significant differences depending on histological type [$F(1.36)=8.66$, $p=0.006$], the depth of invasion [$F(3.34)=3.44$, $p=0.027$] and lymph node metastases [$F(1.36)=7.65$, $p=0.009$], the highest values being present in diffuse type carcinomas with T3–T4 invasion and lymph node metastases (Table 2; Figure 1, G and H). ANOVA tests indicated no significant differences of CD105+ MVD at the advancing edge depending on the analyzed parameters. Also, there were no significant intratumoral and at the advancing edge differences of CD105+ MVD reported to differentiation degree ($p>0.05$, ANOVA test).

In lymph node metastases, CD105+ vessels were present mainly in inter-follicular areas. There were no differences of CD105+ MVD at lymph node metastases level depending on the histological type, intestinal or diffuse ($p>0.05$, ANOVA test).

α-SMA immunoreaction was identified in all cases in smooth muscle fibers, pericytes and myoepithelial cells. The aspect was sometimes discontinuous, especially in small caliber vessels. α-SMA+ MVD analysis indicated no significant differences between intratumoral and advancing edge areas, no matter of analyzed parameters (Table 2). Also, ANOVA tests revealed no significant differences of α-SMA+ MVD in relation to analyzed parameters, excepting histological type, where a significant correlation was found in intestinal type carcinomas at the advancing edge [$F(1.36)=9.92$, $p=0.003$] (Table 2; Figure 1, A–F). For the diffuse type carcinomas, α-SMA+ vessels were almost absent both intratumoral and at the advancing edge level (Figure 1, G and H). The same aspect was reported in cases with lymph node metastases, no matter of histological type.

Pearson correlation index revealed the absence of statistical distribution differences of CD105+ and α-SMA+ vessels both intratumoral ($r(36)=0.19$, $p=0.912$), and advancing edge level ($r(36)=0.172$, $p=0.302$).

VEGF immunoreaction was present in 86.8% of analyzed cases at cytoplasmic level. The immunostain was observed in tumor cells, as well as in endothelial cells and in some stromal cell as plasmocytes, lymphocytes, macrophages and fibroblasts. VEGF score revealed significant differences in relation to histological type [$\chi^2(2,N=33)=7.86$, $p=0.020$], differentiation degree of intestinal type carcinomas [$\chi^2(4,N=33)=8.793$, $p=0.036$] and depth of invasion [$\chi^2(6,N=33)=12.647$, $p=0.049$] (Table 2). Thus, the higher values of VEGF score were reported in diffuse carcinomas, moderate and poor differentiated intestinal carcinomas as well as in invasive cases (Figure 2, A–D). Although in the presence of metastases, the primary tumor VEGF values were higher, it were not statistically significant ($p>0.05$, chi-square) (Figure 2, E and F).

In relation with CD105+ MVD, ANOVA test indicated significant differences at intratumoral level of VEGF score [$F(2.30)=47.374$, $p=0.019$], but not in relation to α-SMA+ MVD ($p>0.05$, ANOVA test).

Discussion

Many studies have followed the mechanisms involved in gastric carcinogenesis, as well as the role of several markers in development and progression of tumors. Most of these studies addressing to prognostic markers have focused on tumor angiogenesis. Thus, several studies revealed the importance of microvessel density and angiogenic factors expression in gastric cancer. Therefore, evaluation of angiogenic profile of gastric cancer may become an important factor for the inclusion of new drugs and angiogenic molecular therapy, besides standard chemo and radiotherapy.

Endoglin (CD105) is a homodimeric membrane glycoprotein with molecular weight 180 kDa [14]. Anti-CD105 antibodies are linked preferentially to endothelial activated cells, which participate to tumor angiogenesis, endothelial cells stained with CD105 being observed both in peritumoral and intratumoral vessels. Therefore, CD105 was proposed as a surrogate marker of malignant tumor neovascularization, being used for assessment of the prognosis [15]. Some studies have followed CD105 expression in several types of solid tumors including gastric cancer [8, 16–18].

