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The Relationship Between Vascular Endothelial Growth Factor Expression in T Cell Subsets and Survival of Patients With Colorectal Cancer

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Abstract

Background/Aim: Angiogenesis and immune modulation are integral to colorectal cancer (CRC) progression. Vascular endothelial growth factor receptor (VEGFR) is expressed not only on endothelial cells but also on lymphocytes, where it may modulates immune activity. However, its distribution on peripheral T-cell subsets and prognostic relevance in CRC remain poorly understood.

Materials and Methods: We prospectively analyzed 52 histologically confirmed patients with CRC and 30 healthy controls. VEGFR2 expression on T-cell subsets [Th1, Th2, Th17, and cytotoxic T lymphocytes (CTLs)] was quantified using flow cytometry. Associations between VEGFR expression and overall survival (OS) were examined using median fluorescence intensity (MFI) and percentage values.

Results: VEGFR expression was significantly elevated in lymphocytes and Th1 cells of patients with CRC compared with controls. Th1 levels were increased, particularly in advanced-stage disease (p=0.01). VEGFR expression in Th1, Th2, and $CD8^+$ CTLs was higher than that in controls (p=0.04, p=0.03, and p<0.001, respectively). Early-stage patients exhibited greater VEGFR expression in Th1 and Th17 subsets than both advanced-stage and control groups (p=0.01 and p=0.04). Patients with Th1 ≤14.7% had longer median OS (43.4 *vs.* 21.7 months, p=0.002), whereas higher Th9 (>10.7%) and Th17 (>11.2%) levels predicted better survival (p=0.001 and p=0.027). Lower VEGFR expression in Th1 (≤212 MFI), Th2 (≤268 MFI) and Th17 (≤285 MFI) subsets correlated with shorter OS (p=0.018, p=0.031, and p=0.031, respectively), indicating that VEGFR over-expression within these subsets may be associated with favorable prognosis.

continued

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Conclusion: VEGFR expression on peripheral T-cell subsets correlates with survival in CRC, suggesting its potential role as a prognostic and immunoregulatory biomarker.

Keywords: Colorectal cancer, T-cell subsets, VEGFR, Th1, Th17, survival.

Introduction

According to 2024 data from the American Cancer Society, colorectal cancer (CRC) is the third most diagnosed cancer in both men and women, and the second leading cause of cancer-related mortality worldwide (1). As the socioeconomic status of countries increases, so does the incidence of CRC (2). Survival rates decline significantly with disease progression. While the 5-year overall survival rate exceeds 90% in Stage I, it falls below 10% in Stage IV disease. Although the Tumor–Node–Metastasis (TNM) staging system is widely used in clinical practice, it does not adequately reflect prognostic heterogeneity. Patients with similar histopathologic features at the same stage can experience markedly different clinical outcomes, likely due to underlying genetic and epigenetic differences. This emphasizes the need for novel prognostic biomarkers in CRC. Angiogenic signaling plays a pivotal role in the development and progression of CRC. Vascular endothelial growth factor (VEGF), a principal mediator of angiogenesis, is also over-expressed in tumor tissues compared to normal colonic epithelium, and is associated with tumor proliferation (3).

