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Article

# A Novel Potential Prognostic Biomarker for Colorectal Cancer: TRPM7

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## Abstract

**Background/Objectives:** Transient receptor potential melastatin 7 (TRPM7), a unique ion channel protein, has important effects for the proliferation of many cancer cell lines and tumor progression in various cancers. This study aimed to investigate whether TRPM7 expression has an effect on the prognosis of patients with colorectal cancer (CRC). **Methods:** TRPM7 expression was evaluated in paraffin-embedded tumor tissues of 259 CRC patients, immunohistochemically. In addition, the ratio of CD4+/CD8+ lymphocytes in the tumor microenvironment was determined semi-quantitatively. **Results:** According to our data, tumor size was clearly higher in cases with high TRPM7 expression than those with low expression. TRPM7 overexpression was closely related to high depth of tumor invasion, increased lymph node metastasis and high distant metastasis rate. These findings exposed that high TRPM7 expression is effective in the progression and aggressiveness of CRC. While there was a negative correlation between TRPM7 overexpression and the density of CD8+ TILs, a positive correlation was revealed between TRPM7 expression and the CD4+/CD8+ TILs ratio. This result aroused curiosity for further studies on what kind of connection is between the TRPM7 channel protein and TILs. Also, high TRPM7 expression was closely associated with low overall (OS) and disease-free survival (DFS) times. Furthermore, multivariate analyses revealed TRPM7 overexpression was independently related with short OS and DFS. **Conclusions:** In conclusion, the high TRPM7 expression and CD4+/CD8+ TILs ratio are independent poor prognostic indicators in CRC. In the future, TRPM7 may be a promising biomolecule for new targeted treatment options for CRC.

**Keywords:** colorectal cancer; TRPM7; TILs; CD4+/CD8+ lymphocyte ratio; survival

## 1. Introduction

Colorectal cancer (CRC) is the third most common malignant neoplasm across the world and is one of the major causes of cancer-related death [1–3]. The vast majority of colorectal cancers are adenocarcinomas that develop from benign adenomatous polyps in the colonic mucosa [1]. Despite the advances in modern surgical methods, radiotherapy and targeted therapies that have increased rapidly in recent years, the survival rate of patients with CRC has remained far from expectations due to the difficulty of early diagnosis and the presence of distant metastases at the time of diagnosis [3]. Tumor stage represented by the tumor-node-metastasis (TNM) system is still considered to be the most validating prognostic indicator [4]. However, patients in the same TNM stage may exhibit quite different clinical features, which makes it difficult to predict prognosis for some patients with CRC. Therefore, new molecular biomarkers are needed for reliable prognostic approaches and more effective targeted therapies [3].

The relationship between ion channels and cancer is one of the most interesting research topics in recent years. Various studies regarding this issue have exhibited significant findings that transient receptor potential (TRP) channels may be correlated with apoptosis, cell proliferation, cancer development, and tumor progression [5–7]. A variety of members of TRP superfamily, especially the TRPM, have been associated with some human cancers including gastrointestinal tract [8–12]. The

TRPM7 protein is an important member of the TRPM subfamily and is a unique molecule joining an ion channel with a functional alpha-kinase domain [13,14]. TRPM7 is widely expressed in a variety of human tissues including liver, spleen, brain, heart, kidney, and lung [5,13]. In addition, TRPM7 expression has recently been displayed in breast cancer, retinoblastoma and gastric cancer [15–17]. TRPM7 channel protein is intensively expressed in various human tumor cells and lack of it inhibits tumor growth. TRPM7 overexpression is shown in breast cancer cells in relation to their proliferative potential [18]. In an another study, TRPM7 played a role in the carcinogenesis of nasopharyngeal carcinomas [19]. Also, it was determined that CRC LoVo cells sensitive to chemotherapy display higher TRPM7 expression compared to drug-resistant ones [20]. Recently, Su et al. reported that reduced TRPM7 expression inhibited CRC cell proliferation, migration, and in vitro invasion. They emphasized up-regulation of TRPM7 in CRC is associated with deeper tumor infiltration, positive lymph node metastasis, distant metastasis, and advanced clinical stage [8].

Since the tumor microenvironment (TME), which is affects all stages of tumor development and growth, is thought to play a key role in tumor behavior, the prognostic significance of tumor-infiltrating lymphocytes (TILs) has been an attracted subject of cancer researches [21–23]. Although there is still no consensus on whether TILs have a good or poor prognostic effect, various clinical and epidemiological investigations have shown that the presence, contents and amount of TILs are significantly associated with survival in CRC. Idos et al. stated that the density of CD8+ T lymphocytes in TME of CRC has a statistically significant prognostic effect on disease-free survival (DFS) and overall survival (OS) [21]. However, the prognostic significance of CD4+ TILs density and CD4+/CD8+ TILs ratio in CRC is still not fully investigated.

There are several in vitro and experimental studies investigating the relationship between TRPM7 and CRC. However, clinical studies involving a large patient population are needed. Therefore, in present study, it was aimed to investigate whether TRPM7 expression and CD4+/CD8+ TILs ratio have an effect on prognosis and survival in CRC. In addition, the relationship between TRPM7 expression and clinicopathological parameters including, histological tumor type, histological grade, depth of tumor invasion (pT), lymph node metastasis (pN), distant metastasis (DM) and TNM stage has also evaluated.

