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Original article

Macrophages polarization and density of tumor-associated dendritic cells correlated with depth of invasion in gastric adenocarcinoma

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Abstract

Introduction: Tumor-associated macrophages (TAM) are the most common inflammatory cells in the tumor microenvironment (TM). As a response to microenvironmental signals, they polarize into tumor resisting M1 or promoting M2 macrophages. TAM and tumor-associated dendritic cells (TADC) can either promote tumor growth and tissue invasiveness or have anti-tumor activity. The aim of the study was the examination of prognostic value in the individual cell population in TM and their correlation with clinicopathological parameters of gastric cancer.

Materials and Methods: The study analyzed 60 samples of gastric cancer, known status of regional lymph nodes and without dissemination at the time of diagnosis. The control group was normal gastric tissue samples. Traditional parameters of biological aggressiveness, tumor size, histological grade, and lymphovascular invasion, are determined after standard hematoxylin-eosin staining. TAM and TADS have been evaluated using the immunohistochemical method with CD68 (TAM), TNFa (TAM-M1), CD163 (TAM-M2), and S100 (TADC) antibodies. Expression evaluation of the tissue antigen was carried out by semiquantitative methods.

Results: There were statistically significant differences of TAM density (P <0.001) with M1 (P <0.001) and M2 (P <0.01) polarization in cancer tissue compared to the control group. Statistically, significant positive linear correlation between the number of CD68 TAM and TAM-M1 was observed (P <0.001). In the cancer tissue samples, with increasing tumor size (pT), increased TADC and TAM M1 density, with no statistically significance (P> 0.05).

Conclusion: TAMs and TADC have shown potential as biomarkers for evaluating the gastric cancer staging and progression. They showed promising prediction in depth of invasion, histological grade of tumor and tumor size.

Keywords: gastric cancer, tumor microenvironment, TAM, TAM-M1/M2, TADC

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Introduction

The interaction between carcinoma cells and their microenvironment is crucial for tumor progression and modulation of invasive activity. Many studies show that numerical and functional variations of tumor-associated macrophages (TAM) and tumor-associated dendritic cells (TADC) may or may not be in correlation with clinicopathological parameters, which means parameters of biological aggressiveness (1). TAM and i TADC show dual potential: they can promote tumor growth and invasion, affect the length of patients survival, but also, they can show antitumor activity. So, overcome the suppressive or immunogenic character, depending on nteraction between these and cancer cells.

TAM has a tendency to be polarized into proinflammatory M1 and anti-inflammatory M2 macrophages. Most of them are M2 polarized (2). They inhibit the inflammation, remodel connective tissue, recruit fibroblasts, promote neoangiogenesis and show tumoricidal activity (3). M1 macrophages show a protective character in tumor-genesis by activating tumor-destroying mechanisms and antagonizing activation of various macrophages M2 which are obviously involved in tumor suppression by adaptive immune response and the promotion of tumor growth, invasiveness, metastasis, stromal remodeling and angiogenesis (2). TAMs are generally considered to be allies in the process of progression of the disease into several types of cancer (4,5,6,7,8), but their role in the gastric and colon cancer is still poorly understood and investigated (9). TADC belong to a monocyte-macrophage cell line which strongly expresses the S100 protein (10). They are a specialized group of cells and play a key role in the induction and maintenance of antitumor immunity, but in the tumor microenvironment (TM) their antigen presenting function may be ineffective or completely lost. In this way, the infiltration of tumor tissue with TADC is reflected in the local immune response. TADC infiltration of primary, esophageal, nasopha-

ryngeal and lung tumors, prolongs patient life and reduces the occurrence of metastatic disease (4,8,9). The development and functional plasticity of TADC are dependent on TM receiving impulses for inducing apoptosis or acceleration of their metabolism (11). The results of interaction, segments of action within the complex paracrine signaling pathway TAM and TADC in gastric cancer that is not yet clarified. Determining the role of TAM and TADC in TM is an important component in the further and possibly different treatment of gastric cancer. In this study, we examined the prognostic value of individual cell population in TM (TAM and TADS) and their correlation with already proven parameters of the biological aggressiveness of gastric cancer: tumor size, grade, the degree of tumor differentiation and presence of lymphovascular invasion. The results could lead to improved prognostic modeling and possibly better planning of therapeutic modalities in patients with gastric cancer.

