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The levels of sICAM-1, sELAM-1, TNFα and sTNFR1 proteins in patients with colorectal adenocarcinoma in tumor and corresponding normal mucosa

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Colorectal cancer is a common malign disease of the gastrointestinal tract. The cancer survival rate depends on the stage of the disease at detection time. It is well known that several molecular mechanisms are involved in cancer and some molecules might affect or modulate cancerogenesis. The aim of the study was to assess the levels of sICAM-1, sELAM-1, TNFα and sTNFR1 protein in tumor and corresponding normal mucosa in a group of patients with colorectal adenocarcinoma and also associations of these parameters with demographic and clinical profiles of the patients. Tissue specimens were obtained during resection of neoplastic lesions. Protein levels were assayed in tissue homogenates by ELISA. The protein level of sICAM-1 in tumor was significantly increased in comparison to the corresponding normal mucosa (80.06 ng/mg *vs* 69.53 ng/mg, p=0.02). Furthermore, a significant positive correlation between sICAM-1 and sTNFR1 proteins levels in tumor (r_s=0.58, p<0.001) and in corresponding normal mucosa (r_s=0.48, p<0.001) was found. Also, significant correlations in corresponding normal mucosa were found between sELAM-1 and sICAM-1 (r_s=0.58, p<0.001) and between sTNFR1 and sELAM-1 (r_s=0.57, p<0.001). Significantly higher level of sTNFR1 in corresponding normal mucosa samples of patients with distant metastases was observed (p=0.04). Obtained results suggest that sICAM-1 protein could be considered as colorectal cancer marker. Furthermore, sTNFR1 also has the potential to become a good prognostic marker used during monitoring of the patients. Nevertheless, a further study in this area to confirm this correlation is required.

Key words: sICAM-1, sELAM-1, TNFα, sTNFR1, proteins, colorectal cancer

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Abbreviations: CAMs, cell adhesion molecules; CD62E, CD62 antigen-like family member E; CRC, colorectal cancer; ELAM-1, endothelial-leukocyte adhesion molecule-1; ELISA, Enzyme-Linked Immunosorbent Assay; ICAM-1, intercellular adhesion molecule-1; ICAMs, intercellular adhesion molecules; IL-1β, interleukin-1β; LECAM2, leukocyte-endothelial cell adhesion molecule 2; NAFs, normal tissue-associated fibroblasts; sELAM-1, soluble endothelial-leukocyte adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; sTNFR1, soluble tumor-necrosis factor receptor 1; TAFs, tumor-associated fibroblasts; TNFα, tumor necrosis factor alfa; TNFR1, tumor-necrosis factor receptor 1; TNFR2, tumor-necrosis factor receptor 2

INTRODUCTION

Colorectal cancer (CRC) is a common malign disease of the colon and rectum (Labianca *et al.*, 2010). In 2002, almost 530 000 death cases of colorectal cancer were reported, which made up to about 8% of all deaths from cancer, although generally the mortality rate in the western countries was declining. Ten years later, the number of deaths was already 700 000. According to predictions, in 2030 the number of deaths will increase to 1 100 000 (Arnold *et al.*, 2017). The cancer survival rate depends strongly on the stage of the disease at detection time. 90% of patients with localized stage survive 5 years, but only 10% with remote metastases manage to survive this period (Haggar & Boushey, 2009). Factors believed to be involved in the risk of developing colorectal cancer can be divided into modifiable ones, such as diet, obesity, lack of physical activity, tobacco use, moderate-to-heavy alcohol use and non-modifiable ones, such as personal or familial history of colorectal polyps or CRC, hereditary conditions such as Lynch syndrome, a personal history of inflammatory bowel disease, racial and ethnic backgrounds, and the presence of type 2 diabetes. Moreover, a significant risk factor is age, since 90% of new cases are reported in individuals over 50 years old (Simon, 2016). It is well known that several molecular mechanisms are involved in cancer development and metastasis. A lot of studies indicate that the following molecules may influence the process of carcinogenesis: intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule-1 (ELAM-1), tumor necrosis factor alfa (TNF-α) and tumor-necrosis factor receptor 1 (TNFR1) (Sawada *et al.*, 1994; Wang & Yong, 2008).