## 2. Materials and Methods

### 2.1. Patients and Pathological Evaluation

The present study was approved by the Firat University Ethical Committee (Date: 14 January 2021, Approval No: 2021/01-06). The study included 259 patients with CRC treated by surgical resection between 2010-2017 at a single institution. The patients treated with adjuvant or neo-adjuvant chemotherapy were excluded. The clinical and pathological data were obtained from hospital medical archives. Pathological slides were evaluated retrospectively by two pathologists at different times. The survival information of the patients was obtained from the medical data processing records of the hospital. OS and DFS was determined as the interval between the dates of surgery and death or recurrence.

### 2.2. Immunohistochemistry

Immunohistochemical staining was carried out using the indirect immuno-peroxidase technique on formalin-fixed paraffin-embedded tissues. In the staining process, below antibodies were used: anti-TRPM7 (bs-9044R, BIOSS antibodies, Woburn, MA, USA), anti-CD4 (SP35, Ventana, Arizona, USA) and anti-CD8 (SP57, Ventana, Arizona, USA). Parafinized slides obtained from pathology blocks containing tumoral tissues were automatically stained with Ventana Bench Mark Ultra coater and ultra-View Universal DAB kit (Ventana, Tucson, AZ-85755, USA) in accordance with the manufacturer's specified procedures. The density and distribution of TRPM7 expression in tumor cells were evaluated immunohistochemically. Additionally, TILs density was evaluated by CD4 and CD8 staining.

### 2.3. The Evaluating of TRPM7 Expression Score

TRPM7 expression in CRC was assessed using the IHC-scoring method confirmed in previous studies [24,25]. While evaluating the preparations, cytoplasmic and membranous staining in tumor cells were taken into consideration. IHC-score was obtained multiplying the staining intensity [0: no staining, 1: weak staining (light yellow), 2: moderate staining (yellow brown), and 3: strong staining (brown)] by the percentage of positive stained cells (0: <5%; 1: 5% -20%; 2: 21% -50%; and 3: >50%). IHC-score ranged from 0 to 9. The mean value of the IHC-score  $3.74 \pm 3.46$  was accepted as the cut-off criterion. Patients with IHC-score below this value were considered as a low-TRPM7 expression group and those above were characterized as a high-TRPM7 expression or overexpression group.

### 2.4. Assessment of TILs Intensity and CD4+/CD8+ TILs Ratio

CD4+ and CD8+ TILs density was assessed holding to earlier studies [22,23]. Briefly, five fields with the intense infiltration of CRC were selected from each staining slide and the percentages of CD4+ and CD8+ TILs were evaluated. The mean of this five areas was put to use as the density of TILs. Firstly, a semi-quantitative staining score was specified according to the following features: 1 (<1% cells); 2 (1-10% cells); 3 (11-33% cells); 4 (34-66% cells); and 5 (67-100% cells). While evaluating CD4 and CD8 positivity, partial or complete positivity in the cytoplasm and/or plasma membrane of inflammatory cells was considered. Secondly, intensity of immunoreactivity was scored as follows: 0 (absent), 1+ (slight), 2+ (moderate), and 3+ (intense). Finally, TILs IHC-scores were calculated by adding the mean staining scores and the intensity for each slide (ranging from 1 to 8). The patients were divided into two groups as a low and high CD4+ and CD8+ TILs density using median value of IHC-score ( $3.69 \pm 2.02$ ,  $3.56 \pm 2.07$ , respectively). In addition, CD4+/CD8+ TILs ratio was determined for each case. The mean value for this ratio was accepted as the cut-off criterion ( $1.67 \pm 1.67$ ). Patients with below this value were considered as a low-CD4+/CD8+ TILs ratio group and those above were characterized as a high CD4+/CD8+ TILs ratio group.

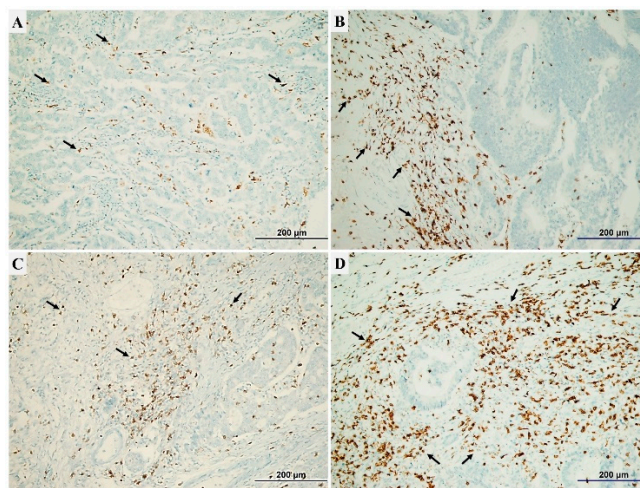
### 2.5. Survival and Statistical Analysis

