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ORIGINAL ARTICLE

Circulating and tissue galectin-1 and galectin-3 in colorectal carcinoma: association with clinicopathological parameters, serum CEA, IL-17 and IL23

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Summary

Purpose: *Galectins are modulators of many processes critical for tumor progression and metastasis but their clinical significance is still unclear. The objective of this study was to analyze the clinical significance of Galectin-1 and Galectin-3 in the tissue and sera of patients with colorectal carcinoma (CRC). Examined were also their association with serum CEA, IL-17 and IL-23 in CRC patients.*

Methods: *One hundred and twenty patients with CRC were included in this study. The expression of Galectin-1 and Galectin-3 in biopsy samples of CRC was determined using immunohistochemistry (N=120). The concentrations of Galectin-1, Galectin-3, IL-17 and IL-23 in the sera of CRC patients (N=38) were determined by Enzyme Linked Immunosorbent Assay (ELISA).*

Results: *Serum Galectin-1 concentrations positively correlated with parameters of malignancy including perineural invasion (p=0.016), lymph node involvement and distant metastases (p=0.029). Higher expression of peritumoral Galectin-1 was associated with both presence of perineural invasion and poor differentiation of CRC. Serum CEA levels positively correlated with circulating Galectin-1, but inversely correlated with peritumoral Galectin-1 expression. There was no correlation between Galectin-3 and clinicopathological parameters of CRC, but it was found that Galectin-3 expression in the tumor tissue positively correlated with serum IL-17 and IL-23. Circulating Galectin-3 levels significantly correlated with IL-17 (p=0.042), but not with IL-23 in the sera of CRC patients.*

Conclusions: *This study suggests that Galectin-1 and Galectin-3 exhibit protumorigenic activity in CRC by affecting different aspects of tumor progression. Galectin-1 facilitates tumor invasion and metastasis while Galectin-3 preferentially modulates tumor-associated inflammatory processes.*

Key words*: CEA, colorectal carcinoma, Galectin-1, Galectin-3, IL-17*

Introduction

Metastatic cascade is the final process in the progression of malignant tumors and includes the detachment of malignant cells from the primary tumor and their attachment to the endothelium and components of extracellular matrix at distal sites. The regulation of adhesion interactions is one of the critical steps in the establishment of metastases. Adhesion molecules are important players in the metastatic cascade involved in the regulation of tumor cell-extracellular matrix, tumor cell-cell and tumor-endothelial adhesion interactions [1].

Galectin-1 and Galectin-3 are members of a protein family that have affinity for β-galactose residues of glycoconjugates. These proteins are involved in many steps of tumor progression such as tumor growth, migration and invasion [2,3]. The direct interaction between tissue-plasmino-

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gen activator (tPA) and Galectin-1 in pancreatic cancer cells and stromal fibroblasts surrounding the tumor [4] has indicated that higher expression of Galectin-1 is involved in tumor cell invasion. It is well documented that Galectin-1 and Galectin-3 bind the plethora of ligands and thus contribute to the adhesion interactions [5,6]. Recently, it has been shown that Galectin-1 binds to adhesion molecules such as CD44 and the epithelial cell adhesion molecule- CD326 [7]. These surface molecules contribute to extravasation of metastatic cells by promoting their attachment to the endothelium of distant organs [8,9]. Thus, it appears that Galectin-1 interaction with CD44 and CD326 may promote establishment of metastases [7].

Carcinoembryonic antigen (CEA) was one of the first serum tumor markers of CRC. CEA is an adhesive molecule previously identified as a ligand of Galectin-1 and Galectin-3 [10-12]. CEA is overexpressed in CRC [13] and promotes the metastatic capacity of colon cancer cells [14]. Thomas et al. [15] have suggested that CEA is a ligand for E- and L-selectin, involved in the adhesion of tumor cells to endothelium at distal sites. CEA has also been linked to processes relevant to tumor metastasis including homotypic cell-cell interactions [16,17], apoptosis resistance [18] and modulation of antitumor immune response [19].

