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ORIGINAL ARTICLE

Distinct Expression Patterns of Two Tumor Necrosis Factor Superfamily Member 15 Gene Isoforms in Human Colon Cancer

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Abstract

Background Tumor necrosis factor superfamily member 15 (*TNFSF15*) gene is involved in development of several cancers. It encodes two proteins: tumor necrosis factor ligand-related molecule 1A (TL1A) and vascular endothelial growth inhibitor 192 (VEGI-192). The main receptor for TL1A is death receptor 3 (DR3).

Aims We investigated expression of *TL1A, VEGI-192,* and *DR3* transcripts in different stages of colon cancer and compared them with survival of patients. We also aimed to reveal possible effects of microsatellite instability (MSI) and selected *TNFSF15* single-nucleotide polymorphisms (SNPs) on expression of this gene.

Methods Forty-five healthy individuals and 95 colon cancer patients were included in the study. Expression of *VEGI-192, TL1A,* and *DR3* was measured by quantitative PCR. SNP and MSI analyses were performed on DNA isolated from normal or cancer tissue.

Results Expression of *VEGI-192* and *TL1A* was elevated in colon cancer, although the level of *VEGI-192* decreased, while the level of *TL1A* increased with the progression of cancer. Patients with low expression of *TL1A* and/or high expression of *VEGI-192* in tumor-transformed tissue showed longer survival. *DR3* expression was decreased in the cancer, but it did not change with the tumor progression. Alleles T of rs6478108 and G of rs6478109 SNPs were associated with elevated expression of the *TNFSF15* gene. There was no relation between the MSI status and *TNFSF15* expression levels.

Conclusions Expression of the *TNFSF15* gene isoforms was associated with the progression of colon cancer. Levels of *TL1A* and *VEGI-192* transcripts can be considered as independent prognostic factors for colon cancer.

Keywords TNFSF15 · TL1A · VEGI-192 · Colon cancer · Advanced gastric cancer

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Introduction

Colon cancer is currently the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths in the world. The global burden of colon cancer is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths in 2030 [1]. Development of colon cancer is a sequential process which on average takes around ten years and involves progression from small polyps through adenoma to carcinoma [2].

One of the genes which appears to be involved in colon cancer pathogenesis is *TNFSF15*. In humans, three different protein isoforms generated from the *TNFSF15* gene were identified—VEGI-174 (174 amino acids), VEGI-192 (192 amino acids), and the full-length product VEGI-251 (251 amino acids, also known as TL1A), although VEGI-174 is most probably a cloning artifact [3–5]. The main receptor for TL1A is death receptor 3 (DR3, TNFRFSF25), a death domain-containing member of the tumor necrosis factor receptor superfamily [3]. Expression of DR3 is present mostly on activated lymphocytes [6], although it has been detected also in human colon epithelium [7]. In humans, there are 11 splice variants of DR3, out of which the transcript variant 1 encodes the full-length transmembrane product that contains the death domain [6]. Studies by Migone et al. [3] and Metheny-Barlow et al. [4] suggest that VEGI-192 may utilize different receptor(s) or signaling pathways than TL1A and at least some effects of VEGI-192 may be induced independently of DR3 signaling. TL1A is a transmembrane or soluble pro-inflammatory molecule which co-stimulates proliferation and effector functions of T cells in the presence of TCR stimulation [6, 8]. Expression of TL1A is localized mostly to activated cells of the immune system—DCs, macrophages, T cells, whereas very little TL1A is present on non-activated immune cells [6]. VEGI-192 on the other hand is expressed mostly on endothelial cells, and its primary function is to inhibit angiogenesis via induction of apoptosis or growth arrest of endotheliocytes [9] or inhibition of endothelial progenitor cells differentiation [10]. Overexpression of VEGI-192 was shown to inhibit tumor neovascularization and progression in a mouse model of lung cancer [11]. In human cancers, higher expression of *VEGI-192* mRNA was found in early stages of clear-cell renal cell carcinoma (ccRCC) than in advanced stages of ccRCC. Furthermore, *VEGI-192* mRNA levels were negatively correlated with tumor histological differentiation grade [12] and epithelial–mesenchymal transition in renal tumor [13]. In human pituitary tumors, high levels of *VEGI-192* mRNA were associated with lower tumor grade and invading pituitary tumors were characterized by lower *VEGI-192* mRNA expression [14]. Currently, little is known about the expression pattern and exact function of TL1A in cancer, although mouse studies showed that ectopic expression of TL1A on tumor cells promotes tumor elimination in a CD8(+) T cell-dependent manner [15]. There is, however, a clear association between enhanced TL1A expression and development of certain autoimmune diseases such as psoriasis or inflammatory bowel disease (IBD) [6], and certain *TNFSF15* single-nucleotide polymorphisms are considered as gastric adenocarcinoma [16], IBD [17, 18], and psoriasis [19] risk factors.

Genetic diversity in colon cancers results from, among other factors, uneven replication of noncoding DNA regions, known as microsatellite instability (MSI) which is caused by defects in the DNA mismatch repair (MMR) system. MSI results in increased susceptibility to additional mutations [20] and may affect expression levels or activity of various genes [21].