In the present study, SPSS version 26 software (IBM Corporation, Armonk, NY, USA) was used for statistical analysis of data. Obtained outcomes from this study were presented as means and standard deviations. Normality of groups was checked by the Shapiro-Wilk test. The expression of TRPM7 and clinicopathological features of the patients were compared according to the normality of the distribution. ANOVA and independent samples t test were used to determine the differences between groups with normal distribution. Chi-square and Mann-Whitney U tests were preferred to determine the differences between groups without normal distribution. Kaplan-Meier method (log-rank test) was used to achieve and compare the survival curves. Univariate and multivariate Cox proportional hazards regression analyses was applied to evaluate independent prognostic indicators associated with survival. For all statistical data, a P value less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. The Correlation Between Clinicopathological Parameters and Survival

Of the 259 patients, 114 (44.0%) were female and 145 (56.0%) were male. The mean age was  $60.3 \pm 13.4$  years (range 24-86). Most of the tumors were on the left side (n=157, 60.6%). Most of the cases consisted of patients with advanced TNM stage (stage III-IV) (n=146, 56.4%). Also, in 131 (50.6%) of the cases in this series, the CD8+ TILs density was low (Figure 1A) and high in 128 (49.4%) (Figure 1B). Again, CD4+ TIL density was determined as low in 129 (49.8%) cases (Figure 1C) and high in 130 (50.2%) cases (Figure 1D).



**Figure 1.** Representative TILs density in colorectal cancer tissues. Arrows show CD8 and CD4 positive T lymphocytes in the tumor microenvironment. (A) low CD8+ TILs density (CD8x200). (B) high CD8+ TILs density (CD8x200) (C) low staining intensity for CD4+ T lymphocytes (CD4x200) (D) high density of CD4+ TILs (CD4x200).

The clinicopathological features are detailed in Table 1. The follow-up period for survival analysis was determined as 5-years. The mean OS was  $49.6 \pm 12.8$  months and the 5-year survival rate was 50.6%. There was recurrence in 163 (62.9%) of the patients, and the mean DFS was determined to be  $44.7 \pm 15.7$  months. It was revealed clinicopathological parameters such as gender, age, tumor size and histopathological type had no effect on survival. Conversely, according to univariate Cox regression analysis, right tumor site, high histological grade, low CD8+ and high CD4+ TILs density, high pT and advanced TNM stage were found to be significantly associated with poor prognosis (Table 1).

**Table 1.** Clinical and pathological features of study cases and their relationship with overall survival [N=259] [univariate Cox regression analysis].

Parameters	Overall survival [Univariate]		
	N [%]	HR [95% CI]	P value
Gender			
Female	114 [44.0]	1.13 [0.79-1,6]	0.491
Male	145 [56.0]		
Age			
19-44	34 [13.1]	1.23 [0.95-1.59]	0.109
45-54	86 [33.2]		
55 ≥	139 [53.7]		
Tumor size [cm]		1.39 [0.97-2.31]	0.070
< 5	105 [40.5]		
≥ 5	154 [59,5]		
Tumor site			
Left	157 [60.6]	0.64 [0.45-0.92]	0.015
Right	105 [39.4]		
Histopathologic type			
Adenocarcinoma	154 [59.5]	1.27 [0.98-1.66]	0.075

Mucinous	82 [31.7]		
Signet-ring	23 [8.9]		
Histologic grade			
Well	44 [17.0]	2.16 [1.56-2.99]	< 0.001
Moderate	173 [66.8]		
Poor	42 [16.2]		
CD8 <sup>+</sup> TILs density			
Low	131 [50.6]	0.58 [0.41-0.83]	0.003
High	128 [49.4]		
CD4 <sup>+</sup> TILs density			
Low	129 [49.8]	0.61 [0.43-0.87]	0.007
High	130 [50.2]		
CD4 <sup>+</sup> /CD8 <sup>+</sup> TILs ratio			
Low	172 [66.4]	2.02 [1.42-2.86]	<0.001
High	87 [33.6]		
Depth of invasion			
Submucosa	34 [3.1]	2.80 [1.98-3.97]	< 0.001
Muscularis propria	68 [26.3]		
Pericorectal tissues	157 [60.6]		
Lymph node status			
Absent	113 [43.6]	3.65 [2.90-4.60]	< 0.001
1-3	97 [37.5]		
≥ 4	49 [18.9]		
Distant Metastasis			
Absent	186 [71.8]	12.93 [8.67-19.28]	< 0.001
Present	73 [28.2]		
TNM staging			
Stage I	59 [22.8]	5.92 [4.45-7.88]	< 0.001
Stage II	54 [20.8]		
Stage III	78 [30.1]		
Stage IV	68 [26.3]		

HR: hazard ratio, CI: confidence interval.

In multivariate analyses, classical prognostic parameters such as histological grade, CD4<sup>+</sup> TILs density, pT and pN were not found to be associated with both OS and DFS (Table 2). However, CD8<sup>+</sup> TILs and TNM stage was determined to be the most valuable independent parameters among all other classical prognostic factors (Table 2). Both OS and DFS times were shown to be significantly reduced in patients with advanced TNM stage (34.0±8.93 months, 25.80±8.09 months, respectively) compared to those with early stage (59.89±0.66 months, 58.45±3.47 months, respectively).