Chronic inflammation has been linked to the initiation and progression of many types of tumors, including sporadic CRC [20,21]. An inflammatory milieu consisting of infiltrated immune cells and released inflammatory mediators can promote the invasive and metastatic capacity of tumor cells [22]. For example, IL-17 and IL-23 are pro-inflammatory cytokines whose expression is elevated in several tumors such as breast cancer and CRC [23-25]. Sobhani et al. [26] have reported differences in the composition of colon microbiota between patients with colon cancer and healthy individuals, suggesting that this imbalance or "dysbiosis" in CRC patients exacerbates inflammation. They revealed significant elevation of the Bacteroides/Prevotella population in cancer patients which are linked to elevated IL-17 producing cells in the colon mucosa [26]. The defects of epithelial barrier induced by cancer-initiating genetic lesions results in microbial pathogens' invasion and release of microbial products that drive IL-23/IL-17 axis activation and promote tumor growth [27]. It appears that the mechanism involved in the pro-tumorigenic activity of IL-17 is related to inflammation-associated signaling pathways. Thus, IL-17 induces IL-6 production, which in turn activates Stat3 and promotes cancer cell survival [28]. Despite the well-defined pro-tumorigenic role of Galectin-3, its involvement in tumor-associated inflammation and consequent tumor progression is not fully elucidated. The link between Galectin-3 and infective and non-infective inflammatory disease is well documented [29]. Galectin-3 acts as an alarmin which increases the inflammatory response [30]. Alarmins are a family of endogenous immunomodulatory molecules that belong to the larger family of danger-associated molecular patterns molecules (DAMPs) [31]. Some investigators illustrate the role of Galectin-3 in the case of stage IV ovarian cancer with underlying inflammatory comorbidities [32]. It seems that circulating Galectin-3 might be a valuable clinical marker of tumor-associated inflammation [32].

The purpose of this study was to analyze the clinical significance of Galectin-1 and Galectin-3 levels in tissue and sera of patients with CRC. Additionally, the association of these galectins with serum CEA, IL-17 and IL-23 levels in CRC patients was assessed. It appears that Galectin-1 may serve as a marker of poor prognosis. Elevated concentrations of Galectin-1 and CEA in the serum of CRC patients positively correlated with metastasis development. In addition, the data acquired indicates the role of Galectin-3 in the CRC progression through increase of the production of pro-inflammatory IL-17 [7].

Methods

Patient characteristics

The tissue specimens from a total of 120 patients with CRC who had undergone surgery from November 2006 to May 2014 at the Center for General Surgery, Clinical Centre of Kragujevac were analyzed in the Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia. The study was approved by the Ethics Committees of these institutions and all patients gave written informed consent for research use of their biological material. The study included 84 males and 36 females with mean age 66.05±10.06 (mean±SD) years (range 31-87). The patient basic characteristics are shown in Table 1.

Histological sections were stained using hematoxylin-eosin and were reviewed by two pathologists to confirm the diagnosis. According to the histological diagnosis all patients had colorectal adenocarcinoma. Patients were staged according to the American Joint Committee for Cancer staging system (AJCC/TNM, 6th Edn). Patients with undefined presence of distant metastases (Mx) were excluded from the analysis of the

Table 1. Basic characteristics of patients with colorectal cancer

Characteristics	*Tissue (N=120)* N	*Serum (N=38)* N
Gender		
Male	77	23
Female	43	15
Age, years		
Mean±SD	66.05 ± 10.06	± 9.96
Range	31 - 87	47 - 86
Tumor location		
Colon	83	15
Rectum	37	23
Tumor depth		
T1/T2	22	11
T3/T4	98	27

Tumor depth: T1: tumor invades submucosa; T2: Tumor invades muscularis propria; T3: Tumor invades through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues; T4: Tumor directly invades other organs or structures and/or perforates visceral peritoneum

association between TNM stage and the level of Galectin-1 and Galectin-3 in tissue and serum.