The fundamental goal of cancer immunotherapy is to induce effective immune effectors – such as antibodies and T cells – against tumor cells, thereby enhancing the patient's intrinsic antitumor response. Tumor-specific T cells are key mediators of adaptive immunity, and tumor infiltration by these cells is associated with favorable prognosis (4). Among these, $CD8^+$ cytotoxic T lymphocytes (CTLs) are considered essential for effective antitumor responses and are supported by $CD4^+$ Th1 and Th17 cells (5). $CD8^+$ T cells recognize and eliminate cancer cells that express tumor-specific neoantigens. Tumor-induced dysfunction of $CD8^+$ T cells occur through a biphasic process. Initially, in early tumorigenesis, T cells may become hyporesponsive due to antigen presentation in a non-inflammatory setting without $CD4^+$ T cell help (phase 1). As the disease progresses, persistent antigen exposure leads to a late dysfunctional state characterized by T cell exhaustion (phase 2), which shares molecular features with chronic infection-induced T cell exhaustion (6, 7). Despite increasing insights into tumor immunology, there is a lack of studies examining how immune responses differ across prognostic subgroups in CRC. In particular, the expression profiles of VEGFR in peripheral lymphocyte subsets and their relationship with established prognostic markers remain unclear. Profiling the distribution of lymphocyte subsets – including Th1, Th2, Th17, and $CD8^+$ CTLs – and characterizing VEGFR expression within these populations may reveal novel prognostic insights. Therefore, this study aimed to evaluate the distribution of T lymphocyte subgroups and VEGFR within these subsets, and to investigate their correlations with clinicopathological features and survival outcomes in patients with CRC.

Materials and Methods

Study population. The study population consisted of 52 patients aged 18 years or older who were diagnosed with CRC based on clinical and pathological evaluations and who presented for the first time to the Medical Oncology Clinic at Necmettin Erbakan University, Meram Faculty of Medicine. All patients were chemotherapy naïve at the time of enrollment. A control group comprising 30 age-matched healthy volunteers, with no history of chronic or metabolic disease or active clinical infection, was also included. All participants provided written informed consent.

Baseline demographic and clinical data were collected for both groups, including age, sex, laboratory findings, tumor type, disease stage, metastatic status, pre-treatment performance status, and treatment modalities. The study protocol was approved by the Local Ethics Committee of Selçuk University Faculty of Medicine (approval number: 2019/126) and financially supported by the Selçuk University Scientific Research Projects Coordination Office (project number: 19202043). Peripheral blood samples were analyzed using flow cytometry at first admission.

Flow cytometric analysis. Helper T lymphocyte (Th) subsets (Th1, Th2, Th9, Th17, Th1Th17) and cytotoxic T lymphocytes (CTL) were identified using the whole blood lysis method *via* fluorescently labeled monoclonal antibodies: CD3-FITC was used for identifying total lymphocytes; CD4-APC/Cyanine7 for helper T cells; and CD8-Alexa Fluor 700 for cytotoxic T cells. VEGFR expression was evaluated using VEGFR-PerCP-Cy5.5 markers. A total of 100 µl of peripheral blood was incubated with the antibodies at room temperature in the dark for 20 min, following the protocol-specified concentrations. Subsequently, 2 ml of lysing solution was added to eliminate red blood cells, and the mixture was incubated again for 15 min at room temperature. The samples were centrifuged at approximately 300 × *g* for 5 min, the supernatant was discarded, and the cells were washed with 2 ml of washing buffer, followed by a second centrifugation under the same conditions. The final cell pellet was resuspended in 500 µl of washing solution and analyzed immediately. Flow cytometric analysis was performed using a 10-color BD FACS Aria III flow cytometer (BD Biosciences, Pharmingen, San Jose, CA, USA), and data acquisition was carried out using BD FACSDiva software version 6.1.3.

Lymphocytes were first gated based on forward scatter (FSC) and side scatter (SSC) characteristics to exclude debris and granulocytes. Within the lymphocyte gate, $CD3^+CD4^+$ helper T cells were further subdivided into Th1, Th2, Th9, Th17, and Th1Th17 subsets based on chemokine receptor expression (CXCR3, CRCR4, CRCR6). $CD3^+CD8^+$ cytotoxic T lymphocytes were identified within the same gate (Figure 1).

Statistical analyses. All statistical analyses were performed using SPSS software version 21.0 (IBM Corp., Armonk, NY, USA). The Kolmogorov–Smirnov test was used to assess the normality of the data, while Levene's test was applied to evaluate the homogeneity of variances. For comparisons between the patient and control groups, the independent samples *t*-test was used for normally distributed data, and the Mann–Whitney *U*-test was used for non-normally distributed data. When comparing more than two groups, one-way analysis of variance (ANOVA) was applied to normally distributed data, whereas the Kruskal–Wallis *H* test was used for non-parametric comparisons.