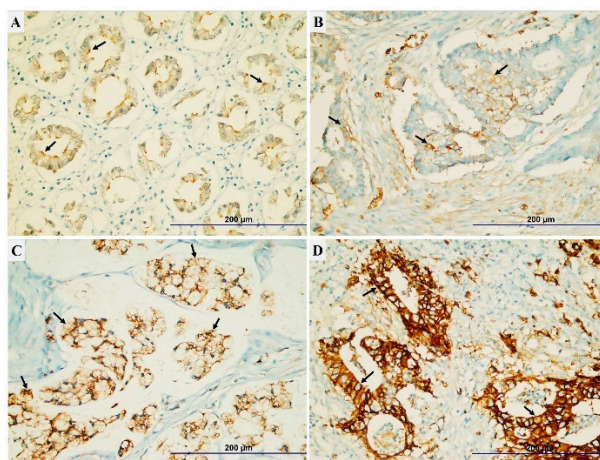
**Table 2.** Classical prognostic parameters and the effect of TRPM7 overexpression on overall and disease-free survival [multivariate Cox regression analysis].

Parameters	Cox regression analyses [Multivariate]					
	Disease-free survival			Overall survival		
	HR	[95% CI]	P value	HR	[95% CI]	P value
Gender [male/female]	0.90	[0.63-1.27]	0.553	1.00	[0.67-1.49]	0.964
Age [19-44/45-54/≥ 55]	1.08	[0.86-1.36]	0.500	1.18	[0.91-1.53]	0.205
Site [left/right]	0.63	[0.45-0.88]	0.007	0.72	[0.49-1.07]	0.107
Type [adeno/mucinous/signet ring]	1.06	[0.83-1.35]	0.626	1.05	[0.81-1.37]	0.677
Grade [well/moderate/poor]	1.24	[0.92-1.69]	0.154	1.12	[0.81-1.55]	0.478
CD8 <sup>+</sup> TILs density [low/high]	0.47	[0.34-0.66]	<0.001	0.59	[0.40-0.85]	0.006
CD4 <sup>+</sup> TILs density [low/high]	0.90	[0.62-1.32]	0.619	1.22	[0.79-1.88]	0.351
CD4 <sup>+</sup> /CD8 <sup>+</sup> TILs ratio [low/high]	1.89	[1.35-2.64]	<0.001	1.77	[1.22-2.56]	0.002
pT [pT1/pT2/pT3]	0.89	[0.62-1.26]	0.517	1.00	[0.63-1.59]	0.978
pN [absent/1-3/≥ 4]	1.37	[0.96-1.95]	0.077	1.42	[0.98-2.07]	0.063
TNM stage [I/II/III/IV]	2.32	[1.49-3.62]	<0.001	3.30	[1.78-6.10]	<0.001
TRPM7 expression [low/high]	4.08	[2.65-6.28]	<0.001	5,81	[3.16-10.65]	<0.001

HR: hazard ratio, CI: confidence interval.

### 3.2. Relationship Between TRPM7 Expression and Clinicopathological Parameters

Totally 259 tumor tissues and 60 adjacent non-neoplastic colorectal tissues were stained with anti-TRPM7 antibody, immunohistochemically. There was lower TRPM7 expression in the normal colonic mucosa compared to tumoral areas (Figure 2A). While low TRPM7 expression (Figure 2B) was observed in 122 (47.1%) of 259 cases, overexpression (Figure 2C and 2D) was observed in 137 (52.9%) of the cases.



**Figure 2.** Representative figures of TRPM7 expression in CRC specimens. Arrows show cytoplasmic TRPM7 expression in normal colonic mucosa and tumor cells. (A) Low staining (normal mucosa adjacent to tumor) (TRPM7x400). (B) low cytoplasmic TRPM7 expression in moderate differentiated tumor (TRPM7x400). (C) high expression of TRPM7 in mucinous adenocarcinoma cells (TRPM7x400). (D) TRPM7 overexpression in tumor cells (TRPM7x400).

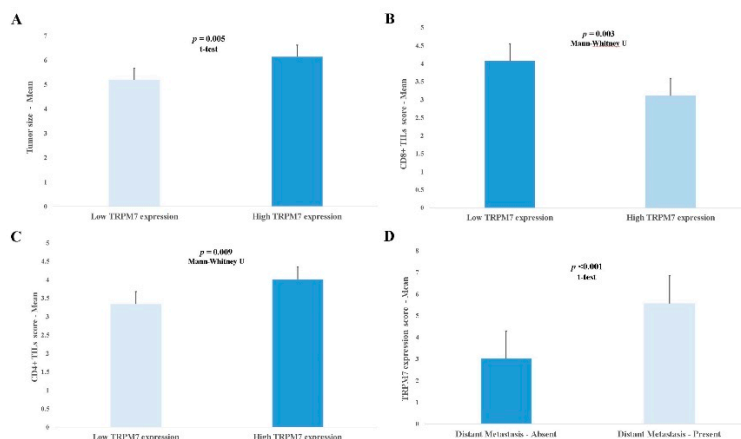
The interrelation between TRPM7 expression and clinicopathological features is shown in Table 3. There was no significant correlation between TRPM7 expression and classical prognostic parameters such as gender, age, and tumor site (not shown). Our results proved high TRPM7 expression was closely related to high histological grade, high pT category, increased pN, high DM rate and advanced TNM stage (Table 3).

