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Abstract

Background/Aim. Gastric cancer (GC) is fourth most frequent malignant tumor worldwide, frequently diagnosed at advanced stages with poor prognosis. The aim of study was to determine expression of IL-32, pro-inflammatory and angiogenic mediators in tumor, peritumor and healthy tissue, in patients with intestinal gastric cancer and the relationship with disease severity. Methods. The tissue samples of intestinal types of tumor of 60 patients with gastric cancer were analyzed. Expression of IL-32, Vascular endothelial growth factor (VEGF), IL-17 and CD31 were measured by immunohistochemistry. Results. IL-32, VEGF and IL-17 expression as well as microvascular density (MVD) were diminished in adjacent tumor tissues compared with tumor tissues. Further, more intense expression of IL-32 and VEGF and enhanced MVD were noticed in patients with severe (TNM stages III and IV) and more progressive GC (lymph vessel invasion). Conclusions. Higher expression of IL-32, VEGF and intense MVD in tumor tissue of GC patients with detectable lymph vessel invasion may be considered as a sign of the tumor’s malignant progression. This emphasizes on protumorogenic and proangiogenic role of IL-32 in biology of intestinal type of gastric cancer.

Key words: gastric cancer, IL-32, VEGF, IL-17, MVD

Apstrakt


Ključne reči: karcinom želuca, IL-32, VEGF, IL-17, MVD
Introduction

Gastric cancer is the fourth most frequent malignant tumor and the second cause of cancer related death worldwide (1). Lauren classified gastric cancer in two major forms: intestinal and diffuse type (2). Helicobacter pylory and chronic inflammation are two primary causes of intestinal gastric cancer (3,4). It is believed that persistent inflammation induces mucosal atrophy and hypochlorhydria, thus increases the risk for development of intestinal metaplasia, dysplasia and finally intestinal type of gastric cancer (4,5). Late diagnosis and mild or absent of symptoms and clinical signs contribute to delayed therapy and high mortality (6).

IL-32 is cytokine known to its involvement in the pathogenesis of diverse allergic, infectious, cancerous, and inflammatory diseases (7,8). Moreover, this pleiotropic cytokine has important role in various biological functions such as cell differentiation, stimulation of proinflammatory cytokines and cell death (8–10). It plays important role in immunomodulation as well in tumor biology (11). But, it’s precise role in this processes is still unknown. IL-32 stimulates production of proinflammatory cytokines including IL-8 and TNF-α, prostaglandin E2 and also stimulates macrophages to produce pro-inflammatory factors (12,13). In line with this, IL32 and IL8 are significantly expressed in patients with ER-positive tumors with detected lymph nodes. It is believed that IL-32 promotes angiogenesis and invasiveness via stimulation of proinflammatory cytokines IL8 and TNF-α and thus contributes to tumor metastasis (14). The other study showed that IL-32 induces development of distant and the lymph node metastasis in patients with colorectal cancer and thus can be treated as the marker of CRC metastasis (15). In opposite, previous study reported an immunosuppressive role of IL-32, by inducing production of anti-inflammatory cytokine IL-10 and immunosuppressive IDO (16). It has been shown that IL-32 expressed in various cancers suppresses cancer cell growth by induction of apoptosis in cancer cells. Moreover, antitumorigenic function of NK cells is stimulated by IL-12 and IL-18, which further induce IL-32 production that stimulates TNF-α synthesis thus enhancing NK-mediated apoptosis (11,17,18).

There are no data about expression of this cytokine in tumor and peritumor tissue in intestinal type of gastric cancer. The aim of this study was to evaluate differences in expression of IL-32 and proangiogenic and proinflammatory molecules VEGF and IL-17 as well as microvascular density in tumor, peritumor and healthy tissue in intestinal form of gastric cancer.

Methods

Ethic approvals

The study was conducted at Center for abdominal surgery, Center for pathology, Clinical Center of Kragujevac and Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia. All patients gave their informed consent and research project was approved by relevant Ethics Committees of the Clinical Center of Kragujevac, Kragujevac, Serbia, and Faculty of Medical Sciences, University of Kragujevac, Serbia. All research procedures were made according to the Principle of Good Clinical Practice and the Declaration of Helsinki.
Patients

The study included total of 60 patients with intestinal form of gastric cancer. Gastric cancer was diagnosed on the basis of gastroscopic and histopathological criteria. The study did not include patients with no well-defined pathology, no adequate clinical document available or with previously diagnosed gastric cancer who were treated with radiation and chemotherapy. Data about age, gender, nuclear grade, well/moderate/poor differentiation and clinical stage by TNM (tumor, nodes, and metastasis) were recorded and analyzed in study.