Post hoc analyses were conducted using the Bonferroni test for data with homogeneous variances and the Tamhane test for data with unequal variances. Pearson's correlation test was used for normally distributed variables, while Spearman's correlation test was applied for non-normally distributed data. A *p*-value less than 0.05 was considered statistically significant. Survival analyses were performed using the Kaplan–Meier method to estimate median overall survival (mOS) across subgroups defined by VEGFR expression levels – quantified either as percentages or mean fluorescence intensity (MFI) values. Optimal cut-off values were determined based on median stratification, and survival comparisons between groups were performed using the log-rank test. A *p*-value <0.05 was considered statistically significant.

Results

Among the CRC cases included in the study, 30 patients (57.7%) were male and 22 (42.3%) were female, with a mean age of 63.17±10.93 years (range=36-82 years). In the control group, 16 individuals (53.3%) were male and 14 (46.7%) were female, with a mean age of 58.27±17.65 years (range=31-86 years). There were no statistically significant differences between the patient and control groups in terms of age or sex distribution ($p>0.05$). Based

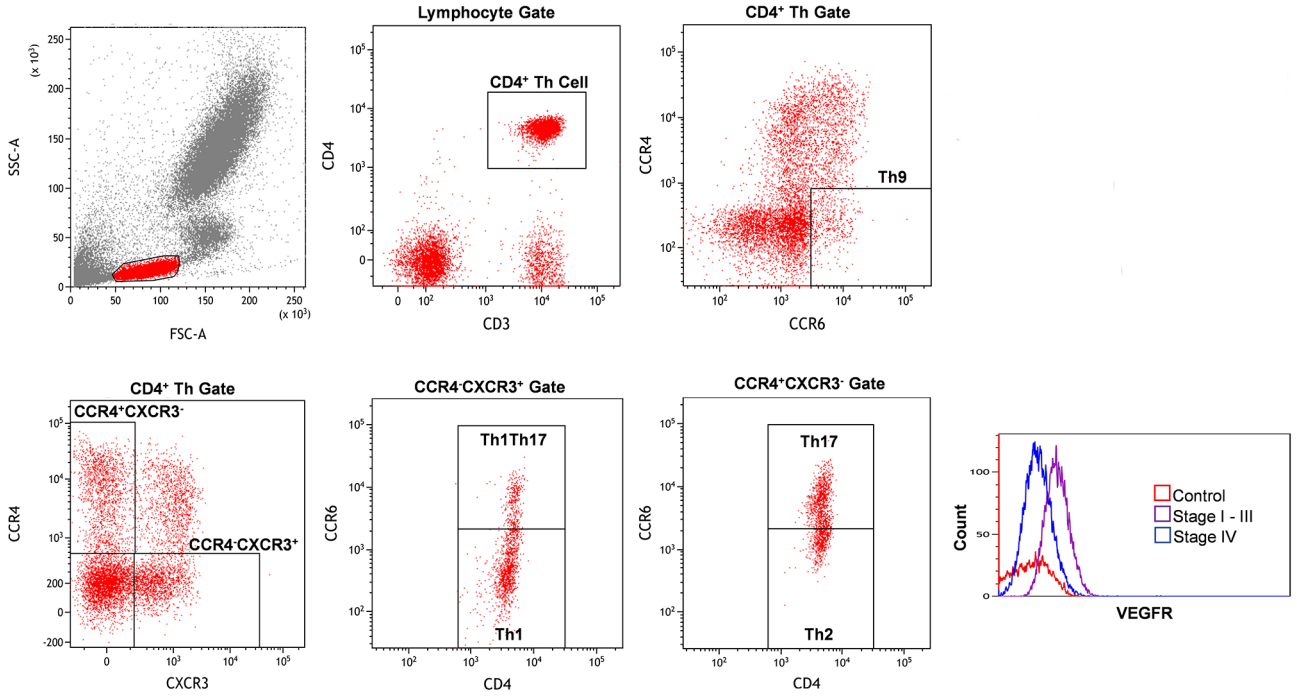

Figure 1. *Flow cytometry analysis of CD4⁺ Th subsets and VEGFR expression in T cells.*

on the TNM classification system, patients were stratified into two groups: early-stage (Stages I-III, n=24) and advanced-stage (Stage IV, n=28). Biochemical parameters, T cell subsets, and VEGFR2 expression levels in T cell populations were statistically compared between the CRC group and healthy controls, as well as between early- and advanced-stage patient subgroups.