**Table 3.** The correlation between TRPM7 expression and classical prognostic parameters.

Parameters	TRPM7 expression Mean $\pm$ SD	P value
Histopathologic type		
Adenocarcinoma	3.79 $\pm$ 2.26	0.010 *
Mucinous	3.30 $\pm$ 2.15	
Signet-ring	4.91 $\pm$ 2.42	
Histologic grade		
Well	2.86 $\pm$ 1.98	0.001 *
Moderate	3.74 $\pm$ 2.30	
Poor	4.64 $\pm$ 2.15	
CD4 <sup>+</sup> TILs density		
Low	3.39 $\pm$ 2.27	0.009 **
High	4.08 $\pm$ 2.23	
CD8 <sup>+</sup> TILs density		
Low	4.14 $\pm$ 2.30	0.003 **
High	3.32 $\pm$ 2.18	
CD4 <sup>+</sup> /CD8 <sup>+</sup> TILs ratio		<0.001 **
Low	3.33 $\pm$ 2.23	
High	4.54 $\pm$ 2.17	
Depth of invasion		
Submucosa	2.05 $\pm$ 1.04	<0.001 *
Muscularis propria	3.39 $\pm$ 2.09	
Pericorectal tissues	4.25 $\pm$ 2.35	
Lymph node status		
Absent	2.31 $\pm$ 1.40	<0.001 *
1-3	4.65 $\pm$ 2.21	
$\geq$ 4	5.20 $\pm$ 2.19	
Distant Metastasis		
Absent	3.02 $\pm$ 2.01	<0.001 ***
Present	5.57 $\pm$ 1.85	
TNM staging		
Stage I	2.35 $\pm$ 1.42	<0.001 *
Stage II	2.22 $\pm$ 1.38	
Stage III	4.24 $\pm$ 2.30	
Stage IV	5.57 $\pm$ 1.83	

SD: Standard deviation, \* ANOVA, \*\* Mann-Whitney U, \*\*\* independent-samples t-test.

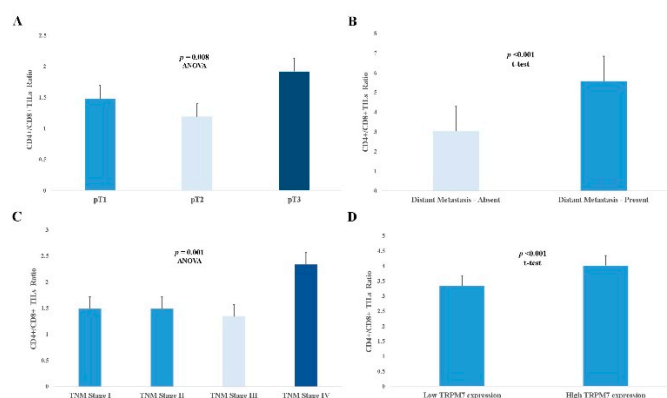
Also, it was found the tumor size was significantly higher in cases with TRPM7 overexpression than those with low expression (Figure 3A). There was a negative correlation between TRPM7 expression and CD8<sup>+</sup> TILs density (Figure 3B). Contrarily, the patients with TRPM7 overexpression had high CD4<sup>+</sup> TILs density (Figure 3C). Consistent with these findings, TRPM7 expression was higher in patients with a high CD4<sup>+</sup>/CD8<sup>+</sup> TILs ratio ( $p < 0.001$ ) (Table 3). In this series, 73 (28.2%) of 259 cases had DM. There was a positive correlation between TRPM7 expression and DM. The TRPM7 expression score was higher in patients with DM than those without (Figure 3D). These data implicated that high TRPM7 expression is effective in the progression and aggressiveness of CRC.



**Figure 3.** Representative images showing the interrelationship between TRPM7 expression and prognostic parameters including tumor size, CD8+ TILs density, CD4+ TILs density and distant metastasis. (A) The TRPM7 expression was higher in tumors with great size ( $3.99\pm 2.34$ ) compared to those with small size ( $3.37\pm 2.14$ ) ( $p=0.005$ , t-test). (B) in tumoral tissues with high TRPM7 expression, the density of CD8+ TILs was significantly low ( $p=0.003$ , Mann-Whitney U test). (C) in tumors with high TRPM7 expression, the density of CD4+ TILs was significantly high ( $p=0.009$ , Mann-Whitney U test). (D) Patients with TRPM7 overexpression had a higher rate of distant metastases ( $p<0.001$ , t-test).

### 3.3. The Effect of High CD4+/CD8+ TILs Ratio on Prognosis

The mean CD4+/CD8+ TILs ratio of all patients was  $1.67\pm 1.67$  (range 0.14-8.0). There was no significant relationship between CD4+/CD8+ TILs ratio and histological type ( $p=0.103$ , ANOVA), histological grade ( $p=0.174$ , ANOVA) and pN ( $p=0.270$ ). Contrarily, high CD4+/CD8+ TILs ratio was positively correlated with pT (Figure 4A), DM (Figure 4B), and TNM stage (Figure 4C). There was also a statistically significant relationship between CD4+/CD8+ TILs ratio and TRPM7 expression. The CD4+/CD8+ TILs ratio was higher in high TRPM7 expressive patients (Figure 4D).