Immunohistochemical staining of VEGF, IL-32, IL-17 and CD31

The tissue samples of stomach from patients were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Four-μm-thick sections from the paraffin blocks were used for IHC. IHC steps were carried out at room temperature. After deparaffinization and rehydration with graded ethanol, the sections were placed into a pressure cooker in 10 Mm sodium citrate buffer (pH 6.0) at full power for 20 min, followed by treatment with 3% hydrogen peroxide solution for 10 min. The primary mono-/polyclonal antibodies against VEGF (ab16883, Abcam, Cambridge, UK, at a 1:200 dilution), IL-32 (ab37158, Abcam, Cambridge, UK, at 10µg/ml), IL-17 (ab79056, Abcam, Cambridge, UK, at a 1:100 dilution) and CD31 (ab79056, Abcam, Cambridge, UK, at a 1:200 dilution) were incubated for 60 min with the tissue sections in a humid chamber, respectively and exposed to EnVision reagent (DakoCytomation, Glostrup, Denmark) for 30 min. The slides were then sequentially incubated with the chromogen reagent for 5 min, counterstained with Meyer’s hematoxylin, and mounted. Negative control staining was performed by using mouse IgG1 isotype antibody. An Olympus microscope (BX50 model) equipped with a digital camera was used to prepare microphotographs with magnifications of 200× or 400×.

IHC scoring

All tissue specimens were investigated by two independent pathologists. They used semi/quantitative modified scoring system, according to (7,19) the percentage of tumor tissue stained with IL32 and intensity of staining. The IHC score was calculated by adding the percentage of positively stained cells to the staining intensity. The percentage of positive cells ranged between 0 and 3, i.e. 0, if less than 10% of tumor cells were stained; 1, if 10–25% of tumor cells were stained; 2, if 25–50% were positive; and 3, if >50% were positive. The staining intensity was scored as: 0, negative immunoreaction; 1, weak intensity; 2, moderate intensity; and 3, strong intensity. The sum of the two parameters varied between 0 and 6.

VEGF scoring was based on the presence, intensity and percent of positive cells, as previously described (19,20). Brown or brown-yellow staining signals found in the cell membrane or cytoplasm were considered to indicate VEGF immunopositivity. The negative controls were unstained. The number of positive cells in 500 tumor cells was counted within 3 randomly selected high power fields (x400). Four grades were defined according to the percentage of positively stained cells: 0, no immunopositive cells; 1, <25% immunopositive cells; 2, 25-50% immunopositive cells; 3, >50% immunopositive cells.
Four grades were defined according to color-staining intensity: 0, no color; 1, weak, pale yellow; 2, medium, brown; 3, strong, dark brown. Single endothelial cells or clusters of endothelial cells positive for CD-31 were considered as a microvessel, by two pathologists. At first, slides were examined at an original magnification of x40. Three „hot spots“ (areas with the highest microvessel density) from each slide were identified and these are as were photographed by a digital camera at an original magnification of x200. The area of this histological field was 0,704µm. MVD (microvessel/HPF) and number of microvessels evaluated according to Weidner et al. MVD of the specimen was estimated as a mean of MVD in three histological fields.

Expression of IL-17 was localized in the cytoplasm of mononuclear cells. Light-microscopic analysis was performed by manually counting positively stained cells in 3 separate areas of intratumor regions under 400x high power magnifications (21).

**Statistical analysis**

The data were analyzed using commercially available SPSS 20.0 software. The results were reported as mean and standard error (SE). In determining statistically significant difference between the means of two groups it was used Student's t-test for independent samples if the data had normal distribution or Mann-Whitney U-test for data without normal distribution. Spearman's correlation evaluated the possible relationship between the expression of IL-32 and presence of lymphatic vessels invasion, in gastric cancer. Strength of correlation was defined as negative or positive weak (-0.3 to -0.1 or 0.1 to 0.3), moderate (-0.5 to -0.3 or 0.3 to 0.5) or strong (-1.0 to -0.5 or 1.0 to 0.5). P-value of 0.05 was considered as statistically significant.