Intercellular adhesion molecules (ICAMs, also referred to as CD45) are cellular ligands for leukocyte β_2-integrins and are responsible for intracellular communication and immune reactions (Gahmberg *et al.*, 1997). There are five already identified representatives of ICAMs which differ in their distribution among various cell types (Gahmberg *et al.*, 1997). ICAM-1 is expressed constitutively at low levels on the vascular endothelium and on some lymphocytes and monocytes. It was found also in other non-hematopoietic cells (Hubbard & Rothlein, 2000; Huang *et al.*, 2004). Up-regulation of ICAM-1 gene takes place in response to such pro-inflammatory cytokines as

IL-1β, TNF-α and IFN-γ (Myers *et al.*, 1992; Huang *et al.*, 2004). A lot of studies showed a significant impact of ICAM-1 on CRC development (Wimmenauer *et al.*, 1997; Alexiou *et al.*, 2001; Maeda *et al.*, 2002; Taglia *et al.*, 2007; Mantur *et al.*, 2009).

ELAM-1 (endothelial-leukocyte adhesion molecule-1), also known as E-selectin, CD62E (CD62 antigen-like family member E) or LECAM2 (leukocyte-endothelial cell adhesion molecule 2), is one of cell adhesion molecules (CAMs) being expressed on inflammatory-activated endothelial cells. E-selectin is responsible for cell adhesion, taking part in recruiting neutrophils, monocytes, and memory T cells to the site of injury (Bevilacqua *et al.*, 1987; Banks *et al.*, 1993, Muraki *et al.*, 1996). ELAM-1 expression is induced by TNF-α or IL-1β and increased further by γ-interferon (Banks *et al.*, 1993). Elevated E-selectin levels have been reported in many tumor types, especially in solid tumors with co-existing ulceration (Banks *et al.*, 1993; Muraki *et al.*, 1996).

TNFα (tumor necrosis factor alfa) also called cachexin or cachectin is a cytokine regulating immune cell survival and function via NF-ϰB and MAPK pathways and produced by many types of cells, including activated macrophages and cancer tissue itself (Zins *et al.*, 2007; Hnatyszyn *et al.*, 2019). It exerts cytotoxicity on many lines of tumor cells, inhibits tumorigenesis and promotes immunological response (Wajant *et al.*, 2003). TNFα has the ability to bind two receptors: TNFR1 (called also TNF receptor type 1, TNF-R, TNF-R-I, CD120a, p55/60) and TNFR2 (TNF receptor type 2, TNF-R-II, CD120b, p75/80). TNFR1 is proved to be expressed constitutively in most tissues, whereas TNFR2 is found mostly in immune system cells. The wide range of TNF-α functions can be explained by the presence of these receptors on nearly all cell types (Zins *et al.*, 2007; Hnatyszyn *et al.*, 2019). TNFR can be found on most human cells and is considered the receptor through which most of the pro-inflammatory effects are elicited. The interaction between TNF-α and TNFR1 triggers important signaling pathways inducing diverse cellular phenomena including inflammation, apoptosis, etc. (Wajant *et al.*, 2003). Soluble forms of TNFR1 and TNFR2, which are proteolytically cleaved from extracellular domains, have TNFα-neutralizing capacity, since they can bind with the ligand and thus counteract biding of TNFα to the cellular receptors (Van Zee *et al.,* 1992).

ICAM-1, ELAM-1 and TNFR1 exist primarily in cell membrane-bound forms where they act mostly as aiders of inflammatory process and help leukocytes infiltrate from the blood stream into the inflammation site (Bevilacqua *et al.*, 1987; Banks *et al.*, 1993; Wajant *et al.*, 2003; Taglia *et al.*, 2007). However, apart from their membrane-bound forms, these molecules have their soluble forms that are present in the serum. Paradoxically, these soluble forms were proven to disrupt the process of leukocytes infiltration as they bind into the same ligands and prevent leukocytes from migrating into the targeted tissue. Thus, instead of impairing cancer development and subsequent metastasis they actually help with this process (Lauri *et al.*, 1991; Van Zee *et al.,* 1992; Sawada *et al.*, 1994; Izumi *et al.*, 1995; Kitagawa *et al.*, 1998; Alexiou *et al.*, 2001; Kang *et al.*, 2005; Mantur *et al.*, 2009). Therefore, the aim of the study was assessing the protein levels of sICAM-1, sELAM-1, TNFα and sTNFR1 in tumor and corresponding normal mucosa in a group of patients with colorectal adenocarcinoma. We also evaluated the association between mentioned protein levels and demographic and clinical factors; such as age, gender, tumor location and size, stage of the tumor according to Duke's staging system, grade (G), nodal metastasis and distant metastasis, and 5-year survival rate.