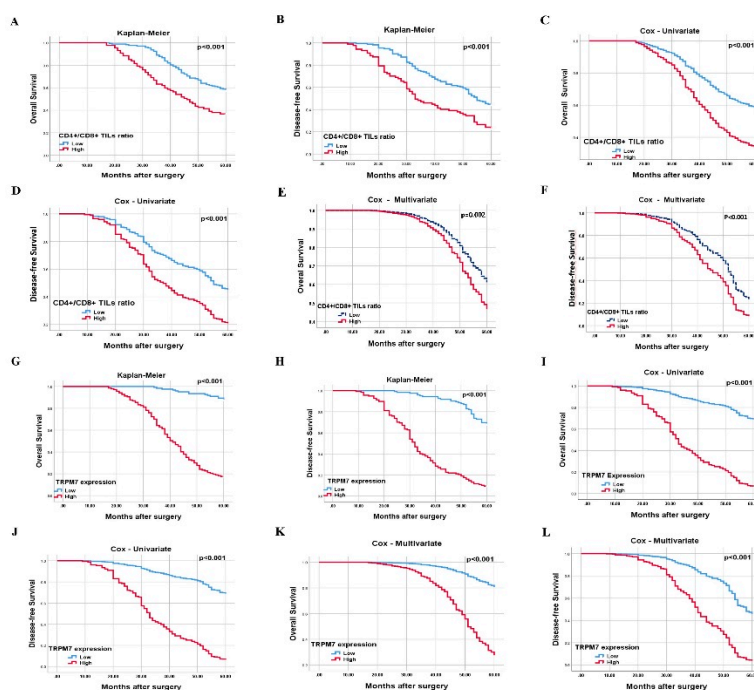
**Figure 4.** The correlation between CD4+/CD8+ TILs ratio and basic prognostic indicators of CRC, and TRPM7 expression. (A) the ratio of CD4+/CD8+ TILs was significantly higher in patients with deeper tumor invasion ( $1.92\pm 1.79$ ) compared to those with more superficial ( $1.48\pm 1.92$ ) ( $p=0.008$ , ANOVA). (B) Distant metastasis was significantly more common in cases with high CD4+/CD8+ TILs ( $p<0.001$ , t-test). (C) The CD4+/CD8+ TILs ratio was significantly higher in patients with advanced TNM stage ( $2.34\pm 2.00$ ) than those with early TNM stage ( $1.49\pm 1.71$ ) ( $p=0.001$ , ANOVA). (D) The CD4+/CD8+ TILs ratio was importantly high in tumors with TRPM7 overexpression ( $p<0.001$ , t-test).

The effect of CD4+/CD8+ TILs ratio on OS and DFS was also investigated in the study. The Kaplan-Meier survival curves displayed CD4+/CD8+ TILs ratio was importantly associated with poor

OS ( $p < 0.001$ , Log Rank) (Figure 5A) and short DFS time ( $p < 0.001$ , Log Rank) (Figure 5B). In the univariate analyses the high CD4+/CD8+ TILs ratio was an importantly correlated with shorter OS (Figure 5C) and DFS time (Figure 5D). Moreover, in multivariate analyses, there was a significant association between high CD4+/CD8+ TILs ratio and worsening OS (Figure 5E) and DFS (Figure 5F). Taken together, the findings support that the high CD4+/CD8+ TILs ratio is a reliable independent marker of tumor progression and poor prognosis in CRC.

### 3.4. TRPM7 Overexpression Can Be Used as an Independent Poor Prognostic Biomarker in CRC

Whether TRPM7 expression could be used as a prognostic biomarker was evaluated using Kaplan-Meier analyses and Cox regression models. The survival curves displayed TRPM7 overexpression was importantly associated with poor OS (Figure 5G) and short DFS time (Figure 5H). The cases with high TRPM7 expression exhibited worsening biological behavior than those with low expression. According to univariate analysis, TRPM7 expression was substantially related with poor OS (HR 6.36, 95% CI 4.33-9.36,  $p < 0.001$ ) and DFS (HR 4.10, 95% CI 3.15-5.34,  $p < 0.001$ ) in CRC. The patients with high TRPM7 expression had significantly shorter mean OS (Figure 5I) and DFS time (Figure 5J) than those with low expression. Furthermore, multivariate analyses revealed TRPM7 overexpression was independently correlated with poor OS (Figure 5K) and DFS (Figure 5L) (Table 2). In short, univariate and multivariate analyses exposed that high TRPM7 expression was a biomarker indicating poor prognosis for CRC patients, independently.



**Figure 5.** Representative Kaplan-Meier survival curves and Cox proportional regression analyses according to CD4+/CD8+ TILs ratio and TRPM7 expression. Patients with high CD4+/CD8+ TILs ratio had a poor (A) overall ( $p < 0.001$ , Log Rank) and (B) disease-free survival rates ( $p < 0.001$ , Log Rank) than those with low CD4+/CD8+ TILs ratio. In univariate Cox regression analyzes, high CD4+/CD8+ TILs ratio was significantly associated with both poor (C) overall ( $p < 0.001$ ) and (D) disease-free survival ( $p < 0.001$ ). According to multivariate analyses, patients with high CD4+/CD8+ TILs ratio had shorter (E) overall (HR 1.77, 95% CI 1.22-2.56,  $p = 0.002$ ) and (F) disease-free survival times (HR 1.89, 95% CI 1.35-2.64,  $p < 0.001$ ). (G) The patients with high TRPM7 expression had a poor overall ( $p < 0.001$ , Log Rank) and (H) disease-free survival rates ( $p < 0.001$ , Log Rank) than those with low expression. In univariate Cox regression analyzes, high TRPM7 expression was significantly associated with both (I) overall ( $p < 0.001$ ) and (J) disease-free survival ( $p < 0.001$ ). According to multivariate Cox regression analyzes, TRPM7 overexpression was significantly correlated with both worsened (K) overall ( $p < 0.001$ ) and (L) disease-free survival time ( $p < 0.001$ ), independently.