**Results**

Sixty adult patients, between 54 and 92 years of age diagnosed and histologically confirmed as intestinal form of gastric cancer were enrolled in this study. There was significant difference in gender distribution: 47 men (78.33%) and 13 women (21.67%). Clinical and pathologic characteristics of these patients are presented in Table 1. We have assessed expression of IL-32, CD31, VEGF and IL-17 in tumor, peritumor and healthy tissue of gastric cancer. Patients with GC were classified in two groups based on TNM stage of disease: I+II and III+IV. Further patients were divided according to the invasion of lymphatic vessels (+ and −). We analyzed values of previously defined markers of interest between defined groups.

**IL-32 expression associated with lymph vessel invasion**

We have assessed expression of IL-32 cytokine in tumor, peritumor and healthy tissue of gastric cancer. Imunohistochemistry data are illustrated in Figure 1C. The results obtained from this experiment have shown that IL-32 is significantly more expressed in tumor tissue in comparison to its expression in peritumor tissue (p=0.001; Figure 1a). Patients with GC were divided into two categories on the basis of TNM stage of disease: I + II and III + IV. There was no significant difference in IL-32 expression between defined groups (data not shown). Further, patients were divided in two groups, based on the invasion of lymphatic vessels (+ and -) and analyzed expression of IL-32. Expression of IL-32 was significantly
increased in patients with detected lymph vessel invasion (p=0.041; Figure 1b). The relationship between IL-32 expression in tumor tissue and the invasion of lymphatic vessels revealed a moderate positive correlation between IL-32 expression and presence of lymphatic vessels invasion (r =0.364; p = 0.040).

**Micro-vascular density associated with TNM system and lymph vessel invasion**

We analyzed microvascular density in tumor, peritumor and healthy tissue of gastric cancer. As the expression of molecule CD31 (PECAM-1) indicates the angiogenesis and the presence of blood vessels, immunohistochemistry was carried out in tumor, peritumor and healthy tissue of all 40 patients with intestinal form of gastric cancer. Our results have shown that MVD was significantly higher in tumor tissue in comparison to peritumor tissue of GC (p=0.001; Figure 2a). Next, patients were divided in two categories on the basis of TNM stage of disease: I+II and III+IV. Patients with TNM stages III+IV revealed significantly higher MVD in tumor tissue in comparison to patients with TNM stages I+II (p=0.018; Figure 2b). Further, we divided patients on the basis of invasion of lymph vessels (+ and -), and analyzed MVD in tumor tissue. MVD was significantly increased in tumor tissue of patients with detectable lymphatic vessels invasion (p=0.012; Figure 2c).

**VEGF expression associated with TNM system and lymph vessel invasion**

Focus of our further research was based on analyzing different proangiogenic soluble factors. Initially, we have investigated expression of VEGF, one of the main proangiogenic molecules. Results obtained from the experiment discovered that VEGF is significantly more expressed in tumor tissue in comparison to peritumor tissue of patients with gastric cancer (p=0.001; Figure 3a). Further, patients were divided based on TNM stage of disease: I+II and III+IV. Patients with TNM stages III+IV had significantly higher expression of VEGF in tumor tissue compared to patients with TNM stages I+II (p=0.018; Figure 2b). Next distribution of patients was created according to existence of lymphatic invasion and analyzed them for expression of VEGF. Expression of VEGF was significantly higher in tumor tissue with lymphatic invasion (p=0.002; Figure 2c).

**IL-17 expression associated with tumor necrosis**

Analyses of the expression of IL-17 revealed that tumor tissue had significantly higher expression of IL-17 in comparison to peritumor tissue (p=0.001; figure 4a). According to presence of necrotic fields in tumor tissue, patients were divided in two groups (+ and -) and analyzed on the expression of IL-17. Results have shown that IL-17 was significantly higher expressed in tumor tissue with detectable necrotic fields (p=0.001; figure 4b).