MATERIALS AND METHODS

Patients and samples. The study group included 47 patients aged 26–82 years (the average 65 years) with a preoperative diagnosis of colorectal cancer based on imaging studies and histopathological examination of specimens. Women constituted 43% (N=20) of the study group, and men 57% (N=27). Tobacco smokers, patients with gastro-intestinal tract, pancreatic and liver diseases, diabetes, lipid metabolism disorders and acute infections were excluded from the research population. Hereditary and family factors associated with the development of colorectal cancer were also excluded. Patients did not receive any pre-operative radio-chemotherapy.

Tissue specimens from both tumor and corresponding normal mucosa, were obtained during the resection of neoplastic lesions in the Clinic of Oncological and Reconstructive Surgery of the Maria Sklodowska–Curie Memorial Cancer Centre & Institute of Oncology, Gliwice Branch, Poland. A corresponding normal mucosa was obtained from a distal segment of resected colon, in a distance of at least 2 cm from the tumor. The mean distance of distal corresponding normal mucosa was 4.49 cm. The specimens were placed on ice and forthwith transported to the laboratory where they were washed twice with cold 0.9% NaCl solution. Subsequently, fragments were frozen at –80°C.

Collected during resection second fragments were examined histopathologically. Histopathological evaluation of the samples was conducted at the Tumor Pathology Department of the Maria Sklodowska–Curie Memorial Cancer Centre & Institute of Oncology, Gliwice Branch, Poland. The presence of colorectal adenocarcinoma was histopathologically confirmed, while the presence of neoplastic cells in the distal corresponding normal mucosa was excluded. In 79% of cases (N=37) the tumor was found in the distal section of the colon, while in 21% of individuals (N=10) tumors were located in the proximal section (the borderline was set at the splenic flexure). Average tumor size in the study group was 4.67 cm. Patients were divided into two subgroups depending on the stage of the tumor according to Duke's scale. The first subgroup consisted of 27 (57%) patients with A and B Duke's stages; the second subgroup consisted of 20 (43%) patients with C and D Duke's stages. Histopathological evaluation revealed that 11 (24%) cases had well differentiated (G1) cancer, 33 (70%) cases had moderately differentiated (G2) cancer, and 3 (6%) poorly differentiated (G3) cancer. The examination did not reveal the presence of any lymphocytic infiltration in the area surrounding the tumors, thus ruling out local inflammation. The research project was approved by the Bioethics Committee of the Maria Sklodowska–Curie Memorial Cancer Centre & Institute of Oncology, Gliwice Branch at the meetings of the Committee Nos. D0/DGP/493-10/05 and KB/493-54/07. All patients familiarized themselves with the protocol and signed informed consent document to participate in the study.

Tissue homogenization and total protein concentration. Fragments of tumor and corresponding normal mucosa were weighted and homogenized using PRO 200 homogenizer (PRO Scientific Inc., USA) at 10000 rpm in nine volumes of PBS (BIOMED, Poland) containing 0.5% Triton® X-100 (Sigma-Aldrich®, USA). Secondly, the suspensions were sonicated with an ultrasonic cell

Table 1. The levels of sICAM-1, sELAM-1, TNFα and sTNFR1 proteins in tissue homogenates from tumor and corresponding normal mucosa in a group of patients with colorectal cancer.
Significant correlations are given in bold.