Material and methods

Tissue samples of gastric cancer of 60 patients with Billroth II resection have been analyzed. Operable tumors, with known status of regional lymph nodes and without evidence distant dissemination at the time of diagnosis were included. Samples of normal gastric tissue, which was taken near the margin of resection, were used as a control group. Tissue samples were fixed in 10% buffered neutral formalin, at room temperature, then incorporated into paraffin blocks, cut at 4-5 microns, mounted and stained with standard hematoxylin-eosin (HE) method. Observation included: tumor size (pT), grade according to the degree of tumor differentiation and the presence of lymphovascular invasion. Immunohistochemical visualization of the protein components of the tested cell type was carried out according to the protocol of the manufacturers: CD68 (Dako, clone 514H12), CD163 (Novocastra ™, clone 10D6), S100 (Dako, Clone Z 0311), TNFα (Santa Cruz, clone 52B83). For immunohistochemical staining, tissue was fixed in neutral, buffered formalin, then put into paraffin and microtomic cut to a thickness of 3 μm. Tissue samples, after immunohistochemical staining, were evaluated by light-microscopy (BX40F4, OLYMPUS, Japan).

Evaluation of TAM was made by the modified method used by Ohno et al. (2003) (12). After identifying the field of the highest cell density in tumor stroma on the microscopic magnification x100, immunoreactive cells were counted in 5 visual fields, under the microscopic magnification x400 within the tumor stroma. The mean value of the five obtained fields was used as the data for the analysis. Numerous macrophages, which were sometimes located near the necrosis field or associated with neutrophil leukocytes groups, were excluded from the evaluation.

Only S100 positive cells, expressing the existence of interdigital cytoplasmic extracts, were evaluated like TADC, by a modified method from Hilly et al. (2013) (13). After identifying the field of the strongest cell density in stromal tumor tissue on the microscopic magnification of x100, immunoreactive cells were counted in 10 visual fields, under the microscopic magnification of x400.

For both types of tumor-associated cellular forms, the principle of scoring by Naita et al. (1998) (14) was used, where the average number of cells was 0-3 (0, 1-19, 20-49, and over 50). Semi-quantitative scoring of the intensity of infiltration is divided into four groups: 0 = no cells; 1 = mild; 2 = moderately and 3 = strong.

Statistics

Statistical analysis was performed in the IBM SPSS Statistics v. 21.0 for Windows. The normal distribution of continuous numerical variables was performed using the Kolmogorov-Smirnov's test. The analysis of categorical variables was carried out using Pearson's χ^2-test or Fisher's exact probability test. Non-parametric distributed numerical variables were analyzed using the Mann-Whitney U test. Analysis of non-parametric values between several groups was performed by Kruskal-Wallis test with subsequent Bonferroni correction. The statistical significance was at the conventional level $\alpha = 0.05$.

Results

The density of TADC and TAM M1/M2 was evaluated by medians which were compared and then correlated between tested and control group of patients.

There was no significance (U = 423.000, z = -0.727, P> 0.05) (Table 1.) between medians of TADC density (Figure 1.) in tissue samples with gastric adenocarcinoma (Me = 3.5 (IQR = 2.3 to 5.0)) and control group (Me = 3.7 (IQR = 2.3 to 7.2)).

Differences in medians of TAM density (Figure 2.) in gastric adenocarcinoma (Me = 14.9, IQR = 5.4 to 26.2) and control group (Me = 4.2; IQR = 1.8 to 7.0) were statistically significant (U = 160.500, z = -4.072, P <0.001) (Table 1.).

There was statistically significant difference (U = 282.000, z = -2.607, P <0.01) between density of TAM-M2 (Figure 3.) in tested (Me = 0.8, IQR = 0.0 to 1.6) and control group (Me = 0.0; IQR = 0.0 to 0.6) (Table 1).

Also, there was statistically significant difference (U = 177.000, z = -3.862, P <0.001) between density of

TAM-M1 (Figure 4.) tissue samples with gastric adenocarcinoma (Me = 9.4 (IQR = 3.8 to 17.5)) and control group (Me = 2.6 (IQR = 1.0 to 4.3)) (Table 1).

It was observed that in the tissue samples of gastric adenocarcinoma, with increasing tumor size (pT), also increased TADC and TAM M1 density, but the differences did not reach statisticaly significance (P> 0.05) (Table 2). The increase of TAM M1 density is recorded only to stage pT3 after which values are stagnating. TAM M2 density was decreasing.