Immunostaining for Galectin-1 and Galectin-3

The primary tumor samples were investigated and processed under identical conditions. Briefly, 4-5 μm-thick tissue sections were cut consecutively from formalin-fixed, paraffin-embedded tissue. Immunohistochemical staining for Galectin-1 and Galectin-3 expression was performed using a commercially available staining kit (Mouse specific HRP/DAB (ABC) Detection IHC Kit, ab64259 and Rabbit specific HRP-AEC detection IHC kit, ab94361, Abcam, Cambridge, UK) [33]. The tissue sections were stained with primary antibodies: rabbit polyclonal anti-Galectin-1 (ab25138, Abcam, Cambrindge, UK, at a 1:2000 dilution) and mouse monoclonal anti-Galectin-3 (ab58086 Abcam, Cambrindge, UK, at a 1:40 dilution), respectively. Negative controls were subjected to the same procedure with the omission of the primary antibody incubation step. Positive controls consisted of tissue known to contain the protein of interest. The tissue sections were examined by conventional light microscopy (Axioskop 40, Carl Zeiss, Oberkochen, Germany).

Assessment of Galectin-1 and Galectin-3 expression in the CRC tissue

The Galectin-1 and Galectin-3 stained tissue sections were assessed semiquantitatively by three independent observers. For each specimen the immunohistochemical analysis consisted of the evaluation of the percentage of positive staining cells and the intensity of staining.

The staining Galectin-3 score was evaluated as the percentage of stained cells out of the total number of evaluated tumor cells. For Galectin-1, the staining score was evaluated as the percentage of stained cells out of the total number of evaluated peritumoral cells. The percentage of positive stained cells per specimen was determined by counting 5 non-overlapping microscopic fields per specimen in tumor areas at x400 magnification. The staining intensity was determined on a four grade scale: 0 (no staining), 1 (weak intensity), 2 (moderate intensity) and 3 (strong intensity).

Determination of concentrations of Galectin-1, Galectin-3, IL-17, IL-23 and CEA in the sera

Venous blood samples (5-10 mL) were taken in vacutainer tubes under sterile conditions from patients with CRC (N=38) just before the operation. Serum concentrations of cytokines and galectins were determined using human sensitive ELISA kits (R&D Systems Minneapolis, MN for IL-17, IL-23 and Galectin-3; Usnc Life Science Inc., China, for Galectin-1). The measurement was performed according to the instructions of the NS manufacturer. Serum CEA levels were measured by chemilluminescence immunoassay on UniCel DxI 600 Analyzer (Beckman Coulter, CA).

Statistics

For data analysis the SPSS Statistics 20 software package was used. Methods of descriptive statistics were used determine measures of central tendency and variability and the results are presented as mean and standard error. For the determination of the strength and direction of correlation between the variables Pearson's and Spearman's correlation coefficients were used. Two-tailed Student's t-test for independent samples was used for determining statistically significant differences between the means of two groups. A p value less than 0.05 was considered statistically significant.

Results

Perineural invasion and metastatic phenotype of colorectal carcinoma is associated with Galectin-1

Among the 120 tumor tissues analysed, more than half were well or moderately well differentiated colorectal adenocarcinomas. Other clinical and pathological parameters of patients are presented in Table 2. In order to determine whether circulating Galectin-1 and Galectin-3 contribute to CRC progression we correlated serum levels of these galectins with clinicopathological parameters. The obtained data showed that serum concentrations of Galectin-1 were significantly higher in the group of patients with positive perineural invasion compared to the group with negative perineural invasion (mean±SE:349.33±48.22 vs 212.64±22.68 ng/ml; p=0.016). In addition, serum concentration of Galectin-1 positively and

Table 2. Clinicopathological characteristics of patients and their relationship with tissue expression or serum levels of galectins

Characteristics	*Tissue*			*Serum*		
	CRC patients (N=120)	*Galectin-1*	*Galectin-3*	*CRC patients (N=38)*	*Galectin-1*	*Galectin-3*
Histologic grade*						
Low	77	p=0.018[b]	NS	17	NS	NS
High	43			21		
TNM classification**						
I	5			5		
II	12	NS	NS	11	r=0.445	
III	8			8	p=0.014[a]	
IV	13			6		
Lymph node metastasis						
No	73	NS	NS	23	NS	NS
Yes	47			15		
Distant metastases						
No	26	NS	NS	25	r=0.393	NS
Yes	13			6	p=0.029[a]	
Vascular invasion						
No	70	NS	NS	18	NS	NS
Yes	50			20		
Lymphatic invasion						
No	20	NS	NS	3	NS	NS
Yes	100			35		
Perineural invasion						
No	60	p=0.007[b]	NS	15	p=0.016[b]	NS
Yes	60			23		
Lymphocytic infiltration		NS	NS		NS	NS
Negative	0			0		
Mild	56	NS		17	NS	
Moderate	51			16		
Strong	13			5		