Currently, there are no published studies on the expression and function of the *TNFSF15* gene in human colon cancer and despite the current state of knowledge, the exact molecular mechanism of colon cancer still remains unknown. Its precise characterization might lead to development of new, effective therapies. Therefore, in this study we investigated expression patterns of *TL1A, VEGI-192,* and *DR3* transcripts in tumor-transformed colon mucosa of patients with different stages of colon cancer and compared it with patients' outcome to determine whether these transcripts can be considered as prognostic factors for colon cancer and to gain a better insight into the role of TNFSF15 isoforms in this malignancy. Our study also aimed to reveal the effects of microsatellite instability and selected *TNFSF15* single-nucleotide polymorphisms (rs6478108 and rs6478109) on expression levels of this gene.

Methods

Patients

A total of 45 control subjects and 95 colon cancer patients were included in the study. Clinical characteristics of subjects included in the study are presented in Table 1. Tissue samples were collected in years 2014–2016. The control group included healthy participants who underwent screening colonoscopy, showed normal colonic mucosa, and had no history of inflammatory bowel diseases or bowel cancer. The control subjects were not treated with any anti-inflammatory or immunostimulatory drugs and were diagnosed at the Department of Gastroenterology and Hepatology (Medical University of Gdańsk, Poland). Colon mucosa biopsies were collected during routine, diagnostic, or surveillance colonoscopy. Colon cancer patients were diagnosed and treated in the Department of

Table 1 Clinical characteristics of patients included in the study

	Colon cancer patients	Control subjects
Total number (*n*)	95	45
Sex (*n*)	F (34)/M (61)	F (23)/M (22)
Age (years ± SD)	61 ± 10.75	52 ± 11.8
Clinical stage		
I	35	N/A
II	21	N/A
III	20	N/A
IV	19	N/A
Metastases to regional lymph nodes		
Absent (N0[a])	54	N/A
Present (N1 and N2[a])	41	N/A
Distant metastases		
Absent (M0[a])	76	N/A
Present (M1[a])	19	N/A

[a]According to the TNM classification

N/A not applicable

General, Endocrine and Transplant Surgery (Medical University of Gdańsk, Poland). Cancer tissue samples were collected during routine surgery, and all cases of colon cancer were confirmed by histopathological examination. The patients were not treated with radiotherapy and/or chemotherapy before the surgery and did not receive any anti-inflammatory or immunostimulatory drugs before the surgery. After the surgery, adjuvant therapy was used in 29 patients. This group involves 26 patients with stage III and IV. (The remaining 13 patients refused adjuvant chemotherapy or were disqualified for comorbidities or poor general performance status.) Three patients with clinical stage II received the adjuvant chemotherapy because of significant risk factors. Chemotherapy regimens were applied according to EURECCA guidelines used in our institution [22].

RNA Isolation and Reverse Transcription

Colon mucosa biopsies and colon cancer samples were immersed in RNA Later fluid (Sigma-Aldrich, Munich, Germany) and stored at − 80 °C until RNA isolation. Total RNA was extracted with RNA Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The extracted RNA was treated with TURBO DNA-free Kit (Ambion, Austin, TX, USA) to remove any possible contamination with genomic DNA. RNA concentration and 260 nm/280 nm absorbance ratio (average 1.95, range 1.90–2.02) were determined using Epoch spectrophotometer (BioTek, Potton, UK). RNA integrity (RIN) was assessed with Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). The average RIN value was 7.2 (range 5.1–9.4). Reverse transcription of 1 µg of total RNA extracted from each sample was carried out with RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, Fitchburg, WI, USA) according to the manufacturer's instructions. The obtained cDNA was stored at − 25 °C until further analyses.

Quantitative PCR

The primers used for quantitative PCR (qPCR) were designed and evaluated using NCBI database and Primer-BLAST software. To amplify cDNA target only and to avoid amplification of any possible genomic DNA contamination, we chose primers that span exon/exon junctions or primer pairs that were separated by at least one intron of more than 1000 bp size on the corresponding genomic DNA. Sequences of the primers, annealing temperatures, efficiency values of qPCR assays, and RefSeq accession numbers are presented in Table 2 [23]. qPCR reactions were performed in StepOnePlus instrument (Life Technologies, Grand Island,

Table 2 Sequences of primers used in the study

Gene (isoform)	Forward primer	Reverse primer	Annealing temperature (°C)	qPCR efficiency (%)	RefSeq accession number
IPO8	5′-TTGGAAGAAACCGCG CTTGAGG-3′	5′-ACCAGGCTGCATCTC GACTCTG-3′	59	98.75	NM_001190995
PPIA	5′-CTTGGGCCGCGTCTC CTTTGAG-3′	5′-GCTTGCCATCCAACC ACTCAGTC-3′	59	97.25	NM_001300981.1
TNFSF15 (TL1A)	5′-AAGGACAGGAGTTTG CACCTTCA-3′	5′-AAGTGCTGTGTGGGA GTTTGTCT-3′	62	98.56	NM_005118.3
TNFSF15 (VEGI-192)	5′-AAGGGCCGTCTTCAT TTCAGT-3′	5′-TCGGCCTGCTTGTCT GATTT-3′	60	97.25	NM_001204344.1
TNFRSF25 (transcript variant 1) [23]	5′-ATGGCGATGGCTGCG TGTCCTG-3′	5′-AGCGCCTCCTGGGTC TCGGGGTAG-3′	63	94.23	NM_148965.1