There was no statistical difference in density of TADC and TAM in carcinoma samples with or without lym-

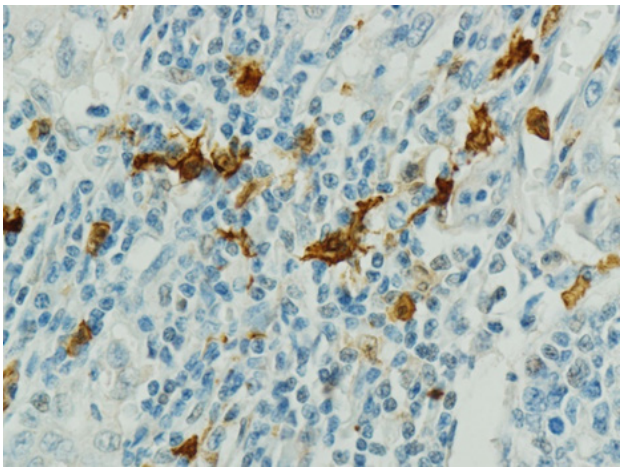

Figure 1. Tumor associated, S100 positive, dendritic cells in tumor microenvironment (400x).

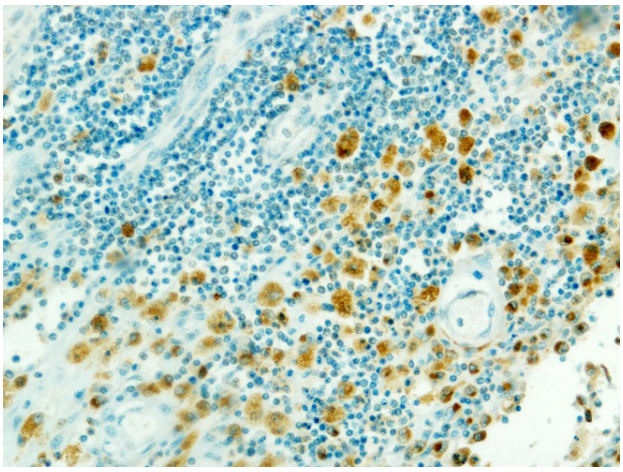

Figure 2. Tumor-associated, CD68 positive, macrophages in the tumor microenvironment (200x).

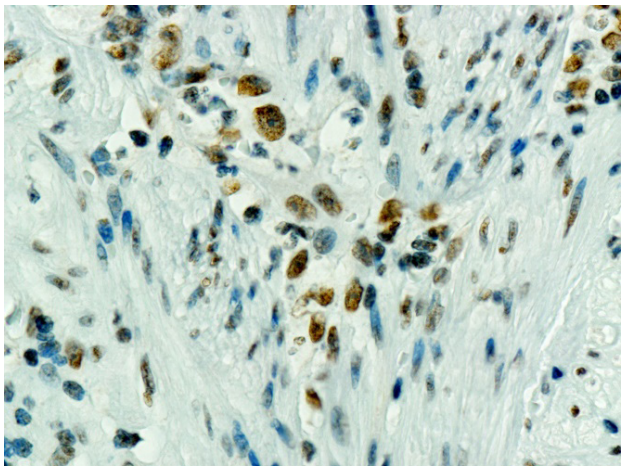

Figure 3. Tumor-associated, CD 163 positive, macrophages-M2 in the tumor microenvironment (400x).

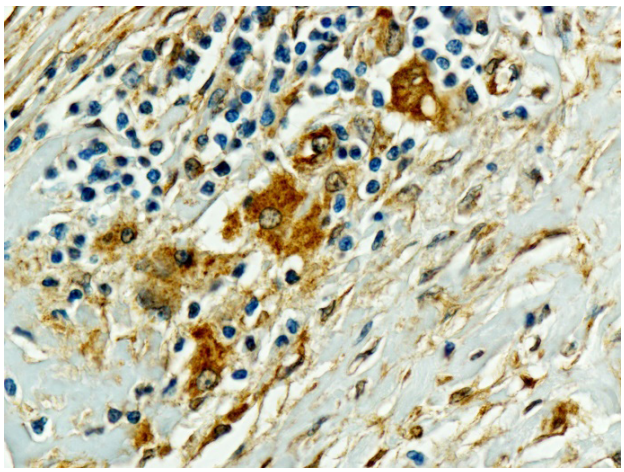

Figure 4. Tumor-associated, TNFα positive, macrophages-M1 in the tumor microenvironment

Table 1. The density of tumor-associated dendritic cells and tumor-associated macrophages.