T cell subsets. The distribution of $CD4^+$ helper T cell subsets (Th1, Th2, Th9, Th17, and Th1Th17) was evaluated according to disease stage. Th1 cells were found to be higher in patients with CRC compared to the control group (p=0.04). In advanced-stage patients, Th1 cells were higher compared to controls (p=0.01) (Table I). The data illustrated that Th1 cell percentages are increased in patients with CRC, particularly in those with advanced-stage disease. While Th2 and Th9 percentages appear similar between early- and advanced-stage patients, a modest reduction in Th17 cell percentage is observed in the advanced-stage group (Figure 2).

VEGFR expression. In patients with CRC, VEGFR expression was increased in Th1, Th2, CD8+ and compared to controls (p=0.04, p=0.03, p=0.00, respectively). VEGFR expression in Th1and Th17 was increased in early-stage patients compared to both advanced-stage patients and controls (p=0.01, p=0.04, respectively) (Table II, Figure 3). A heat map showing VEGFR expression in lymphocyte subpopulations and disease stages is presented. In patients with early-stage CRC, VEGFR expression in $CD8^+$ T lymphocytes was lower compared to advanced-stage patients (p=0.04). In contrast, VEGFR expression in $CD8^+$ T lymphocytes was prominent in advanced-stage patients (p=0.04) (Figure 4).

Correlation analyses between hematological and biochemical parameters and surface VEGFR expression on T cell subsets in patients with colorectal cancer were evaluated. Lymphocyte count was negatively correlated with VEGFR expression in lymphocytes. $CD3^+$ T cells, and Th2 cells (p=0.04, p=0.03, and p=0.04, respectively). A positive correlation was found between neutrophil count

Table I. *Comparison of T cell subsets of patients with colorectal cancer.*

Mean±SD (min – max) (%)	All patients (N=52)	Control (N=30)	*p*-Value *	Early stage (n=24)	Advanced stage (n=28)	*p*-Value**
$CD3^+$ T Cell	68.00±8.90 (46.40-83.00)	68.41±7.36 (51.10-80.80)	0.83	69.05±7.84 (48.80-83.00)	67.09±9.77 (46.40-80.90)	0.69
$CD4^+$ Th	37.20±8.78 (14.50-65.70)	41.25±9.23 (23.20-59.60)	0.05	36.95±8.01 (23.60-53.80)	37.40±9.53 (14.50-6570)	0.15
Th1	13.26±7.10 (4.50-51.40)	10.47±3.82 (3.70-21.20)	**0.04**	11.64±4.52 (4.50-20.50)	1,465±857 (4.50-51.40)	**0.045**[a]
Th2	9.78±3.76 (1.60-19.80)	9.75±4.16 (2.20-20.10)	0.97	9.70±3.84 (2.70-16.40)	9.85±3.77 (1.60-19.80)	0.87
Th9	16.01±10.87 (4.10-56.20)	16.00±12.40 (4.10-51.10)	0.83	17.69±9.07 (7.10-43.80)	14.57±12.17 (4.10-56.20)	0.08
Th17	12.47±3.95 (5.80-22.20)	13.19±4.09 (5.40-21.10)	0.43	13.41±4.22 (5.80-22.20)	11.65±3.57 (5.90-21.80)	0.21
Th1Th17	8.98±6.38 (2.10-39.80)	7.82±4.15 (1.70-18.90)	0.71	9.12±4.04 (3.90-16.60)	8.87±7.93 (2.10-39.80)	0.38
$CD8^+$ TL	26.05±8.18 (7.90-38.60)	22.74±8.87 (10.90-50.70)	0.09	27.38±7.49 (11.90-38.60)	24.92±8.70 (7.90-38.30)	0.14