*Low: well and moderately well differentiated adenocarcinoma; High: poorly differentiated and undifferentiated.
**Stage 0 – Tis, N0, M0; Stage I – T1/T2, N0, M0; Stage II – T3/4, N0, M0; Stage III – any T, N1/2, M0; Stage IV – any T, any N, M1. Patients with undefined presence of distant metastasis (mx) were excluded from the analysis of the association of TNM stage and level of Galectins and are not shown in the Table.
NS: not significant.
p values were assessed by [a]Pearson's test, as well as by [b]Independent Samples t-test, where appropriate.
r: correlation coefficient shows the strength and direction of the relationship between paired data.

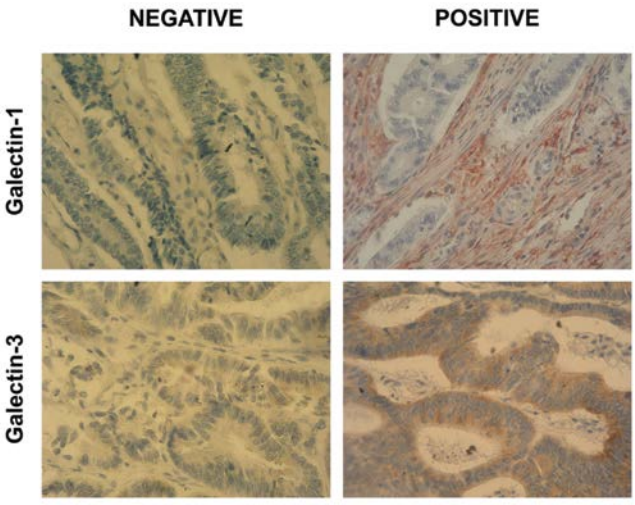

Figure 1. Immunohistochemical staining of galectins in CRC tissues. Representative tissue sections of negative and positive Galectin-1 and Galectin-3 expression are shown (original magnification ×200). Positive Galectin-1 expression was predominantly detected in peritumoral stroma of colorectal carcinoma (upper right panel). Galectin-3 expression was identified predominantly in the cytoplasm of the tumor cells (lower right panel).

significantly correlated with TNM stage and distant metastases (Table 2). Namely, serum Galectin-1 concentrations were significantly increased in patients with lymph node metastases as well as patients with distant metastases (p=0.029). We did not find significant association between circulating Galectin-3 and any of the clinicopathological parameters of CRC patients examined (Table 2).

The next aim of this study was to investigate the pattern of expression of Galectin-1 and Galectin-3 in CRC tissue by immunohistochemistry. As shown in Figure 1, Galectin-1 and Galectin-3 had different patterns of expression in CRC tissue.

In line with the findings in the serum, the percentage of Galectin-1 positive peritumoral cells was higher in CRC patients with positive perineural invasion when compared to the group with negative perineural invasion (mean±SE: 75.32±3.61% vs 60.16±4.10%; p=0.007). The percentage of Galectin-1 positive peritumoral cells was significantly higher in poorly differentiat-

Table 3. Correlation between galectins' expression and serum level of CEA in CRC patients

	Galectin-1		*Galectin–3*	
	Tissue expression [a]*extent of staining*	*Serum values* [b]*staining intensity*	*Tissue expression* [a]*extent of staining*	*Serum values* [b]*staining intensity*
Serum CEA	r= -0.406 p= 0.024*	NS	r= 0.635 p= 0.000**	NS

[a]extent of staining was evaluated as the percentage of stained cells out of the total number of evaluated cells.
[b]staining intensity was scored as 0 (no staining), 1 (weak intensity), 2 (moderate intensity) and 3 (strong intensity).
p values were assessed by *Pearson's or **Spearman's tests depending on distribution of variables
r: correlation coefficient showing the strength and direction of the relationship between paired data
NS: not significant

ed in comparison to well and moderately well differentiated CRC (mean±SE: 63.29±3.72% vs 76.75±4.06% ; p=0.018) (Table 2). There was no correlation between Galectin-3 positive tumor cells and clinicopathological characteristics of CRC patients (Table 2).