TM Cells	Group	N	M	SD	Min.	Max.	Percentiles			p-value
							25th	50th	75th	
S100+ TADC	Tested gr.	60	4.2	2.7	1.2	13.4	2.3	3.5	5.0	0.468
	Control gr.	16	4.9	3.0	1.4	12.2	2.3	3.7	7.2	
CD68+ TAM	Tested gr.	60	19.4	18.0	1.8	88.0	5.4	14.9	26.2	‹0.001
	Control gr.	16	4.5	3.2	0.0	9.6	1.8	4.2	7.0	
CD163+ TAM-M2	Tested gr.	60	1.5	2.4	0.0	11.8	0.0	0.8	1.6	‹0.01
	Control gr.	16	0.4	0.9	0.0	3.2	0.0	0.0	0.6	
TNFα+ TAM-M1	Tested gr.	60	11.8	10.3	0.0	47.2	3.8	9.4	17.5	‹0.001
	Control gr.	16	2.9	2.1	0.0	7.4	1.0	2.6	4.3	

N- number, M-mean, SD-standard deviation

phovascular invasion (LVI) (P> 0.05) (Table 3.), nor in the mean value of TADC and tumor grade within the tested group (P> 0.05) (Table 4.).

A statistically significant difference in the mean value of TAM-M1 density and tumor grade (P <0.01) was noted (Table 4). Post-hoc analysis after Bonferroni's correction (P <0.008), showed statistically significant differences between the grade 1 and 2 (P = 0.001) and the grades 2 and 3 (P = 0.006). Other grade comparisons did not reach the statistical significance threshold P <0.008 (Figure 5.).

There was no statistically significant difference in TADC and TAM density according to regional lymph node status and histological type of tumor (p > 0.05). However, a statistically significant positive linear cor-

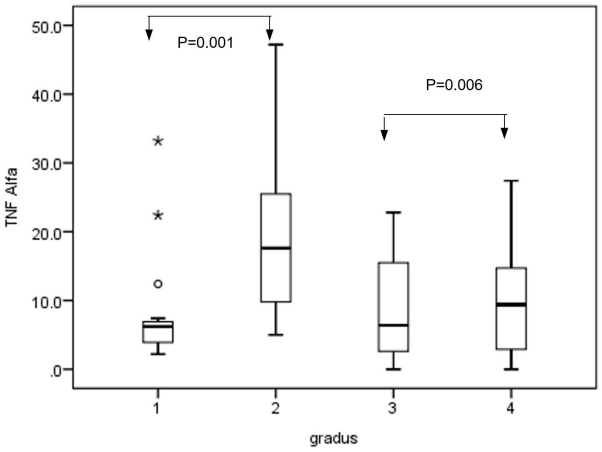

Figure 5. TAM-M1 expression compared to the histological grade of gastric cancer

Table 2. Correlation between tumor size and density of tumor-associated macrophages and dendritic cells

Tested cells	pT	N	M	SD	Min.	Max.	Percentiles			p-value
							25th	50th	75th	
S100+ TADC	1+2	20	4.0	2.7	1.4	9.5	2.2	3.0	4.6	>0.05
	3	27	3.9	2.3	1.2	12.3	2.6	3.4	4.7	
	4	13	5.0	3.3	1.6	13.4	2.3	4.4	6.2	
CD68+ TAM	1+2	20	14.6	11.8	1.8	44.2	5.2	11.5	20.5	>0.05
	3	27	22.4	20.4	2.2	88.0	8.4	9.4	26.2	
	4	13	20.2	20.1	1.8	65.6	5.0	12.4	29.1	
CD163+ TAM-M2	1+2	20	1.7	2.6	0.0	10.4	0.1	1.2	1.6	>0.05
	3	27	1.0	1.2	0.0	4.8	0.0	0.6	1.6	
	4	13	1.9	3.8	0.0	11.8	0.0	0.4	1.6	
TNFα+ TAM-M1	1+2	20	10.2	11.1	1.0	47.0	2.7	6.9	11.8	>0.05
	3	27	13.9	9.4	0.0	33.0	5.4	14.0	20.2	
	4	13	10.1	10.7	0.0	39.0	3.0	4.8	16.2	

N- number, M-mean, SD-standard deviation

Table 3. Correlation between the lymphovascular invasion of gastric adenocarcinoma and density of tumor associated macrophages and dendritic cells.