*For the statistical comparison between the patient and control groups, the Independent Samples *t*-Test was used for data following a normal distribution, and the Mann-Whitney *U*-Test was used for data not following a normal distribution. **One-way ANOVA was used for data following a normal distribution, and the Kruskal-Wallis H test was used for data not following a normal distribution. [a]Control - Early Stage: 0.34; Control – Stage 4: 0.01; Early Stage – Stage 4: 0.16. Statistically significant *p*-values are shown in bold.

and VEGFR expression in Th2 and Th9 cells (p=0.03 and p=0.04, respectively). The neutrophil-to-lymphocyte ratio was positively correlated with VEGFR expression in lymphocytes. $CD3^+$ T cells, $CD4^+$ Th cells, and Th2 cells (p=0.01, p=0.01, p=0.01, and p=0.00, respectively). LDH levels were positively correlated with VEGFR expression in Th9 cells (p=0.01). Alkaline phosphatase levels were positively correlated with VEGFR expression in lymphocytes. $CD3^+$ T cells, Th2, and Th17 cells (p=0.02, p=0.04, p=0.04, and p=0.04, respectively) (Table III).

Overall survival analysis. In the survival analysis of patients with colorectal cancer, significant differences in median overall survival (mOS) were observed based on lymphocyte subset percentages. Patients with a lymphocyte percentage >29.2% had significantly longer mOS compared to those with lower values [51.8 months; 95% confidence interval (CI)=43.0-60.6 *vs*. 30.5 months; 95%CI=23.5-37.6; p=0.004). Similarly, a neutrophil-to-lymphocyte ratio (NLR) ≤1.87 was associated with improved survival outcomes (49.5 months; 95%CI=39.1-59.9 *vs*. 32.0 months; 95%CI=25.1-38.9; p=0.015). When evaluated by T-helper cell subtypes, patients with Th1 levels ≤14.7% demonstrated significantly prolonged mOS (43.4 months; 95%CI=36.5-50.4) compared to those with higher levels (21.7 months; 95%CI=12.6-30.9; p=0.002). In contrast, no significant survival difference was observed for Th2 levels (p=0.205). Higher levels of Th9 (>10.7%) and Th17 (>11.2%) were both associated with significantly improved mOS (Th9: 45.3 *vs*. 24.6 months; p=0.001; Th17: 44.1 *vs*. 28.8 months; p=0.027). For $CD8^+$ cytotoxic T lymphocytes (CTLs), lower levels (≤20.6%) were associated with a numerically longer mOS, although the difference did not reach statistical significance (44.5 *vs*. 35.2 months; p=0.186) (Figure 5).

In patients with CRC, mOS was further analyzed according to VEGFR expression levels across lymphocyte subsets. Patients with low VEGFR expression in Th1 cells (≤212 MFI) had significantly shorter survival compared to those with higher expression levels (41.5 months; 95%CI=33.5-49.6 *vs*. 30.7 months; 95%CI=22.1-39.3; p=0.018). Similarly, low VEGFR expression in Th2 (≤268