NY, USA). Each reaction mixture contained 1 µL of undiluted cDNA, 0.2 µM of forward and reverse primers, 10 µL of SensiFast No-Rox Kit (Bioline, London, UK; containing Sybr® Green fluorophore, Taq DNA polymerase, and a mixture of deoxynucleotides), and water to 20 µL. Conditions of qPCR reactions were as follows: denaturation for 2 min at 95 °C followed by 40 cycles of denaturation for 5 s at 95 °C, annealing for 10 s at 59–63 °C, and an extension step (with fluorescence reading) for 20 s at 72 °C. The presence of a single, specific PCR product in each reaction was confirmed by melting curve analysis. All qPCR reactions were performed in triplicates, and the geometric means of Ct values were used for data analysis. Gene expression data were normalized to mean expression levels of *PPIA* and *IPO8* genes which showed the highest stability in the tested biological samples among other evaluated housekeeping genes, including *HPRT1, GAPDH, RPLP0,* beta-2-microglobulin, and beta-actin (own data).

Single-Nucleotide Polymorphism Analysis

Genomic DNA from normal colon tissue collected from colon cancer patients during tumor resection was extracted using Extractme® DNA Tissue Kit (Blirt, Gdańsk, Poland) in accordance with the manufacturer's instructions. Single-nucleotide polymorphism (SNP) genotyping was carried out with TaqMan allelic discrimination assay from Applied Biosystems (Foster City, CA, USA). The SNP assays were also purchased from Applied Biosystems. Every reaction in a 96-well plate was conducted in the total volume of 25 µL per well, including 11.25 µL purified genomic DNA sample with DNase-free water (15 ng/µL extracted DNA per well), 12.5 µL TaqMan Universal PCR Master Mix (2×), and 1.25 µL SNP Genotyping Assay (20×). All of the samples were analyzed in duplicates. A negative control was included in each assay. The amplification protocol consisted of 10 min at 95 °C and 40 cycles of amplification (95 °C for 15 s and 60 °C for 60 s). All TaqMan real-time PCR runs were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the StepOne™ Software v 2.2.2.

Microsatellite Instability Analysis

Microsatellite instability (MSI) status was determined according to the National Cancer Institute Workshop and Familial Predispositions and was based on polymorphism analysis of five markers: BAT26 and D2S123 for MSH2, BAT25 for the c-kit oncogene, APC-D5S346 for APC gene, and MFd15 D17S250 for BRCA1 gene [24]. MSI status was assessed with the use of high-resolution melting qPCR according to [25] with certain modifications. The referenced [26] primers were purchased from Sigma-Aldrich (Sigma-Aldrich, Munich, Germany). DNA of 15 ng extracted from paired tumor and normal colon was used for PCR amplification with SensiFast HRM (BioLine, London, UK) and 200 nM each primer in the final volume of 10 µl. All reactions were run in duplicates. First, the reactions were run in qPCR 8-tube stripes (4titude, Wotton, UK) in MyGo Pro Real-Time PCR System (IT-IS International Ltd., Stokesley, UK) at the following conditions: 95 °C for 3 min, 35 cycles of 95 °C for 10 s, 51 °C for 10 s and 72 °C for 15 s (fluorescence reading). HRM profile: from 60 °C to 97 °C with fluorescence reading every 0.05 °C. MSI pattern was assessed by auto high-resolution melt with the use of MyGo Pro PCR Software ver. 3.3 (IT-IS International). Furthermore, HRM analysis was duplicated (by transferring the PCR stripes with PCR products) at StepOnePlus apparatus (Life Technologies—Applied Biosystems, Grand Island, NY, USA) with the following profile: from 60 to 95 °C with fluorescence reading every 0.2 °C. HRM analysis at StepOnePlus apparatus was assessed by high-resolution Melting software ver. 3.0 (Life Technologies—Applied Biosystems) using manual of auto alignment of melt curves. The occurrence of microsatellite instability was noted if the additional melting spike appeared in tumor samples. If the results of analyses at MyGo and StepOnePlus were different, the PCR reaction was repeated. If the MSI occurred at 1–2 markers, low-grade MSI (MSI-L) was noted; if the mutation was observed at 2–5 markers, high-grade MSI (MSI-H) was acknowledged.

Data Analysis

For relative gene expression, Ct values of the tested genes were first normalized to mean Ct values of reference genes (*IPO8* and *PPIA*) (ΔCt) and $2^{-\Delta Ct}$ values of individual patients in colon cancer groups were then normalized to the average $2^{-\Delta Ct}$ value in control patients. Multivariate analysis was performed with Cox proportional hazards regression model. All statistical calculations were performed with Prism 6 software (GraphPad, San Diego, CA, USA) or XLSTAT (Addinsoft, New York, USA). Normality of gene expression data was checked with D'Agostino and Pearson omnibus test and Mann–Whitney U test, Friedman's ANOVA with Bonferroni post hoc test or log-rank (Mantel–Cox) test were used to compare gene expression data. P values lower or equal to 0.05 were considered as statistically significant.