## 4. Discussion

Recently, there are various studies claiming that TRPM7 plays a role in carcinogenesis and could be a potential target in cancer treatment [9,17,26]. TRPM7, a special member of the super family of TRP ion channels, has permeability for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions. Because the  $\text{Ca}^{2+}$  ion is an efficient regulator of cell cycle and proliferation, the regulation of TRPM7 functions is thought to be vital for cancer cell growth and tumor biology [26,27]. TRPM7, which is basically a bivalent cation-permeable channel with an intrinsic kinase, accomplishes its physiological effects by doing as a cellular sensor and signal transducer thanks to its ion channel and kinase activity [9,28]. TRPM7 channel functionality depends on the activity of the endogenous kinase capable of phosphorylating serine and threonine residues of the protein. The TRPM7 channel is activated when the TRP domain of TRPM7 interacts with phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>). However, since stimulation of phospholipase C (PLC) leads to hydrolysis of PIP<sub>2</sub>, the channel is inactivated [9]. Additionally, the interaction between the kinase domain and the PLC arranges the activity of TRPM7 channel in an addicted way to cAMP and protein kinase A. TRPM7 kinase also has the capable of auto-phosphorylation, and this is regulated via the intracellular  $\text{Mg}^{2+}$  and ATP levels [29].

TRPM 7, widely expressed in normal tissues, has been shown to be overexpressed in various types of cancer, abnormally [24,28,30,31]. Studies investigating the effect of TRPM7 on pancreatic cancers revealed TRPM7 expression significantly increased in pancreatic adenocarcinoma cells and tissues [9,32,33]. TRPM7 expression in pancreatic cancer tissues was clearly demonstrated to positively associate with the tumor size and tumor stages [33]. These results shown high TRPM7 expression is related with tumor growth, progression and metastasis in pancreas cancer. Additionally, there are studies showing TRPM7 is overexpressed in cell lines and tissues of breast cancer and glioblastoma [34,35]. According to Guilbert et al., TRPM7 expression plays an important role in the proliferation of MCF7 breast cancer cells, and TRPM7 protein levels in breast cancer suggest its overexpression may be a feature of high-grade, highly proliferative breast cancer [34]. In the study of Wan et al., it was claimed TRPM7 regulate miR-28-5p expression, which represses proliferation and invasion of glioma cells by targeting Rap1b signaling. According to Wan et al., While TRPM7's channel activity is necessary for the growth of glioma cells, its kinase domain contributes to tumor progression through cell migration and invasion [33]. These results show that TRPM7 has an important contribution to the proliferation of cancer cells and tumor growth. Compatible with previous studies, we determined the tumor size was significantly larger in cases with TRPM7 overexpression compared to those with low expression. In recent study, Pugliese et al. reported TRPM7 expression to be higher in tumoral areas than adjacent non-neoplastic tissue in both IBD-related and sporadic cases [36]. A higher TRPM7 expression was determined in adenocarcinomas than in colonic adenomas. Also, TRPM7 expression was positively associated with histological grade [36]. Similarly, we found that tumors with high TRPM7 expression had significant loss of differentiation and high histological grade. Especially, this positive relationship between high histological grade and TRPM7 overexpression supports the view that TRPM7 may play a promotor role in the acquisition of a malignant phenotype.

TRPM7 is thought to have a regulatory role in epithelial-mesenchymal transition (EMT), which is one of the important steps in tumor metastasis [28]. Epidermal growth factor (EGF) is associated with a temporary elevation of intracellular  $\text{Ca}^{2+}$  and activation of signal transducer and activator of transcription 3 (STAT3). TRPM7 gene silencing in a breast cancer cell line (MDA-MB468) given rise to suppress of EGF-induced Vimentin expression and phosphorylation of STAT3, which are markers of EMT [37]. According to Yee et al., in pancreatic adenocarcinoma, TRPM7 expression is necessary for  $\text{Mg}^{2+}$  dependent cell migration and cell invasion [33]. These results demonstrate that the  $\text{Ca}^{2+}/\text{Mg}^{2+}$  homeostasis and associated signaling system provided by TRPM7 is essential for the migration and invasion of cancer cells and backs up the potential role of TRPM7 channels in tumor metastasis [28,33,37]. Su et al. evidently observed the up-regulation of TRPM7 expression in CRC tissues compared to non-tumoral areas [8]. They stated patients with TRPM7 overexpression had aggressive pathological features such as deeper tumor infiltration, positive lymph node metastasis,

distant metastasis, and advanced clinical stage compared to those with low expression. Furthermore, they reported the down-regulation of TRPM7 expression in CRC cells reversed the EMT process with a decrease in N-cadherin and an increase in E-cadherin. And so, in vitro, they clearly demonstrated the reduced TRPM7 expression inhibits CRC cell proliferation, migration and invasion [8]. The results obtained in our study support the in vitro studies mentioned above. We showed both lymph node metastasis and distant metastasis rates in CRC patients with TRPM7 overexpression were significantly higher than those with low expression. One of the most important parameters affecting the prognosis in colorectal cancer is the occurrence of distant metastasis. Therefore, new studies are needed to determine whether the effect of TRPM7 on metastasis is only through  $Ca^{2+}/Mg^{2+}$  homeostasis or through other pathways.