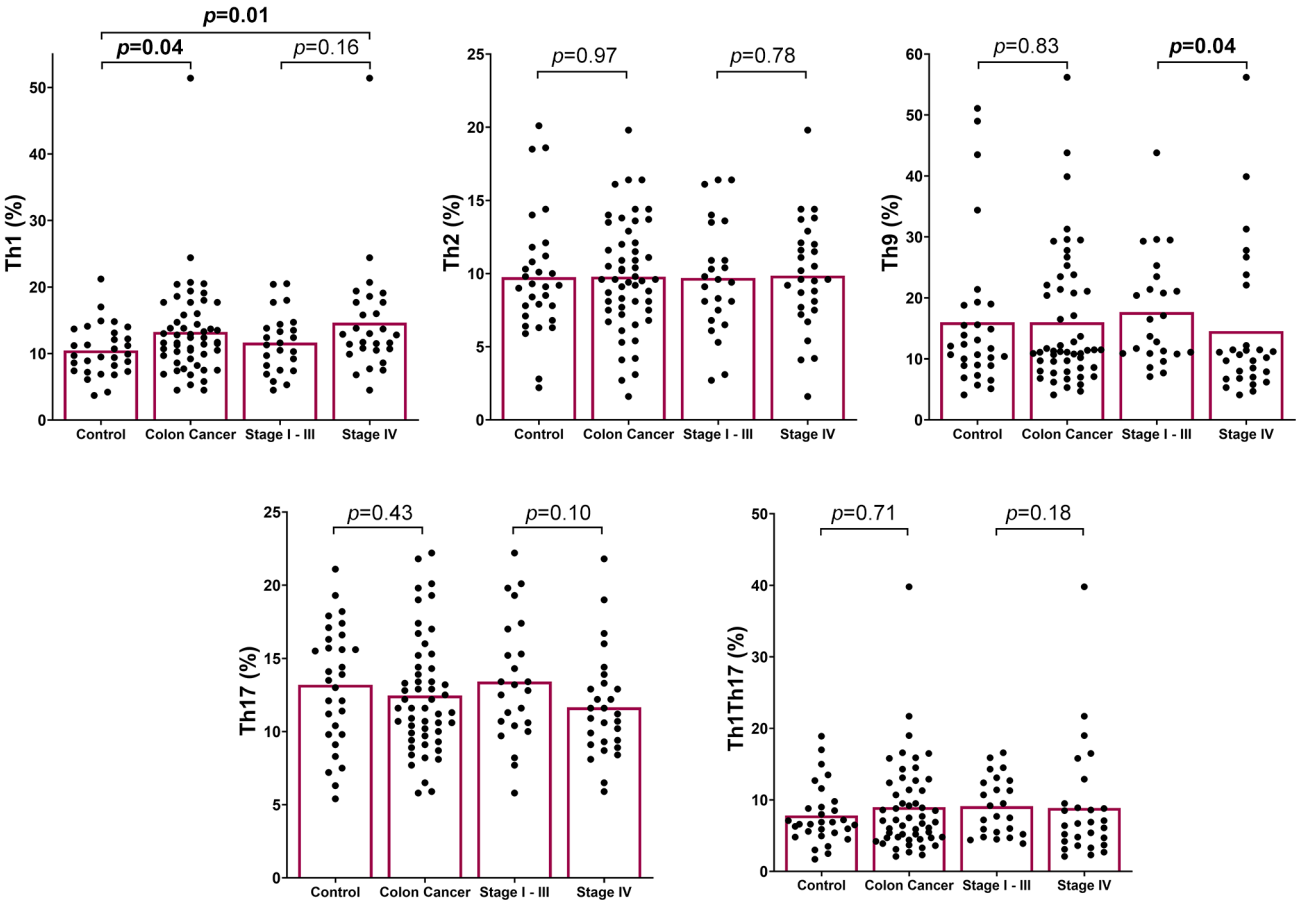

Figure 2. *The distribution of helper T cell (Th) subsets according to stages of colorectal cancer. The percentages of Th1, Th2, Th9, Th17, and Th1Th17 cells were compared between the healthy control group, all patients with colorectal cancer (CRC). and subgroups of early-stage (Stage I-III) and advanced-stage (Stage IV) patients. Compared to controls, Th1 cell percentages are significantly higher in patients with CRC (p=0.04), with advanced-stage patients showing the largest increase (p=0.01). Advanced-stage patients have a slightly lower Th17 cell percentage, but there is no discernible difference in Th2 or Th9 cell percentages between the early and advanced-stage groups. The Th1 and Th17 percentages do not show any statistically significant variations. Individual samples are represented by each dot, while mean percentage values are shown by bars, p-values that are statistically significant are displayed.*