In this study, intratumoral CD105+ MVD analysis has indicated significant differences related to histological type ($p=0.006$), depth of invasion ($p=0.027$) and lymph node metastases ($p=0.009$). The most higher values were present in diffuse carcinomas, with T3–T4 invasion and lymph node metastases. ANOVA tests indicated no significant differences of CD105+ MVD at advancing edge related to parameters analyzed. Also, there were no differences of CD105+ MVD in lymph node metastases depending on histological type, intestinal or diffuse.

MVD is an important instrument in evaluation of prognosis of patients with gastric cancer [5], being reported a direct correlation between metastases incidence and number and density of new vessels in gastrointestinal tumor [3, 4, 7, 8]. However, some studies proved that MVD is involved in tumor progression and metastasis [19–21], but other studies indicated that MVD did not seem to influence survival rate [22]. Thereby, a study found an obvious correlation between MVD and TNM stage especially for advanced stages of local disease, MVD being assessed after CD34 immunostain [23]. In other studies, patients in first and second stage of disease had a representative MVD, being involved in lymph node metastases [20], but its role was more obvious in cases with distant metastases [19, 21]. Another recent study indicated that MVD identified by CD105 may be useful as a predictor for resected gastric cancer recurrence and may present a specific association with local-regional and hematogenous metastasis [24]. Tanigawa N et al. (1996) investigated the association between high values of MVD and metastases presence in 110 patients with curative gastrectomy in the absence of oncological therapy [4]. The authors reported the enhancing of hematogenous metastases incidence correlated with increased number of vessels, MVD values being significant higher for metastatic tumors than for non-metastatic ones.

Angiogenesis is not only dependent of proliferation and invasion of endothelial cells, but also by the presence of pericytes in neof ormation vessels to stabilize their walls. Pericytes plays an important role in the regulation and maturing vessels, and also in the production of VEGF which stabilize the endothelial cells. Although the role of pericyte in cancer pathology is still unclear, it is considered that signal abnormalities of endothelial cells – pericytes could contribute to tumor angiogenesis and metastasis [25].
Assessment of CD105, α-SMA and VEGF expression in gastric carcinomas

Figure 2 – VEGF immunoexpression, gastric adenocarcinoma, ×100: (A) Well-differentiated, intestinal type; (B) Moderately differentiated, intestinal type; (C) Poorly differentiated, intestinal type; (D) Diffuse type; (E) Lymph node metastasis, intestinal type; (F) Lymph node metastasis, diffuse type.

A variety of signal factors mediates the interaction between pericytes and endothelial cells, including VEGF, PDGF-B and Ang/Tie2. Pericyte recruitment by the tumor neovessels is dependent on signaling through the PDGFB/PDGFRB and Ang-1/Tie2 network [26–28]. Thus, some authors have hypothesized that the maturation of vessels is critically involved in the response to antiangiogenic tumor therapy [29].

In this study, α-SMA+ MVD analysis indicated significant differences between intratumoral and the advancing edge areas, regardless of the analyzed parameters, excepting histological type where there was observed a significant relationship in favor of intestinal type carcinoma, at advancing edge \((p=0.003)\). Moreover, statistic tests indicated the absence of statistical distribution differences between CD105+ MVD and α-SMA+ MVD both intratumoral \((p=0.912)\) and at advancing edge \((p=0.302)\).

Immature microvessels, which are not covered with pericytes are irregular and permeable, so that they could be more easily penetrated by tumoral cell, compared to mature vessels [30]. Studies of colorectal cancer which followed MVD values and pericyte coverage index (PCI) have reported that immature vascularization was observed in low differentiated tumors and was correlated with metastases, thus with a poor prognosis [30]. Moreover, the authors found that tumors with a low pericytes coverage index had a lower
survival rate. A similar study including urothelial carcinomas of urinary bladder indicated that microvessels of these tumors had a significant loss of pericycle coverage compared to normal urinary bladder mucosa [31]. Data from this study indicated that the survival without disease progression was shorter in patients whose tumors had a larger superficial coverage of microvessel with pericytes.