Serum CEA negatively correlates with peritumoral tissue expression of Galectin-1, but positively correlates with circulating Galectin-1

Next, serum levels of CEA were been compared with serum levels and tissue expression of Galectin-1 in patients with CRC. The data showed significant positive correlation between serum levels of CEA and Galectin-1. There was statistically significant inverse correlation between serum CEA concentrations and peritumoral tissue expression of Galectin-1. The association between CEA and Galectin-1 is presented in Table 3 which shows no correlation between CEA and Galectin-3 in CRC patients.

Both tissue and serum Galectin-3 positively correlate with IL-17 levels in the sera of CRC patients

The level of expression of Galectin-3 (weak, moderate and strong intensity) was positively correlated with serum IL-17 (p=0.036; r=0.635) and IL-23 levels (p=0.019; r=0.639) (Figure 2A). The results further showed that increased serum Galectin-3 levels positively correlated with serum IL-17 concentration in CRC patients (p=0.042; r=0.336) (Figure 2B), but not with IL-23 (data not shown).

Discussion

In the current study, circulating Galectin-1 positively correlated with perineural invasion, lymph node involvement and distant metastases. In contrast to Galectin-3 which is expressed in tumor cells, Galectin-1 was predominantly located in the stroma surrounding tumor cells and to a very small extent in the CRC cells. Galectin-1 overexpression in the peritumoral stroma was significantly associated with both perineural invasion and poor differentiation of CRC. In addition, serum CEA positively correlated with circulating Galectin-1 levels, but inversely correlated with peritumoral Galectin-1 expression. Galectin-3 expression in the tumor tissue positively correlated with serum IL-17 and IL-23.

Galectin-1 overexpression in the oral cancers and lung adenocarcinoma promotes tumor invasion and metastasis by increasing the expression of matrix metalloproteinases, including MMP-1 and MMP-9, and reorganizing cytoskeleton [34]. Neoplastic perineural invasion is one of the metastatic spreading routes and is reported that perineural invasion is an important characteristic of many malignancies, including CRC [35,36]. The present study showed that increased serum levels of Galectin-1 positively correlated with perineural invasion in CRC patients. Previous studies demonstrated that circulating Galectin-1 levels were significantly increased in CRC patients compared to healthy individuals, and its elevated levels markedly decreased after surgery [37]. In line with a recent report [38], the obtained data indicate the positive correlation between increased serum levels of Galectin-1 and lymph node and distant metastases.

Increased Galectin-1 expression has been reported in various malignant tumors [39,40]. In this study Galectin-1 was predominantly expressed in the stroma surrounding tumor cells and in a very small extent in the CRC cells. This is in agreement with results from several studies showing marked increase in Galectin-1 expression mainly in stromal cells during progression of CRC [41-43]. In line with these data, higher expression of Galec-