Protein level	Tumor Me (Q1-Q3)	Corresponding normal mucosa Me (Q1-Q3)	*p* value
sICAM-1 (ng/mg protein)	80.06 (50.99–146.20)	69.53 (38.62–99.39)	**0.02**
sELAM-1 (ng/mg protein)	3.18 (2.71–4.62)	3.19 (2.16–5.34)	0.81
TNFα (pg/mg protein)	7.16 (4.39–10.29)	6.26 (3.78–8.26)	0.24
sTNFR1 (ng/mg protein)	0.76 (0.53–1.21)	0.76 (0.59–1.10)	0.85

Me stands for median, Q1 stands for lower quartile, Q3 stands for upper quartile

disrupter (UP 100, Hilscher, Germany). Subsequently, the homogenates were centrifuged at 12000 rpm for 15 minutes at +4°C. The total protein concentration was determined using pyrogallol-red method by reagent kit for direct colorimetric measurements of total protein (Sentinel Diagnostics, Italy). Readings were taken at 600 nm wavelength at 37°C using Technicon RA-XT™ biochemical analyzer (Technicon Instruments Corporation, USA).

Enzyme-Linked Immunosorbent Assay (ELISA). Protein levels were assayed in homogenates by Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's procedure. The sICAM-1 and sELAM-1 (CD62E) levels were assayed by the Diaclone ELISA Kit (France), the TNFα level by the Immundiagnostik TNFα ELISA Kit (Germany), and sTNFR1 level by human sTNFR1 (60kDa) ELISA BMS203CE (Bender MedSystems, Austria). Absorbance readings were obtained with ELISA PowerWave XS™ (BioTek®, USA) at 450 nm wavelength and calibrated according to the standard curve in ng/ml (TNFα in pg/ml). The obtained results were recalculated to the corresponding total protein concentration. All samples were analyzed in duplicates.

Statistical analysis. Statistical analysis was performed using STATISTICA v. 13.36.0 (StatSoft, Cracow, Poland). Statistical significance was set at a *p*-value below 0.05. Data was presented as mean value ± standard deviation in case of normal distribution and with median and lower/upper quartile in other cases. Distribution of variables was evaluated by the Shapiro-Wilk's test, and homogeneity of variances was assessed by the Levene's test. In case of skewed data distribution, logarithmic transformation was done before analysis. Categorical variables were compared using χ^2 test. T-Student test for normal data distribution and U Mann-Whitney test for other type of data distribution were done for comparison of quantitative data. Dependable values were compared by *t*-Student or Wilcoxon test depending on the data distribution. The assessment of association between clinical status and protein levels was done with the multivariable linear regression and the backward-stepwise procedure. Spearman's rank correlation coefficient (r_s) was calculated to evaluate association between examined parameters.

RESULTS

The results of comparison of protein levels between the tumor and corresponding normal mucosa are presented in Table 1. The protein level of the adhesion molecule sICAM-1 was significantly increased in tumor samples with the median value of 80.06 ng/mg against 69.53 ng/mg in the corresponding normal mucosa samples, (p=0.02; Table 1).

Significant positive correlation between sICAM-1 and sTNFR1 proteins levels was found in tumors and in corresponding normal mucosa (r_s=0.58, p<0.001, r_s=0.48, p<0.001; respectively, Table 2 and Table 3). Furthermore, significant positive correlations between sICAM-1 and sELAM-1 (r_s=0.58, p<0.001) and sTNFR1 and sELAM-1 (r_s=0.57, p<0.001) were found in the corresponding normal mucosa (Table 3).

No association was found between levels of selected proteins and demographic and clinical parameters; such as age, gender, tumor location and size, stage of the tumor according to Duke's staging system, grade (G), nodal metastasis and distant metastasis, and 5-year survival rate, with the exception of sTNFR1. It was found that patients with distant metastases had significantly higher sTNFR1 level in corresponding normal mucosa in comparison to patients without distant metastases [1.22 (0.76–1.49) *vs* 0.65 (0.52–1.03); p=0.04, Mann-Whitney U-test].