Results

TL1A and VEGI-192 but Not DR3 mRNA Expression Levels Change with the Progression of Colon Cancer

To investigate the expression pattern of *TL1A*, *VEGI-192,* and *DR3* in colon cancer, we measured the levels of their

transcripts in tumor-transformed colon mucosa and normal colon mucosa collected from healthy subjects. Initial analysis of gene expression showed that levels of *TL1A* and *VEGI-192* transcripts were significantly elevated in tumor-transformed tissue as compared to control samples (on average 6.54-fold and 54.99-fold, respectively; Fig. 1). Interestingly, the level of *DR3* transcript variant 1 was slightly but significantly lower in cancer tissue than in control colon mucosa (on average 0.70-fold; Fig. 1). Further analysis of gene expression data revealed that expression of *TL1A* mRNA increased with the progression of colon cancer. Compared to healthy control tissue, in colon cancer the level of *TL1A* transcript was on average 3.54-fold higher in stage I, 3.97-fold higher in stage II, 5.85-fold higher in stage III, and 11.21-fold higher in stage IV (Fig. 2a). Moreover, *TL1A* mRNA levels were significantly elevated in tumors that metastasized to regional lymph nodes (on average 2.22-fold higher than in tumors without metastases to regional lymph nodes; Fig. 2b) and formed distant metastases (on average 2.79-fold higher than in tumors without distant metastases; Fig. 2c). An opposite expression pattern of *VEGI-192* mRNA was observed in colon cancer tissue. Even though overall expression of *VEGI-192* transcript was higher in tumor-transformed tissue than in healthy colon mucosa, its expression decreased with the progression of colon cancer. Compared to healthy tissue, the level of *VEGI-192* transcript was on average 86.26-fold higher in stage I, 33.36-fold higher in stage II, 16.79-fold higher in stage III, and 14.17-fold higher in stage IV colon cancer (Fig. 2a). The expression of *VEGI-192* mRNA was significantly lower in tumors that formed metastases to regional lymph nodes (on average 0.22-fold; Fig. 2b) and distant metastases (on average 0.24-fold; Fig. 2c) than in tumors that did not form any metastases. Interestingly, expression of *DR3* transcript variant 1 did not change with the progression of colon cancer (Fig. 2a–c).

Expression Levels of TL1A and VEGI-192 mRNA Are Associated with Single-Nucleotide Polymorphisms rs6478108 and rs6478109

Our study showed that colon cancer patients with C/T and T/T genotypes of rs6478108 have higher expression of *TL1A* transcript than colon cancers with C/C genotype of rs6478108 (2.65-fold and 3.20-fold, respectively). Expression of *VEGI-192* transcript was 1.83-fold higher in colon cancer samples with T/T rs6478108 genotype as compared to C/T genotype, although we did not find any differences between samples with other genotypes (Fig. 3a). Also, colon cancers with A/G and G/G genotypes of rs6478109 had higher expression of *TL1A* mRNA than colon cancers with genotype A/A of the same SNP (2.72-fold and 3.01-fold, respectively). Colon cancers with genotype G/G of rs6478109 had 1.96-fold higher expression of *VEGI-192* transcript as compared to A/G genotype, and there were no significant differences in *VEGI-192* mRNA expression between other genotypes (Fig. 3b).

Microsatellite Instability Does Not Affect Levels of TL1A and VEGI-192 mRNA Expression in Patients with Colon Cancer

Since dysfunction of the DNA mismatch repair (MMR) system, leading to microsatellite instability (MSI), may result in altered expression levels of certain genes, such as *BAX, FAS,* and others [21], we decided to check whether MSI status can be associated with expression levels of the two isoforms of the *TNFSF15* gene. However, having compiled *VEGI-192* and *TL1A* mRNA expression data with the MSI status of the investigated patients, we did not find any correlation between the MSI status and expression levels of *VEGI-192* and *TL1A* transcripts (Fig. 4).

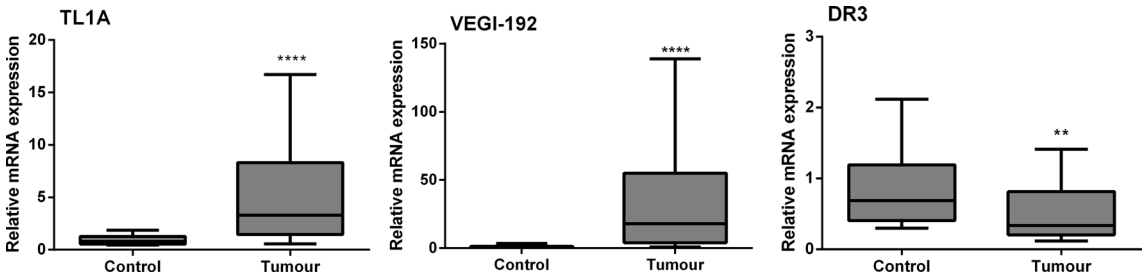


Fig. 1 Relative expression of TL1A, VEGI-192, and DR3 mRNA in colon cancer tissue. The data are presented as a fold change versus the mean value in the control healthy colon mucosa. $^{**}p = 0.005$; $^{****}p < 0.0001$. p values were calculated with Mann–Whitney U test. Box and whisker plots represent median values and 5–95 percentiles