There are very few clinical studies investigating the effect of TRPM7 on the prognosis and survival of cancer patients [25,30]. Furthermore, the results of these studies are incompatible. Nakashima et al., demonstrated TRPM7 expression is negative correlated with pT, pN category and stage in esophagus cancer. They indicated the overall survival rate of the cases with high TRPM7 expression was importantly higher than that of those with low expression, also TRPM7 expression was an independent prognostic indicator for esophageal cancer [30]. However, Rybarczyk et al. revealed expression of TRPM7 was a potential biomarker of poor prognosis of pancreatic ductal adenocarcinoma [38]. In a recent study by Lee et al., which contained conflicting results with our study, it was determined that TRPM7 overexpression was negatively correlated with age and p53 in patients with rectal cancer [39]. They also found a positive correlation between TRPM7 expression and KRAS expression in rectal cancer. On the other hand, survival analysis in their study showed that TRPM7 overexpression had no prognostic significance in colon and rectal cancers. Contrarily, in our study, no significant difference was observed between TRPM7 expression and tumor site. In this study TRPM7 overexpression was closely related to aggressive histopathological features such as high histological grade, high pT, high pN category and distant metastasis rates in CRC. In addition, high TRPM7 expression in patients with CRC was associated with low OS and DFS rates according to univariate analysis. Moreover, in multivariate analysis, we established TRPM7 overexpression is a strong independent poor prognostic indicator.

TILs, particularly CD3+ and CD8+ T cells, are regarded as markers of the host immune response to the tumor, and many studies have indicated they are a strong independent positive predictor of relapse and OS in various cancers, including CRC [21–23,40,41]. However, CD4+ T cells are less defined as a prognostic marker in CRC [40,41]. It has been reported that the density of CD4+ TILs is a negative prognostic parameter in some malignant tumors such as breast, kidney, and lung cancer [42,43]. However, conflicting results were revealed about the effects of CD4+ TILs density on prognosis in CRC [40,41]. According to Kuwahara et al., while increased CD8+ TILs and CD4+ TILs density in CRC were closely associated with longer survival duration, low CD4+ TILs density had a markedly poor prognostic effect [40]. Contrarily, Lavotshkin et al. shown that longer DFS time was associated with higher CD8+ TILs density and lower CD4+/CD8+ ratios [44]. Diederichsen et al. stated patients with low CD4+/CD8+ ratios had a better clinical behavior with significantly higher 5-year survival, regardless of Dukes stage and age [41]. Consistent with previous studies, high CD8+ TILs rate was found as an independent good prognostic parameter in present study. High CD4+ TILs density and CD4+/CD8+ TILs ratio were closely associated with aggressive pathological features such as high pT, distant metastasis, and advanced TNM stage. Patients with high CD4+/CD8+ TILs ratio had shorter DFS and OS times compared to those with low. Except these, a negative relationship between TRPM7 expression and CD8+ TILs density was determined in present study. In contrast, patients with TRPM7 overexpression had high density of CD4+ TILs. Also, the high CD4+/CD8+ TILs ratio was found to be higher in patients with high TRPM7 expression compared to those with low expression. In light of these findings, hypothetically, it can be argued TRPM7 overexpression reduces the density of CD8+ T lymphocytes by reversing the function of T lymphocytes in host defense against the tumor thereby may adversely affect the prognosis. In our opinion, the role of TRPM7, which is

known to have important effects on the regulation of lymphocyte functions, in the composing the TME and in the regulation of host defense against the tumor is worth investigating.

By the way, it would be useful to point out that our study has some limitations in order to positively influence future studies. First of all, this study is a retrospective study including cases from a single health center. Therefore, it should be taken into consideration that it may have the potential for selection bias. Secondly, patients who received chemotherapy are excluded from our study. However, in recent years, chemotherapy and targeted therapies have been widely used in the treatment of CRC. Therefore, it may be appropriate to include patients receiving chemotherapy in the study population in further studies to investigate whether potential biomarkers have therapeutic or prognostic value. Moreover, studies investigating the effects of agonist and antagonist agents for TRPM7 on CRC cell lines can be added to clinical studies.

Consequently, we have defined the pattern of TRPM7 expression in human CRC tissues, for the first time. According to our data, TRPM7 overexpression is closely associated with aggressive histopathological features and progression of CRC. The patients with high TRPM7 expression had shorter survival period. In multivariate analyses, TRPM7 overexpression, CD8+ TILs density and CD4+/CD8+ TILs ratio have been shown to be independent poor prognostic biomarkers. We also predicted TRPM7 may adversely affect the host immune response to the tumor by decreasing CD8+ TILs density. Therefore, the effects of TRPM7 on host defense in patients with CRC and its potential to be a target for new treatments are worth investigating.

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