DISCUSSION

Many molecular events involving a process of initiation and formation of colorectal cancer have already been well described. They comprehend primarily genetic and epigenetic alterations that activate oncogenes and inactivate tumor suppressor genes. The most common gene expression changes seen in colorectal cancer include genes involved in the following signaling pathways: WNT signaling, MAPK signaling, PI3K signaling, TGFβ signaling, and p53 signaling. It is also postulated that gene mutations result in the formation of cancer stem cells, which are essential for initiation and maintenance of a tumor (Kuipers *et al.*, 2015). A detailed analysis that is supposed to be used for prediction of tumor progression ability has to include not only analysis of tumor cell

Table 2. Spearman rank correlation coefficient (r_s) values for sICAM-1, sELAM-1, TNFα and sTNFR1 protein levels in tumors.
Significant correlations are given in bold.

Variable	sICAM-1	sELAM-1	TNFα	sTNFR1
sICAM-1	1.00	0.36	-0.02	**0.58**
sELAM-1	0.36	1.00	0.06	0.22
TNFα	–0.02	0.06	1.00	–0.11
sTNFR1	**0.58**	0.22	–0.11	1.00

Table 3. Spearman rank correlation coefficient (r_s) values for sICAM-1, sELAM-1, TNFα and sTNFR1 protein levels in corresponding normal mucosa.
Significant correlations are given in bold.

Variable	sICAM-1	sELAM-1	TNFα	sTNFR1
sICAM-1	1.00	**0.58**	0.20	**0.48**
sELAM-1	**0.58**	1.00	0.32	**0.57**
TNFα	0.20	0.32	1.00	0.37
sTNFR1	**0.48**	**0.57**	0.37	1.00

phenotype, but also tumor microenvironment-related information. Inflammation is a one of the important factors in the development and progression of a cancer. Tumor-infiltrating inflammatory cells secrete a variety of proteins that can induce growth-promoting and angiogenesis-promoting factors (Galon *et al.*, 2014; Kuipers *et al.*, 2015). An extensive body of research works underlines the crucial influence of a host immune system on colorectal cancer development (Pages *et al.*, 2005; Galon *et al.*, 2006; Galon *et al.*, 2007; Pages *et al.*, 2009; Mlecnik *et al.*, 2011; Angell & Galon, 2013).

A lot of prognostic molecular and cellular biomarkers of colorectal cancer have already been proposed and tested, such as microsatellite instability; LINE-1 hypomethylation; mutations of *BRAF*, *KRAS*, and *APC* genes; characterization of $CD3^+$, $CD8^+$ and $CD45RO^+$ tumor infiltrating cells (Galon *et al.*, 2014; Mármol *et al.*, 2017), as well as many adhesion molecules such as vascular endothelial growth factor, E-cadherin, CD24, CD44, osteopontin and epithelial cell adhesion molecule (Broll *et al.*, 2001; Chai *et al.*, 2015; Seo *et al.*, 2015). In our study, we aimed to assess the utility of the following protein levels as prognostic factors: sICAM-1, sELAM-1, TNFα, sTNFR1. Colorectal cancer is associated with inflammatory process and other anti-tumor reactions not only in the tumor but also in the surrounding tissue, called corresponding normal mucosa (Wittig *et al.*, 1997; Maurer *et al.*, 1998). Therefore, we decided to compare levels of these potential prognostic proteins both in tumor and surrounding tissue. The interaction between inflammatory processes and the development of malignancy seems to be connected with the soluble forms of the studied molecules, as well as molecular mechanisms involved in cancer metastasis (Wittig *et al.*, 1997; Maurer *et al.*, 1998). Due to a low number of corresponding studies, various approaches and methodology used in other research works, we decided to compare our results also with studies presenting other cases (other biological material and other types of cancer) yet, still focused on proteins of our interest (sICAM-1, sELAM-1, sTNFα and sTNFR1).