**Discussion**

Gastric cancer is the fourth most common cancer throughout the world behind lung, breast and colorectal cancers and the second major cause of cancer-related death (22,23). Around 90% of all gastric cancers are adenocarcinomas, created from the glands of stomach mucosa (24). According to Lauren’s classification, there are two major histological types of
gastric cancer: intestinal and diffuse type (2). Intestinal type of gastric cancer consists of tubular or glandular metaplastic cell formations (25). It is more frequent in elder males, with a lower TNM stage and a low risk of lymph node metastasis (26).

IL-32 is cytokine known to its important biological functions. Due to its proinflammatory function, IL-32 induces production of different chemokines and pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, IL-8, and macrophage inflammatory protein-2 (MIP-2) and activation of the p38 mitogen-activated protein kinase (MAPK), nuclear factor κB (NF-κB), and activator protein-1 (AP-1) signaling pathways (27). IL-32 plays role in genesis and progression of gastric cancer. In the present study, we analyzed expression pattern of IL-32 in tumor and peritumor tissue. We found significantly higher expression in tumor tissue in comparison to peritumor tissue (Figure 1a). Moreover, IL-32 expression in tumor tissue was significantly higher in patients with more progressive GC (lymph vessel invasion; Figure 1b). These results are in line with previous studies claiming that IL-32 is higher in sera of gastric cancer patients (28,29) and that IL-32 is linked to development of Helicobacter pylori-associated gastric cancer (30). We obtained a positive correlation between the IL-32 expression in tumor tissue and disease severity (lymph vessel invasion), indicating on its pro-tumorogenic role.

Moreover, IL-32 facilitates angiogenesis trough induction of production of matrix metalloproteinase and VEGF thus facilitating invasion and migration of tumor cells (31). According to these data, further step was focused on analyses of microvascular density, proangiogenic and proinflammatory soluble molecules in tumor and peritumor tissue of gastric cancer. CD31 is one of the most useful markers for detection of microvascular density. Platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD31) has pleiotropic effects such as transendothelial migration of leukocytes and inflammation as well as endothelial cell biology (32). Moreover, CD31 plays important role in tumor biology in few ways. It is one of the most abundant junctions set deep between endothelial cells thus supporting the integrity of endothelial membrane and regulating leukocyte migration and vascular permeability (33,34). We found increased MVD in tumor tissue in comparison to peritumor tissue (Figure 2a). Moreover, MVD was significantly more explicit in patients with severe (TNM stages III and IV; Figure 2b) and more progressive disease (lymph vessel invasion; Figure 2c). MVD may be one of the important prognostic factors for gastric cancer patients and MVD value and lymph node metastasis represent independent prognostic factors (35).

Analyze of VEGF expression revealed higher expression in tumor tissue in comparison to peritumor tissue of patients with gastric cancer (p=0.001; Figure 3a), as well as more intense expression in patients with severe (TNM stages III and IV; Figure 3b) and more progressive disease (lymph vessel invasion; Figure 3c). In line with this finding, tumors with lymph node metastasis were associated with a high VEGF-A, VEGF-B and VEGF-C, mRNA in lung adenocarcinoma (36). The VEGF expression positively correlate with gastric cancer progression (TNM stage, tumor size, positive lymph nodes and lymphovascular invasion) (37).

As it is known that IL-32 promotes angiogenesis and inflammation, our further investigations was focused on analyses of proangiogenic and proinflammatory cytokine IL-17, in tumor and peritumor tissue of gastric cancer. Tumor tissue had significantly higher expression of IL-17 in comparison to peritumor tissue (Figure 4a). Interestingly, we found increased IL-17 in tumor tissue with detectable necrotic fields (Figure 4b). Only a few
studies evaluated IL-17 in gastric cancer, mainly describing IL-17 as promoter of cancer progression (38).

The selective process of metastasis requires active cross-talk between tumor cells and peritumor tissue, which is mediated by direct tumor cell-stromal cell contact or paracrine cytokine and growth factor signaling (39). The peritumor environment should be fully taken into account in assessing the process of tumor progression. Therefore, our goal was to evaluate peritumor expression of IL-32, VEGF, IL-17 and MVD. We found lower expression of IL-32, VEGF and IL-17 as well as decreased MVD in adjacent tumor tissues compared with tumor tissues. Most research has focused on the intratumor environment, and the potential roles of angiogenesis and immunomodulation in the peritumor environment remain unclear. To our knowledge, this is the first study investigating peritumor IL-32 in any localization. In line with our findings, analysis of tumor and peritumor tissue of eyelids revealed that VEGF and MVD are highly expressed in the tumors (40). Interestingly, recent study revealed significantly higher peritumor expression of VEGF in hepatocellular carcinoma (41), opposite of our results. In other study peritumor expression of IL-17 corresponded with a significantly lower overall survival and may present independent prognostic factors of patients with intrahepatic cholangiocarcinoma (42).