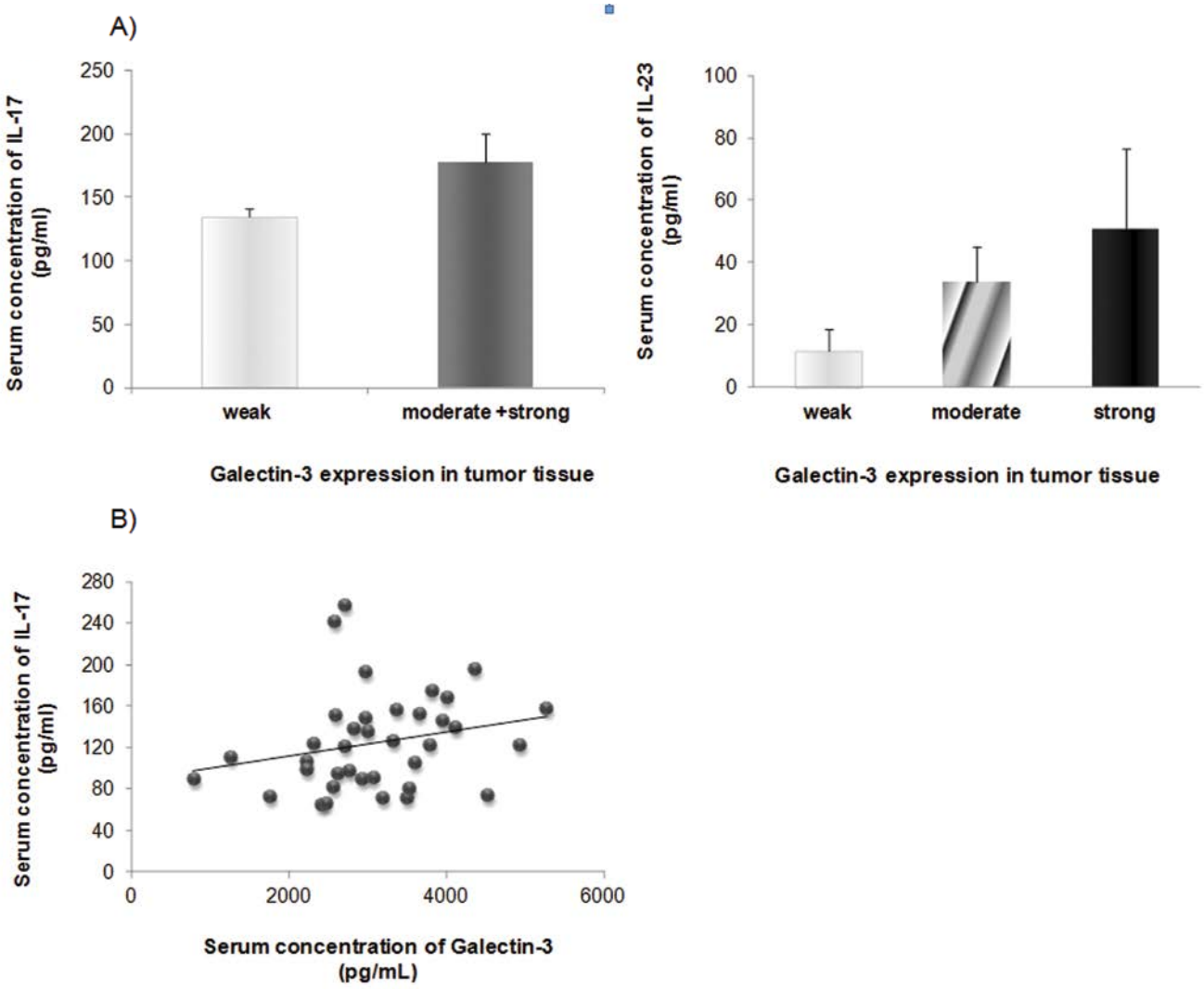

Figure 2. The association between tissue expression and serum levels of Galectin-3 with serum IL-17 and IL-23 levels in CRC patients. **A**: Positive correlation between the intensity of Galectin-3 expression (weak, moderate or strong) in tumor tissue and serum concentrations of IL-17 (p=0.036; r=0.635) and IL-23 (p=0.019; r=0.639); **B**: Serum Galectin-3 was elevated and the levels positively correlated with serum IL-17 concentration in CRC patients (p=0.042; r=0.336). P values were assessed by Spearman's method. Values are shown as means and standard errors.

tin-1 in the peritumoral stroma was significantly associated with both perineural invasion and poorly differentiated CRC. In ovarian carcinoma, Galectin-1 is also preferentially expressed in the adjacent peritumoral stroma. Namely, cancer-associated fibroblasts which immediately surround ovarian cancer cells have more Galectin-1 at both the mRNA and protein levels compared with distant fibroblasts [44]. It appears that the malignant cells may be responsible for higher expression of Galectin-1 in the stromal cells, suggesting that Galectin-1 is involved in the crosstalk between malignant cells and the tumor-associated stroma and also in the modulation of tumor cell behavior. The obtained data suggests that both peritumoral Galectin-1 expression and circulating Galectin-1 levels may reflect the aggressiveness of CRC.