TM Cells	Lympho-vascular invasion (LVI)	N	M	SD	Min.	Max.	Percentiles			p-value
							25th	50th	75th	
S100+ TADC	1	33	4.2	2.6	1.4	12.3	2.4	3.6	5.5	>0.05
	2	27	4.1	2.9	1.2	13.4	2.3	3.2	4.8	
CD68+ TAM	1	33	21.8	21.2	1.8	88.0	5.0	19.4	26.3	>0.05
	2	27	16.4	12.9	1.8	53.2	7.4	12.8	21.0	
CD163+ TAM-M2	1	33	1.8	3.0	0.0	11.8	0.0	0.6	1.8	>0.05
	2	27	1.1	1.5	0.0	7.4	0.0	1.0	1.4	
TNFα+ TAM-M1	1	33	12.0	9.9	0.0	39.0	3.8	9.4	18.3	>0.05
	2	27	11.6	10.9	0.0	47.0	3.6	8.4	15.2	

N- number, M-mean, SD-standard deviation

Table 4. Correlation of gastric cancer grade and density of tumor-associated macrophages and dendritic cells

Tested cells	pT	N	M	SD	Min.	Max.	Percentiles			p-value
							25th	50th	75th	
S100+ TADC	1	15	4.4	2.7	1.7	9.5	2.3	3.2	5.8	>0.05
	2	15	4.4	3.2	1.6	12.3	2.6	3.3	5.2	
	3	15	2.9	1.1	1.4	4.8	1.6	2.9	3.9	
	4	15	4.9	3.1	1.2	13.4	2.3	4.6	5.8	
CD68+ TAM	1	15	16.1	18.3	3.6	73.6	5.0	8.4	19.6	>0.05
	2	15	25.2	25.2	6.2	65.6	17.6	25.0	27.6	
	3	15	19.5	19.5	2.2	54.6	3.6	17.8	26.2	
	4	15	16.6	16.6	1.8	88.0	4.6	12.4	21.0	
CD163+ TAM-M2	1	15	1.0	0.7	0.0	2.4	0.4	1.0	1.4	>0.05
	2	15	2.3	3.6	0.0	11.8	0.4	1.2	1.8	
	3	15	1.7	2.7	0.0	8.6	0.0	0.2	1.8	
	4	15	0.8	1.3	0.0	4.8	0.0	0.4	1.2	
TNFα+ TAM-M1	1	15	8.3	8.5	2.0	33.0	3.4	6.2	7.4	0.005
	2	15	20.1	11.9	5.0	47.0	9.8	17.6	26.6	
	3	15	9.1	7.5	0.0	23.0	2.6	6.4	15.8	
	4	15	9.8	8.7	0.0	27.0	2.4	9.4	15.4	

N- number, M-mean, SD-standard deviation

and/or produce tumor destructive reactions and induce both, positive and negative effects on tumor growth, depending on the stimulus from tumor microenvironment. Such a high presence of TAM correlates with longer survival in patients with squamous cell carcinoma of the esophagus, endometrial cancer, non-microcellular lung cancer, and nasopharyngeal carcinoma, while their role in gastric and colorectal cancer is still insufficiently clarified (20).

One of the possible reasons for the previous contradictory results is that the above studies were performed only by immunohistochemical detection of TAM using CD68 antibodies. Using the CD68 antibody, it is not possible to estimate the presence of two polarization forms of macrophages, which have almost completely opposite biological characteristics. Only two studies using dual immunohistochemical color analyzed the relationship between macrophages M1 and M2 and the prognosis of the disease. They report a significant direct correlation between M1 infiltrating phenotype and survival time in patients with non-microcellular lung carcinoma (NSCLC) (21).

Therefore, we used double immunohistochemical staining to separate polarization forms of TAM M2 (CD163) and TAM M1 (TNF alpha) morphologically from total TAM (CD 68).

In this study, a statistically significant difference between the investigated and the control group was found, by analyzing the TAM (CD 68 positive cell) medium-TAM-M1 (TNFα-positional cell) and TAM-M2 (CD 163 positivity cells). Only two recently published studies described that gastric cancer in patients with a high number of TAM has a worse outcome than those with low TAM number (22,23).