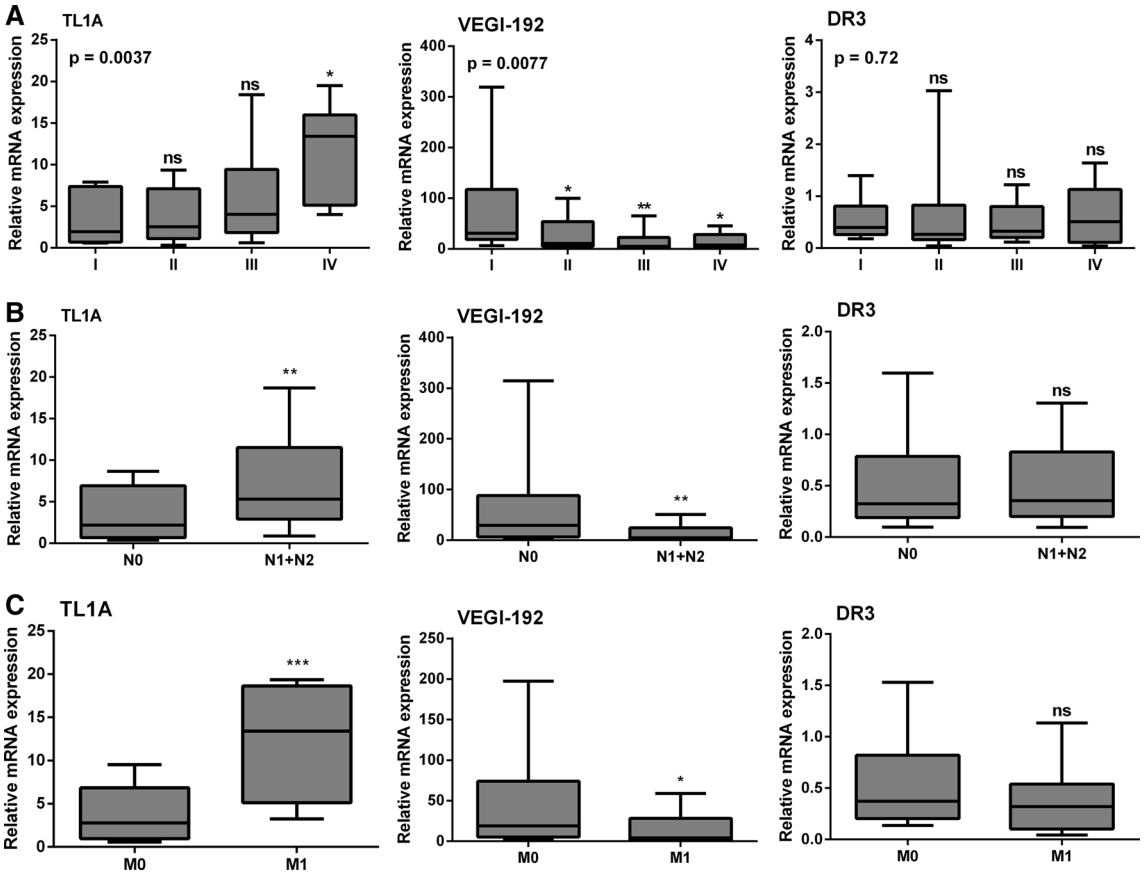
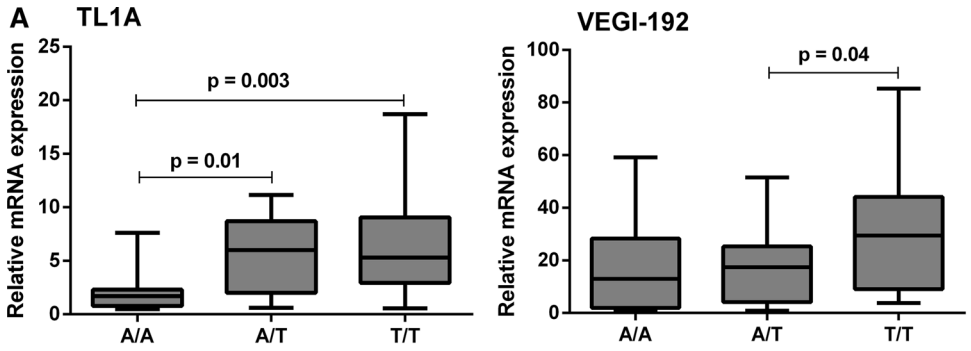


Fig. 2 Relative expression of TL1A, VEGI-192, and DR3 mRNA in different stages of colon cancer (**a**); in cancers with (N1 + N2) or without (N0) metastases to regional lymph nodes (**b**); and in cancer with (M1) or without (M0) distant metastases (c). The data are presented as a fold change versus the mean value in the control healthy colon mucosa. $*p = 0.01$; $**0.003 > p > 0.001$; $***p = 0.0001$; $^{ns}p \geq 0.05$. *p* values in (**a**) presented as alphanumeric values were calculated with Friedman's ANOVA with Bonferroni post hoc test; *p* values in (**a**) presented as symbols were calculated with Mann–Whitney U test versus group I. In (**b**, **c**), *p* values were calculated with Mann–Whitney *U* test. Box and whisker plots represent median values and 5–95 percentiles