**Conclusion**

In summary, increased local expression of IL-32, in GC patients with detectable lymph vessel invasion may be considered as a sign of the tumor’s malignant progression and, consequently, of a poor prognosis for patients. Increased IL-32, as well as VEGF and MVD in severe and advanced gastric cancers, may implicate on protumorogenic and proangiogenic role of IL-32 in intestinal type of gastric cancer. These observations point on possible facilitating role of IL-32 in biology of intestinal form of gastric cancer and its potential use as therapeutic target.

**Declaration of interest**

The authors declare that they have no conflict of interests.

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REFERENCES


### Table 1.

**Baseline characteristics of patients with intestinal type of GC**

<table>
<thead>
<tr>
<th>Intestinal type gastric cancer</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>47/13</td>
</tr>
<tr>
<td>Age (mean (range))</td>
<td>75 (54-92)</td>
</tr>
<tr>
<td>TNM Classification</td>
<td></td>
</tr>
<tr>
<td>(I and II/III and IV)</td>
<td>27/33</td>
</tr>
<tr>
<td>Nuclear grade (I/II/III)</td>
<td>5/41/14</td>
</tr>
<tr>
<td>Histological differentiation rate (well/moderate/poor)</td>
<td>11/31/18</td>
</tr>
<tr>
<td>Lymph vessel invasion (absent/present)</td>
<td>10/50</td>
</tr>
<tr>
<td>Necrosis (absent/present)</td>
<td>21/39</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1: IL-32 expression in tumor, peritumor and healthy tissue of intestinal GC.
A: Significantly higher IL-32 expression in tumor tissue in comparison to its expression in peritumor tissue in intestinal gastric cancer (0.001). B: Patients with detected lymph vessel invasion had significantly higher expression of IL-32 compared to patients without lymph vessel invasion (p=0.041). P values were assessed by Mann–Whitney Rank Sum test.
C: H&E staining of representative tumor and peritumor tissue of intestinal GC. Representative IL-32 staining in tumor, peritumor and healthy tissue of intestinal GC (200x and 400x magnification).

Figure 2: MVD in tumor, peritumor and healthy tissue of intestinal GC.
A: CD31 expression was significantly higher in tumor tissue in comparison to its expression in peritumor tissue in intestinal gastric cancer (0.001). B: Patients with higher TNM stage (stage III+IV) had significantly higher expression of CD31 compared to patients with lower TNM stage (stage I+II) (p=0.018). C: Patients with detected lymph vessel invasion had significantly higher expression of CD31 compared to patients without lymph vessel invasion (p=0.012). P values were assessed by Mann–Whitney Rank Sum test.
D: Representative CD31 staining in tumor, peritumor and healthy tissue of intestinal GC (200x and 400x magnification).

Figure 3: Immunohistochemical analysis of VEGF in tumor, peritumor and healthy tissue of intestinal GC.
A: Significantly higher VEGF expression in tumor tissue in comparison to its expression in peritumor tissue in intestinal gastric cancer (0.001). B: Significantly higher expression of VEGF in tumor tissue of patients with TNM stages III+IV compared to patients with TNM stages I+II (p=0.018). C: Expression of VEGF was significantly higher in tumor tissue of patients with detected lymphatic invasion in comparison to patients with no detected lymphatic invasion (p=0.002). P values were assessed by Mann–Whitney Rank Sum test.
D: Representative VEGF staining in tumor, peritumor and healthy tissue of intestinal GC (200x and 400x magnification).

Figure 4: IL-17 expression in tumor, peritumor and healthy tissue of intestinal GC.
A: significantly higher expression of IL-17 in tumor tissue in comparison to peritumor tissue (p=0.001). B: significantly higher IL-17 expression in tumor tissue of patients with detectable necrotic fields compared to patients without detectable necrosis (p=0.001).
C: Representative IL-17 staining in tumor, peritumor and healthy tissue of intestinal GC (200x and 400x magnification).