Changes in the aggressiveness of tumor including the ability of tumor cells to metastasize are most probably related to their adhesive properties. Galectin-1 binds various glycoconjugate ligands in the extracellular milieu [6] and thus modulates the adhesion between tumor cells or between tumor cells and the extracellular matrix (ECM) [45]. For example, Galectin-1 increases the adhesion of prostate and CRC cell lines to components of ECM [44,46]. CEA is one of the Galectin-1 ligands on human colon carcinoma cells [10]. The presented data show that serum CEA positively correlated with circulating Galectin-1, but negatively correlated with Galectin-1 expression in the peritumoral stroma. A previous study indicates that Galectin-1 is involved in homotypic cell aggregation of human melanoma cells [47], suggesting that Galectin-1 may promote the formation of tumor emboli and thus allow survival of disseminating tumor cells in the circulation. Significant correlation between the degree of cell aggregation and CEA expression by

CRC cells was demonstrated, indicating that CEA also mediates the homotypic aggregation of CRC cells in malignant effusions [16]. The capacity of different CRC cell lines to metastasize in the liver of a nude mouse model positively correlates with CEA production [48].

Simultaneous elevated serum concentrations of Galectin-1 and CEA indicate the possibility of their cooperation during CRC metastasis.

Our previous study indicates that Galectin-3 is an important facilitator of melanoma lung metastasis by promoting tumor cell adhesion and by suppressing the antitumor immune response [49]. It has been shown that strong Galectin-3 expression in CRC is associated with tumor progression, distant metastases and poor prognosis [50]. However, no correlation between Galectin-3 expression and the progression of CRC was observed. These inconsistencies, observed even in human tumors of the same origin, may be the consequence of the heterogeneity of tumor cells. The correlation between Galectin-3 and malignancy is therefore unlikely. In this study, Galectin-3 expression in the tumor tissue positively correlated with serum IL-17 and IL-23 levels. Additionally, serum Galectin-3 levels were associated with IL-17, but not with IL-23 in the sera of CRC patients. Previous studies have shown elevated serum concentrations of IL-17 in patients with CRC in comparison to healthy individuals [33] or patients with ulcerative colitis [51]. Forsman et al. [52] indicated that Galectin-3 contributes to the pathogenesis of rheumatoid arthritis by promoting the production of proinflammatory IL-17, IL-6 and TNF and also by increasing the number of IL-17–producing cells. It seems that inflammation is involved in sporadic colon carcinoma [21]. In the current study, the patients with sporadic CRC were analyzed. Although there are many differences between colitis-associated and sporadic CRC, there is overlap in the mechanisms that drive both conditions [21]. On the basis of the above findings, we speculate that one of the possible mechanisms that underlie Galectin-3 dependent modulation of CRC progression, is based on the proinflammatory properties of Galectin-3 to upregulate the production of IL-17. The importance of IL-23 activity in sporadic CRC is not clear, although it could involve the effects of IL-23 on the maintenance of Th17 immune response. Most reports suggest that IL-17 is an important modulator of CRC initiation and progression [53,54].

In summary, the presented data support the role of Galectin-1 and Galectin-3 in the progression of CRC. Firstly, we showed that increased serum and peritumoral Galectin-1 levels are significantly linked to perineural invasion, lymph node involvement and distant metastases of CRC. Secondly, simultaneous elevated concentrations of Galectin-1 and CEA in the sera of CRC patients indicate the possibility of their cooperation during tumor metastasis. Thirdly, it appears that Galectin-3 is involved in inflammation during CRC progression by increasing the production of proinflammatory IL-17. Further studies are necessary to clarify the role of Galectin-3 in IL-17 dependent inflammation during CRC progression.

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Conflict of interests

The authors declare no confict of interests.

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