Expression Levels of TL1A, VEGI-192, and DR3 mRNA Are Associated with Different Survival of Colon Cancer Patients

To investigate whether expression levels of *TL1A*, *VEGI-192,* and *DR3* transcripts have any relationship with survival of colon cancer patients, we grouped them as "high" and "low" depending on the mean expression value of a given transcript. Survival of the patients was monitored for 30 months. Patients with *TL1A* mRNA expression lower or equal to 6.54-fold increase over healthy controls ("low"; $n = 62$) showed higher survival rate than patients with high *TL1A* mRNA expression ($n = 33$). Patients with *DR3* mRNA expression lower or equal to 0.70-fold decrease under healthy controls ("low"; $n = 60$), and patients with *VEGI-192* mRNA expression lower or equal to 54.99-fold increase over healthy controls ("low"; $n = 69$) had lower survival rate than patients with high expression levels of the respective transcripts (DR3 high: $n = 35$; VEGI-192 high: $n = 26$) (Fig. 5). In the next step, we chose patients with both low expression of *TL1A* and high expression of *VEGI-192* mRNA in tumor tissue ($n = 13$) and compared their survival rate with patients with both high expression of *TL1A* and low expression of *VEGI-192* mRNA in tumor tissue ($n = 21$). The survival rate of the colon cancer patients with both low *TL1A* and high *VEGI-192* mRNA expression was significantly higher than of patients with both high *TL1A* and low *VEGI-192* mRNA expression (Fig. 5). Interestingly, we did not find any differences in survival rates of colon cancer patients with low *TL1A*, high *VEGI-192,* and high *DR3* mRNA expression ($n = 4$) and the patients with high *TL1A*, low *VEGI-192,* and low *DR3* mRNA expression ($n = 16$) (Fig. 5). Multivariate analysis, which included patients receiving and not receiving adjuvant therapy after the surgery, revealed that high expression of TL1A and low expression of VEGI-192, but not expression level of DR3, were independent risk factors in the investigated patients (Table 3).



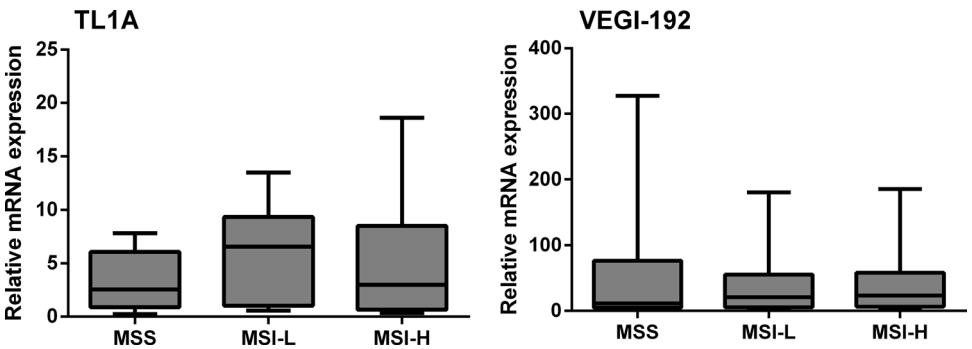


Fig. 3 Relative expression of TL1A and VEGI-192 mRNA in patients with different genotypes of rs6478108 (**a**) or rs6478109 (**b**) single-nucleotide polymorphisms. The data are presented as a fold change versus the mean value in the control healthy colon mucosa. *p* values were calculated with Mann–Whitney *U* test. Box and whisker plots represent median values and 5–95 percentiles

Fig. 4 Relative expression of TL1A and VEGI-192 mRNA in patients with stable microsatellites (MSS), low-grade microsatellite instability (MSI-L), or high-grade microsatellite instability (MSI-H) in colon cancer tissue. The data are presented as a fold change versus the mean value in the control healthy colon mucosa. Comparison of groups did not show any statistically significant differences ($p \geq 0.05$). *p* values were calculated with Mann–Whitney *U* test. Box and whisker plots represent median values and 5–95 percentiles

Discussion

Most of the previous studies on expression of the *TNFSF15* gene in human and animal malignancies did not differentiate between VEGI-192 and TL1A isoforms, and did not specify precisely enough which isoform was detected or focused on total expression of the *TNFSF15* gene. Therefore, it is difficult to compare the results obtained by our group with the studies of other researchers. This study is, to our best knowledge, the first one that reports that *VEGI-192* and *TL1A* transcripts have distinct expression patterns in human colon cancer and that their levels in tumor tissue are associated with tumor invasiveness. Expression levels of both *TL1A* and *VEGI-192* transcripts were significantly elevated in colon cancer tissue as compared to healthy colon mucosa; however, high *TL1A* mRNA expression seems to be linked to progression and invasiveness of colon cancer, whereas *VEGI-192* mRNA expression pattern is opposite. Moreover, colon cancer patients with low expression of *TL1A* and/or high expression of *VEGI-192* mRNA in tumor tissue showed longer survival. In our study, RNA was isolated from either colon mucosa (control subjects) or whole colon cancer mass (tumor patients). Therefore, it is difficult to determine exactly which cells were producers of TL1A, VEGI-192, and/or DR3, since the investigated tumor tissue fragments contained not only