VEGF is one of the most important promoters of angiogenesis in gastrointestinal tumors [32], its expression being a useful marker for angiogenesis evaluation [33–35] and tumor prognosis [21, 36, 37].

VEGF immunoexpression analysis indicated a positivity in 86.8% of investigated cases. VEGF score revealed significant differences depending on histological type (p=0.020), differentiation degree of intestinal carcinomas (p=0.036) and depth of invasion (p=0.049). The highest values were observed in diffuse type gastric cancers, moderate and poor differentiated intestinal carcinomas, as well as in invasive tumors (T4).

VEGF expression in gastric cancer was associated with a variety of clinico-pathological parameters, such as differentiation degree [4], intestinal type [7], lymphatic and hematogenous invasion [8]. Yamamoto S et al. suggested that VEGF has an important role in angiogenesis and gastric carcinomas progression, especially in well-differentiated type carcinomas [38]. Other studies reported that there is not a statistically significant relationship between VEGF and differentiation degree, although it is highly expressed in poor differentiated carcinomas [39].

Moreover, it was noticed that for intestinal and diffuse gastric carcinomas are different angiogenic phenotypes [7, 38]. Intestinal type carcinomas are more dependent to angiogenesis compared to diffuse type of gastric carcinomas. Intestinal type carcinomas, but not diffuse type, express high levels of VEGF-A, VEGF-A, expression level being significant correlated with the number of vessels [7, 38]. These findings suggest that VEGF-A promotes angiogenesis and gastric cancer progress, especially in intestinal type gastric cancer.

VEGF overexpression in advanced stage tumors observed in our study is reported in other similar studies [19]. Furthermore, Maehara Y et al. proved that VEGF expression is an independent risk factor for vascular invasion that explaining the large number of metastases [40].

In this study, statistical analysis with ANOVA test indicated significant differences at intratumoral level of VEGF score in relationship with CD105+ MVD (p=0.006).

Several groups of investigators reported a significant correlation between VEGF-A expression and CD105+ in gastric cancer, as well as MVD values [7, 38, 41]. Konno H et al. found a poor correlation between VEGF staining intensity and MVD, respectively negative stain 12.1±10.51, low positive 16.67±10.41 and high positive 19.7±9.97 [42]. Another study reported that maximum expression of VEGF was associated with similar expression of endoglin [15], high expression of VEGF/CD105 being correlated only with lymph node metastases (p=0.028), the conclusion of this study being that both markers are valuable prognostic indicators. Fondevila C et al. examined 156 patients with curative gastrectomy [43] and they reported: the association of VEGF expression with a shorter global survival and disease-free survival time (RR=2.48 [95% CI, 1.16–5.28], p=0.02); significant correlation of VEGF expression with MVD [OR] = 2.65 [95% CI, 1.33–5.29], p=0.005); high MVD value associated with a shorter disease-free survival time, MVD≥100 being significant correlated with lymph node metastases (p<0.003); univariate analysis of VEGF expression and MVD were independently and significantly associated with tumor recurrence and disease-free survival. The differences between the various studies may be due to polymorphisms of VEGF, which may contribute to gastric tumor characteristics [44].

**Conclusions**

In this study, the angiogenic phenotype of gastric diffuse carcinomas, invasive moderate and poor differentiated intestinal carcinomas and in the case of metastasis was characterized by high values of CD105+ MVD and VEGF, respectively low values of α-SMA+ MVD. Angiogenesis, assessed by VEGF and MVD (CD105+ and α-SMA+) expression is related to the progression and metastasis of gastric cancer and could be considered a prognostic indicator of the tumors.

**Contribution Note**

All authors contributed equally to this paper.

**References**