MFI) and Th17 (≤285 MFI) subsets was associated with poorer survival outcomes (Th2: 48.1 months; 95%CI=35.7-60.6 *vs*. 35.3 months; 95%CI=28.6-41.9; p=0.031; Th17: 49.9 months; 95%CI=36.9-62.8 *vs*. 35.3 months; 95%CI=28.6-41.9; p=0.031). No statistically significant differences in overall survival were observed based on VEGFR expression in total lymphocytes, $CD3^+$, $CD4^+$, Th9, or $CD8^+$ cytotoxic T lymphocyte (CTL) subsets (p>0.05). For example, patients with low VEGFR expression in $CD3^+$ T cells (≤203 MFI) had an mOS of 30.7 months (95%CI=18.9-42.6; p=0.121), whereas those with higher expression had an mOS of 39.0 months (95%CI=32.0-45.9) (Figure 6).

Discussion

Our study evaluated peripheral T-cell subsets and the expression levels of VEGFR in these cells in patients with CRC. It is the first to examine the significance of VEGFR expression on peripheral T-lymphocyte subsets in CRC. In patients with CRC, Th1 cell levels were found to be higher, particularly in those with advanced-stage disease. The

Table II. *Distribution of VEGFR expression (MFI) across T cell subsets in colorectal cancer patients: comparison between control, early-stage, and advanced-stage groups.*

Mean±SD (min – max)	All patients (N=52)	Control (N=30)	*p*-Value*	Early stage (n=24)	Advanced stage (n=28)	*p*-Value**
Lymphocyte VEGFR (MFI)	231.60±42.83 (121-344)	224.13±32.99 (146-303)	0.41	240.71±28.91 (200-299)	223.80±51.14 (121-344)	0.17
$CD3^+$ T Cell VEGFR (MFI)	231.15±43.04 (121-343)	222.83±32.53 (146-302)	0.36	241.21±29.79 (198-298)	222.54±50.76 (121-343)	0.14
$CD4^+$ Th VEGFR (MFI)	233.69±43.39 (120-337)	224.33±33.55 (147-302)	0.15	244.25±30.10 (204-318)	224.64±50.99 (120-337)	0.09
Th1 VEGFR (MFI)	222.94±44.28 (115-317)	206.83±26.79 (142-269)	**0.04**	239.25±34.38 (194-304)	208.96±47.51 (115-317)	**0.01**[a]
Th2 VEGFR (MFI)	238.08±39.85 (121-332)	227.07±30.09 (159-302)	**0.03**	247.08±30.64 (197-308)	230.36±45.45 (121-332)	0.11
Th9 VEGFR (MFI)	225.27±45.23 (121-356)	225.20±61.31 (134-496)	0.39	223.75±35.40 (190-356)	218.00±51.75 (121-349)	0.43
Th17 VEGFR (MFI)	243.58±47.54 (61.00-349)	237.73±27.51 (181-290)	0.18	258.38±30.01 (206-313)	230.89±56.04 (61-349)	**0.04**[b]
Th1Th17 VEGFR (MFI)	231. 65±52.02 (118-349)	243.43±100.68 (134-635)	0.34	236.54±33.32 (194-330)	227.46±64.23 (118-349)	0.56
$CD8^+$ TL VEGFR (MFI)	259.21±51.84 (181-446)	238.77±88.70 (179-696)	**0.00**	271.92±54.71 (224-446)	248.32±47.53 (181-383)	**0.00**[c]