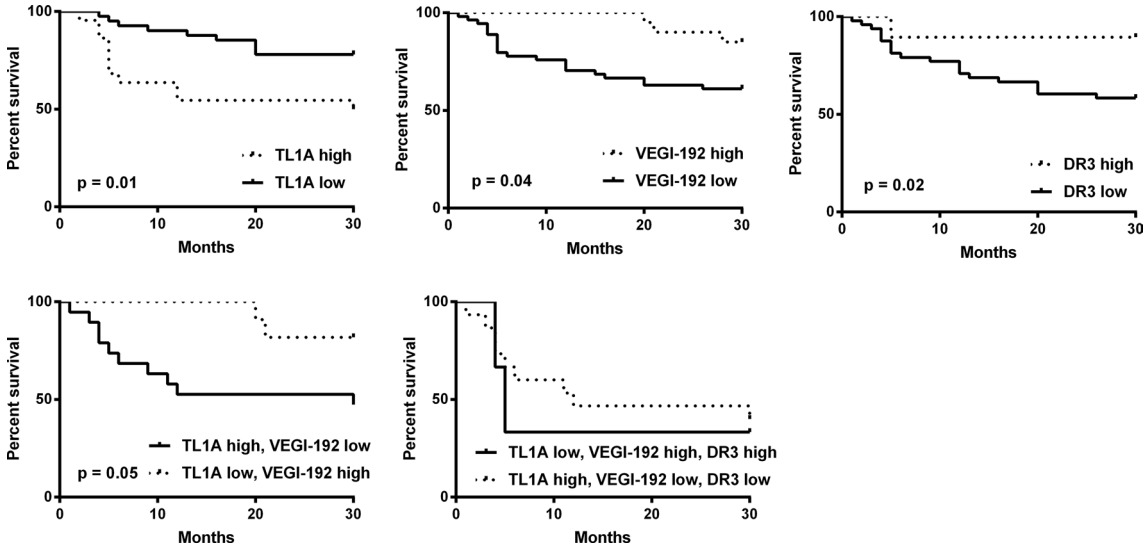


Fig. 5 Survival curves of patients with high and/or low expression of TL1A, VEGI-192, and DR3 mRNA. If no *p* values are presented on a graph, $p \geq 0.05$. *p* values were calculated with log-rank (Mantel–Cox) test

Table 3 Identification of independent prognostic factors by Cox multivariate analysis

Variable	HR (95% CI)	*p*
Age	1.060 (1.020–1.102)	0.003
Stage	2.037 (0.640–6.487)	0.229
N stage (N1 + N2 vs. N0)	0.549 (0.123–2.451)	0.432
M stage (M1 vs. M0)	4.159 (0.901–19.201)	0.068
TL1A expression (low vs. high)	0.420 (0.000–0.966)	0.041
VEGI-192 expression (low vs. high)	2.374 (1.081–5.215)	0.031
DR3 expression (low vs. high)	0.647 (0.279–1.498)	0.309
Adjuvant therapy (present vs. absent)	0.472 (0.171–1.301)	0.148

cancer cells but also cells of tumor stroma and infiltrating mononuclear cells of the immune system.

Our findings stand in agreement with studies conducted by Zhang et al. [12] who showed that levels of *VEGI-192* mRNA were higher in clear-cell renal cell carcinoma of earlier stages than in cancers of advanced stages. Interestingly, in the cited study [12] expression of *TL1A* transcript in cancer tissue samples was similar and that of *VEGI-192* transcript was lower than in normal tissue. This discrepancy is difficult to explain since the laboratory technique used by Zhang et al. [12] to measure levels of *TL1A* and *VEGI-192* transcripts (quantitative PCR) was similar to the method used by us and the only difference was the type of tumor. Therefore, we believe that this difference may be tumor dependent. The role of VEGI-192 in tumor progression appears to be associated with its anti-angiogenic function. The growth of solid tumors strictly depends on angiogenesis, and several studies demonstrated that the degree of tumor vascularity correlates positively with the disease stage and the likelihood of metastases formation [27]. Since VEGI-192 is a typical anti-angiogenic molecule [9], its decreased expression in advanced tumors may trigger formation of blood vessels in tumor tissue and in this way facilitate the progression of the disease.

TL1A, on the other hand, is a pro-inflammatory molecule that interacts with the death receptor 3 (DR3) which results in activation of the transcription factor NF-κB [28] and can boost anti-tumor immunity by mounting T cell immunity. Therefore, TL1A shows a high degree of functional similarity with tumor necrosis factor (TNF) signaling via TNF receptor 2 (TNFR2). It is well established that prolonged stimulation with pro-inflammatory molecules contributes to elevated risk of tumor transformation [29]. Indeed, TNF promotes tumor progression via induction of pro-inflammatory cytokines and matrix metalloproteinases [30], and elevated expression of TNF is associated with poor prognosis and cachexia [31]. Several authors suggest that the link between inflammation and cancer is the transcription factor NF-κB since its pathway promotes tumor growth due to the activation of anti-apoptotic genes in malignant cells and pro-inflammatory cytokines' expression by myeloid and lymphoid cells which in turn act on epithelial cells promoting their proliferation and survival [29, 32, 33]. Interestingly, inhibition of NF-κB activity at early stages of tumor did not affect tumor transformation of epithelial cells. Instead, it suppressed tumor growth and progression at advanced stages of cancer development [33]. This finding is supported by studies conducted by Moorchung et al. [34] who showed that in colon cancer, higher nuclear translocation of NF-κB was detected in lymphocytes infiltrating malignant rather