In our report, we have found elevated sICAM-1 protein level in the colorectal cancer tissue in comparison with the corresponding normal mucosa sample. Up-regulation of ICAM-1 in cancer tissue has already been described by many authors. Schellerer and others (Schellerer *et al.,* 2014) compared an expression pattern of tumor-associated fibroblasts (TAFs) and normal tissue-associated fibroblasts (NAFs) isolated respectively from colorectal cancer and healthy colorectal tissue in terms of ICAM-1 expression. The number of ICAM-1-positive fibroblasts was significantly higher in TAFs than in NAFs. Moreover, interleukin-1β (IL-1β) stimulation resulted in significantly greater increase of ICAM-1-positive cells in TAFs group when compared to NAFs. Furthermore, isolated TAFs displayed a higher than NAFs adhesion capacity for tested monocytic cells. To confirm increased expression of ICAM-1 in original tumor tissue, Schellerer and others (Schellerer *et al.,* 2014) conducted also immunohistochemical staining which indicated significant margin of ICAM-1-positive fibroblast residing tumor environment when compared to normal mucosa. Presented findings and our results support the hypothesis that inflammation process (detected here by increased ICAM-1 expression) fosters host's reaction against the tumor by adhesion of infiltrating antitumor immune cells. The presence of peritumoral inflammation in colon carcinoma was shown to be positively correlated with the expression of ICAM-1. Moreover, Kelly and others (Kelly *et al.,* 1992) concluded that ICAM-1 might have played a role in leukocyte trafficking and epithelium-leukocytes interplay (Kelly *et al.*, 1992). It was confirmed by Maurerand others (Maurer *et al.,* 1998), who also found that the presence of ICAM-1 in small blood vessels and matrix of the tissue within colorectal cancer could favor extravasation and adhesion of cytotoxic lymphocytes to neoplasmal cells which was responsible for anti-tumor reaction in the host. Furthermore, ICAM-1-positive fibroblasts may stabilize the tumor and thus, reduce its progression (Schellerer *et al.*, 2014). A few reports have already showed that increased membrane-bound ICAM-1 was correlated with decreased lymph node metastases and better differentiated tumors as well (Wimmenauer *et al.*, 1997; Maeda *et al.*, 2002; Taglia *et al.*, 2007). Yang's *et al.* (2015) study indicates that a decrease in ICAM-1 mRNA expression may influence the malignancy and aggressiveness of the colorectal cancer. The above presented findings may suggest a dependence of ICAM-1 expression on a tumorigenesis process and the protein itself might be used as an indicator to predict the course of that process.

Membrane-bound ICAM-1 is believed to impair metastasis *via* preventing cells from detaching from the primary tumor. Moreover, membrane-bound ICAM-1 expression by cancer cells in CRC correlates positively with tumor differentiation level (Taglia *et al.*, 2007). On the other hand, many researchers indicate the important role of soluble form of ICAM-1 (sICAM-1). The level of sICAM-1 is significantly positively correlated with tumor stage and the appearance of metastasis (Kitagawa *et al.*, 1998; Alexiou *et al.*, 2001; Kang *et al.*, 2005; Mantur *et al.*, 2009). The mechanisms involved in sICAM-1 formation have not been completely elucidated. It is proposed that sICAM-1 may be generated by proteolytic cleavage from membrane-bound ICAM-1, although several studies demonstrated the presence of specific mRNA transcripts coding for sICAM-1 in cells (Wakatsuki *et al.*, 1995; Champagne *et al.*, 1998; Lyons & Benveniste, 1998; Witkowska & Borawska, 2004). Interesting results were showed by Maruo and others (Maruo *et al.,* 2002) who studied ICAM-1 expression and the level of soluble ICAM-1 (sICAM-1) in gastric patients at different stages of the disease. They reported that the serum ICAM-1 level of gastric cancer patients was significantly higher

than that of healthy volunteers. They also found that the sICAM-1 level was significantly higher in patients with liver metastasis than in patients without liver metastasis. Such results suggested that ICAM-1 was overexpressed in cancer cells and subsequently released in a form of sICAM-1, which would then promote hematogenous metastasis by suppressing local anticancer immunity (Maruo *et al.,* 2002). What is more, Komatsu and others (Komatsu *et al.,* 1997) proved that plasma concentration of ICAM-1 does not accurately reflect the level of its expression on the cells. Nevertheless, the level of serum ICAM-1 seems to be a good diagnostic feature, as our studies confirm its significantly elevated levels in tumors in comparison to normal mucosa. The presence of elevated sICAM-1 may be related to disease-associated differences in the regulation of inflammatory process in colorectal cancer.