In this study, we noticed that the density and polarization of TAM were statistically significantly more pronounced in the carcinoma tissue compared to the control group tissue. The difference in TADC density between carcinoma's and normal tissue samples was not observed. TAM density type M1 is the most intense in gastric adenocarcinoma with histological grade 2. We noticed, in patients with an infiltrative form of gastric cancer, a positive linear correlation between tumor size (pT) and density of TAM type M1, but without statistical significance. Also, increasing tumor size was in correlation with increase in TADC density. The increase in TAM-M1 densities, as statistically significant, was recorded only to the pT3 stage. The TAM type M2 is inversely proportional to M1, i.e. decreases with increasing pT tumor stages. TAM density did not show a statistically significant difference in correlation with lymphovascular invasion, regional lymph node status, and histological type of tumor in infiltrative gastric adenocarcinoma, while the mean value of TAM M1 densities showed a statistically significant difference in

relation to the histological grade of the tumor. Statistically significant differences were found between tumor grade 1 and 2 and grade 2 and 3. A statistically significant positive linear correlation between the number of CD68 + and M1-TNFα + tumors of associated macrophages was found.

In 2013, Pantano and al. did the first study in which they analyzed two types of polarized macrophages in gastric cancer. Multivariate analysis has led to the finding that the density of M1 macrophage is a prognostic factor, and that only the M1 / M2 ratio can be an independent prognostic factor, indicating that the cellular and molecular interaction between M1 and M2 population plays an important role in determining prognosis in patients with gastric cancer, opening new therapeutic horizons, including strategies that will aim to change TAM phenotypes in patients with gastric cancer. No association was found between the density of macrophage M2 and disease prognosis, as well as the statistical significance with tumor stage, histological type, lymph node involvement and tumor grade (2), which is partly opposite to our results.

Some studies have shown that the degree of macrophage infiltration of the carcinoma tissue is a significant survival predictor in patients with gastric cancer (22,24,25). It has been shown that the removal of macrophages by genetic mutations has reduced tumor progression and metastasis (26). Wu et al. (2015) concluded that the increase in macrophage density was associated with disease progression and a worse prognosis in gastric cancer undergoing radical resection, with the density of CD68 + macrophages being particularly high in tumor stage 4 (pT4). One possible explanation is that macrophages can induce invasiveness of cancer by activating β-catenin pathways and as such be a tumor invasion promoter (27). Yamaguchi et al. (2016) investigated the TAM type M2 in patients with gastric cancer, in whom peritoneal dissemination of the disease was present, and found that the number of macrophages found in the free fluid of the abdominal cavity polarized in type M2 (CD163 + or CD204 +) was significantly higher in patients with gastric cancer with peritoneal dissemination of the disease compared to patients without dissemination, and concluded that M2 can contribute to tumor proliferation and progression (28). Similar results were also made by Zhang et al. (2016) who reported that a high level of TAM infiltration is associated with aggressive malignancies and represents an independent, poor, prognostic factor in patients with gastric cancer (29). In their study, high expression of CD68 correlated with pTNM disease, lymph node involvement, metastasis, Borrmann's type of cancer, the degree of differentiation and tumor size, but not with the sex and age of the patient, nor with the depth of tumor invasion.

In this study, the circumferential distribution of peripheral S100 dendritic cells was detected. Their density was expressed through correlated median control and investigated a group of patients. No statistically significant differences were observed. Further statistical analysis showed that in patients with an infiltrative form of adenocarcinoma with an increase in pT tumor stage, had an increase in the density of tumors of infiltrating dendritic cells. The mean value of dendritic cell density did not show statistical significance in correlation with lymphovascular invasion, regional lymph node status, histological gradient, and type of tumor. Saito and al. (1998), in the multivariate analysis showed that infiltration of dendritic cells and tumor size are two independent prognostic factors for gastric carcinoma (10). Tsujitani et al. (1993) have reported that infiltration by dendritic cells is not correlated with the depth of tumor invasion across the gastric wall, which is contrary to our results (30). Also, the infiltration of dendritic cells does not prevent tumor invasion. The relationship between infiltration of dendritic cells and the occurrence of metastases in regional, perigastric lymph nodes is explained by the authors so that infiltration of dendritic cells can prevent further spread of tumor cells outside the primary node in advanced gastric cancer (31).

Conclusion

The density of tumor-infiltrating dendritic cells, tumor-associated macrophages and their polarization forms, correlate with the depth of tumor invasion in the gastric wall and histological tumor grade, while with other pathological parameters of biological aggressiveness of gastric cancer is not correlated.

Declaration of interest

The authors declare no conflicts of interest.

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