than benign tumors. In a recent article, Niu and colleagues showed in a mouse model of colitis-induced colon cancer that TL1A contributes to progression of this disease via activation of Wnt/β-catenin pathway [35]. These observations are consistent with our findings that elevated expression of TL1A transcript is associated with the progression of colon cancer in humans. Therefore, we speculate that this cytokine, similarly to TNF, may contribute to colon cancer development by enhancing inflammation in the tumor microenvironment and the balance between its protective and pro-tumorigenic function most probably depends on local concentration and time of stimulation.

At present, the role of DR3 in cancer development and progression still remains unclear. An in vitro study conducted on human colon cancer cell lines HT29 and LoVo showed that DR3 expressed by cancer cells binds to E-selectin present on human umbilical vein endotheliocytes (HUVEC) and acts as a metastasis trigger, allowing cancer cells to evade apoptosis and activate survival and migratory events [36]. Other authors reported that DR3 expression is reduced in hepatocellular carcinoma cell lines [37] and human breast cancer in which it also was associated with worse prognosis [38]. On the contrary, it was found that DR3 expression levels did not correlate with human pituitary tumor invasion [14]. To our best knowledge, expression of *DR3* mRNA levels in human colon cancer has not been studied to date. Even though our study showed that *DR3* mRNA levels were lower in colon cancer tissue than in control samples and colon cancer patients with high expression of *DR3* mRNA in colon cancer tissue showed longer survival rates, we did not find any association between *DR3* expression levels and progression of colon cancer. Due to very sparse available literature data concerning expression and role of DR3 in human cancers, currently it is very difficult to unambiguously define its function in cancer development.

There are many known single-nucleotide polymorphisms of the *TNFSF15* gene which are associated with inflammatory bowel diseases or certain types of cancer. To our best knowledge, currently there are not any published studies on expression levels of *TL1A* and *VEGI-192* mRNA in colon cancer in relation to *TNFSF15* single-nucleotide polymorphisms. Therefore, we have decided to investigate association of two common *TNFSF15* SNPs, rs6478108 and rs6478109, with transcriptional activity of this gene in colon cancer patients. Studies conducted by other authors showed that these SNPs are associated with elevated risk for developing gastric adenocarcinoma [16], ulcerative colitis [18], or Crohn's disease [39, 40]. In this study, we showed that elevated expression of *TL1A* mRNA is associated with C/T and T/T genotypes of rs6478108 single-nucleotide polymorphism (SNP) and A/G and G/G genotypes of rs6478109 SNP, while increased expression of *VEGI-192* mRNA is associated with T/T genotype of rs6478108 SNP and G/G genotype of rs6478109 SNP. Therefore, we suppose that the alleles responsible for enhanced expression of the *TNFSF15* gene are T for rs6478108 SNP and G for rs6478109 SNP. This finding is consistent with the observations of other authors who showed that allele T of rs6478108 SNP is a risk allele for ulcerative colitis [18], while C/C genotype of rs6478108 SNP and A/A genotype of rs6478109 are protectively associated with Crohn's disease [39]. Moreover, in vitro studies by Hedl and colleagues showed that the allele A of rs6478109 SNP is related to enhanced expression of the *TNFSF15* gene in macrophages [41]. Even though rs6478108 and rs6478109 SNPs are clearly associated with expression levels of the *TNFSF15* gene, it is difficult to speculate at this moment which of the alleles is the risk factor for colon cancer since TL1A and VEGI-192 appear to play opposite roles in the progression of this disease. Our studies suggest also that expression levels of the *TNFSF15* gene do not depend on microsatellite instability status of the patients' genome isolated from the tumor-transformed tissue.

During the course of the studies presented in this article, we made an attempt to detect TL1A and VEGI-192 proteins in colon cancer and control tissue by western blotting. However, expression levels of these proteins were close to detection limit of the technique that we used (data not shown); therefore, it was impossible to use the obtained results for quantitative analysis of TL1A and VEGI-192 expression. Also, detection of these proteins in the collected tissues by immunohistochemistry was very problematic due to the lack of commercially available antibodies that unambiguously detect the two isoforms separately. In fact, there are very few published studies that show the presence and clearly distinguish the expression of native TL1A or VEGI-192 proteins in human tissue [42, 43] which is most probably caused by low expression of these proteins and technical limitations.

In summary, we show here for the first time that *TL1A* and *VEGI-192* transcripts have opposite expression patterns in human colon cancer, related to the progression of this malignancy and survival of colon cancer patients. For these reasons, the levels of *TL1A* and *VEGI-192* transcripts in colon cancer tissue can be considered as prognostic markers for this disease.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interests.

Ethical statement All procedures were performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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