Another protein of our interest was sELAM-1. We did not find any significant difference between the level of sELAM-1 in the tumor and corresponding normal mucosa of colorectal cancer patients. A few studies showed relatively higher serum levels of E-selectin in colorectal cancer patients and suggested that the E-selectin-mediated binding of colorectal cancer cells to human endothelium correlates with tumor progression and the formation of blood-transferred metastases (Lauri *et al.*, 1991; Sawada *et al.*, 1994; Izumi *et al.*, 1995). In a report of Alexiou and others (Alexiou *et al.,* 2001), significantly higher ELAM-1 (as well as ICAM-1) serum level in colorectal cancer patients when compared to healthy individuals was showed. Moreover, E-selectin serum levels correlated positively with ICAM-1 serum levels, disease stage and the presence of both lymph node and visceral metastatic disease (Alexiou *et al.,* 2001). Another study by Wittig and others (Wittig *et al.,* 1997), who investigated sICAM-1 and sELAM-1 expression in tumor as well as nonmalignant cell lines, showed sICAM-1 upregulation as a response to sELAM-1 pretreatment in tumor cell lines, but not in related nonmalignant cells, indicating a tumor-specific mechanism. In our study, we also show a significant positive correlation between sICAM-1 and sELAM-1 (r_s=0.58, p<0.001) but only in normal mucosa. In the cancer tissue, the levels of both proteins display a positive correlation as well (r_s=0.36), however, statistical significance is missing. Interesting results were presented by Maurera and others (Maurer *et al.,* 1998) who showed elevated levels of ICAM-1, V-CAM-1 and ELAM-1 mRNA within colorectal cancer tissue compared to normal tissue. It was explained that elevated expression of ICAM-1 may be preventing cell–cell disruption and as a result the tumor dissemination. They also stated that elevated expression of ICAM-1 and VCAM-1 but not ELAM-1 might be favoring the host anti-tumor defense by trafficking of lymphocytes (Maurer *et al.*, 1998). The problem is that the study deals with the membrane-bound forms of these proteins, which makes it difficult to compare to our studies. Nevertheless, it confirms the complexity of ELAM-1 involvement in the process of carcinogenesis or metastasis.

The reason for discrepancies between the above presented literature data on the levels of ELAM-1 and our results might be explained by the study by Sawada and others (Sawada *et al.,* 1994), who found that not so much the concentration of ELAM-1 as the presence and expression of suitable ligands for this protein, such as sialyl Lewis x and LAMP molecules, are important for its activity. Another problem is that two different forms of this protein, soluble and membrane-bound, present quite different physiological activity (Wittig *et al.*, 1997). Therefore, the level of its expression might not be sufficient enough to be a good prognostic or diagnostic factor on its own. Our results indicate that the problem of ELAM-1 involvement is more complicated and needs to be studied further.

TNFα is known to trigger oncogenic signal pathways in epithelial cells regulating their growth and survival (Wang *et al.*, 2008). It displays a pro-tumorigenic activity in the development of colorectal cancer (Klampfer, 2011). Activation of TNFα provides, through the expression of ELAM-1 and a surface sialyl carbohydrate Lewis x and a, an adhesion of cancer cells to vascular endothelium. Subsequently, initial adhesion molecules, like ICAM and VCAM, deliver solid adhesion in a chronic inflammation during tumor-promoting process (Dianzani *et al.*, 2008). So far, few studies proved overexpression of TNFα in CRC tissues as well as a positive correlation between TNFα levels and the CRC progression and reduced patient survival. Obeed *et al.* (2014) confirmed a significantly higher expression of TNFα (at both mRNA and protein level) in colorectal cancer tissue in relation to adjacent normal tissue (N=30). Researchers showed a strong correlation between elevated TNFα expression and advanced tumor stages (stage III and IV). Just as in our study, all the patients having undergone any additional therapy collaterally to surgical treatment were excluded from the study (Obeed *et al.*, 2014). Zins and others (Zins *et al.,* 2007) confirmed there was a correlation between the expression of TNFα mRNA and the occurrence of colorectal cancer within the colon tissue. TNFα influenced many processes such as, cell death regulation, cell proliferation and inflammation. Despite the fact that TNFα is able to initiate apoptosis within the tumor, these capabilities are repeatedly deactivated. In some cases, TNFα stimulates the survival of cancer cells, and that property is known as tumor promoting. Moreover, an over-expressed TNFα can enhance the metastatic activity of tumor cell lines (Zins *et al.*, 2007).

TNF receptors (including TNFR1) can be found on most human cells and their interaction with TNFα presents wide range of effects (Wajant *et al.*, 2003; Zins *et al.*, 2007; Hnatyszyn *et al.*, 2019). TNFR1 is known to be responsible for tumor-suppressive activity of TNFα, whereas TNFR2 role seems to be quite different and still underestimated. It is found mostly on suppressive immune cells, including regulatory T cells and myeloid-derived suppressor cells and some tumor cells. Contrary to TNFR1, TNFR2 seems to be tumor-promoting instead of tumor-supressing factor (Wajant *et al.*, 2003; Sheng *et al.,* 2018). We are planning to include this protein, among others in further research and analyses. Equally important is soluble form of TNFR1, since its interaction with TNFα can give different effects to its cell-bound form (Van Zee *et al.,* 1992), thus sTNFR1 was another protein included in our analysis. We found significantly higher level of sTNFR1 in corresponding normal mucosa samples of patients with distant metastases. Significantly higher serum concentration of soluble TNFR1 in patients with colorectal adenomas in comparison with control group was found by Hosono and others (Hosono *et al.,* 2012) as a promising biomarker for that tumor. Moreover, it was also confirmed that TNFR1 presents high levels of diagnostic sensitivity and specificity in patients with colorectal cancer (Hosono *et al.*, 2012). A correlation between soluble forms of TNFR1 and ICAM-1 in tumor has been found in our studies, we have also found a significant correlation between these molecules also in the normal mucosa. Viac and others (Viac *et al.,* 1996) also found correlation between soluble forms of

TNFR1 and ICAM-1 in malignant melanoma. A strong positive correlation was found in the tumors, while it was weak in the normal mucosa. These correlations may suggest a physiological dependence of those molecules on one another and their participation in either tumorigenesis or cancer development. sELAM-1 might be involved as well, as positive significant correlations between this protein and both sTNFR1 and sICAM-1 were found in the corresponding normal mucosa. Most probably the development of cancer takes place when dynamic balance between the expression levels of these three proteins is disrupted. Moreover, sTNFR1 might be used as a prognostic factor of the severity of the disease and a possibility of metastases occurrence, as its level was found to be higher in patients with distant metastases. Nevertheless, further studies in this area to confirm these correlations are required.

Based on the results of our research the level of sICAM-1 molecule was significantly increased in tumors in comparison to normal mucosa. It might possibly be used as an additional indicator to help distinguish between the tumor and corresponding normal mucosa. It seems that none of the studied proteins can be an efficient marker on its own and when considering their levels only. In order to increase the efficiency of diagnosis and prognosis, the whole microenvironment should be analyzed. This would have to include the levels of not only soluble forms but also membrane-bound forms of these proteins, as well as their ligands (especially in the case of sELAM-1) and some additional proteins. Nevertheless, further studies are needed to confirm whether this course of action would be the right one and also to study further the involvement of these and similar proteins in the process of tumor formation, development and metastasis occurrence, as still our knowledge in this regard seems not efficient enough. The differences in the regulation and activity of the selected molecules can be factors in defining cancer but additional work is needed to determine the specificity of these potential biomarkers. Further studies would need to include, apart from already mentioned factors, such as levels of expression of both membrane-bound and soluble forms of the proteins and their ligands, also other proteins, such as VCAM-1 or TNFR2, comparative material from healthy individuals, as well as sample analysis from the same patients after tumor removal, bigger study group might be needed too.

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Authors' contributions

JKS – research concept and design; JKS, ŁK – collection and/or assembly of data; JKS, PC, JS, AJO – data analysis and interpretation; JKS, PC, BB, KG, JS – writing the article; AW, ZO – critical revision of the article. All authors – final approval of the article.

Competing interests

The authors declare that they have no competing interests.

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