*For the statistical comparison between the patient and control groups, the Independent Samples t-Test was used for data following a normal distribution and the Mann-Whitney *U*-Test was used for data not following a normal distribution. **One-way ANOVA was used for data following a normal distribution and the Kruskal-Wallis H test was used for data not following a normal distribution. [a]Control - Early Stage: 0.003; Control - Advanced Stage: 0.67; Early Stage – Advanced Stage: 0.01. [b]Control - Early Stage: 0.02; Control - Advanced Stage: 0.90; Early Stage – Advanced Stage: 0.03. [c]Control - Early Stage: 0.00; Control - Advanced Stage: 0.11; Early Stage – Advanced Stage: 0.11. Statistically significant *p*-values are shown in bold. VEGFR: Vascular endothelial growth factor receptor; MFI: median fluorescence intensity; $CD3^+$ T cell: total T lymphocytes; $CD4^+$ Th: helper T lymphocytes; Th1, Th2, Th9, Th17, Th1Th17: helper T-cell subsets; $CD8^+$ TL: cytotoxic T lymphocytes; All patients: entire study cohort; Control: healthy controls; Early stage: stage I-III disease; Advanced stage: stage IV disease; SD: standard deviation; min–max: minimum and maximum values.

infiltration of Th1 cells and the levels of secreted cytokines in CRC tissue may enhance cancer cell apoptosis, reduce angiogenesis, and attract cytotoxic $CD8^+$ T cells to the tumor microenvironment, which is generally associated with a better prognosis (8, 9). The observation that Th1 cells were higher in patients with advanced-stage CRC may initially seem contradictory, given that Th1 responses are generally associated with a favorable prognosis. Similarly, Lee *et al.,* reported that patients with advanced-stage CRC exhibited higher levels of peripheral Th1 cells compared with those with early-stage disease (10). However, this finding may reflect a compensatory immune mechanism whereby the host attempts to mount a stronger Th1-mediated response against increasing tumor burden. In this context, elevated Th1 cell levels in advanced disease may not signify effective tumor control, but rather a reactive immune activation attempting to counteract tumor progression. These results suggest a potential shift toward Th1-mediated immune responses with disease progression, which may reflect immune activation

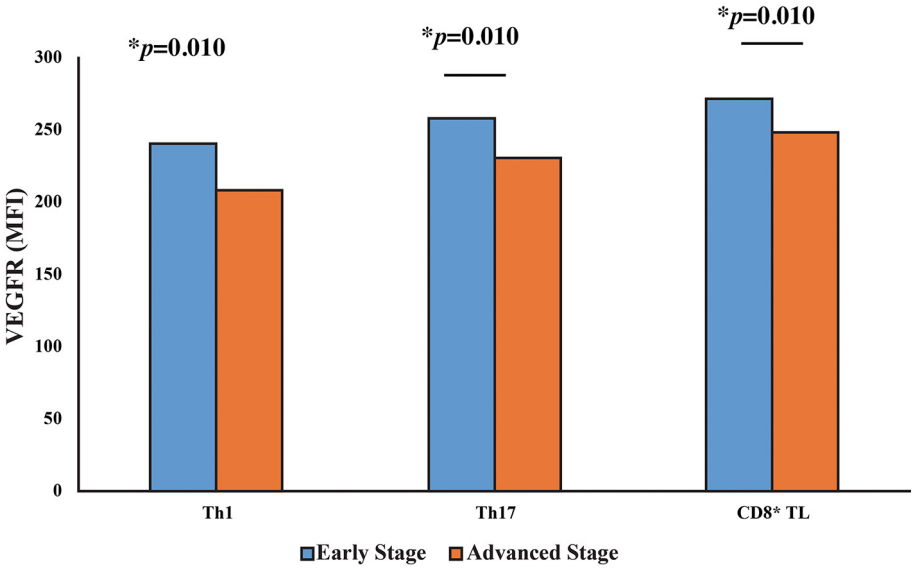

Figure 3. *Comparison of VEGFR expression (MFI) in T cell subsets according to disease status and stage in colorectal cancer. *p<0.05.*

or exhaustion patterns in tumor microenvironment dynamics. Th1 and functional CD8⁺ T cells are crucial for anti-tumor immunity; however, immunosuppressive cytokines such as TGF-β and IL-10 can diminish CD8⁺ T cell cytotoxicity, leading to functional exhaustion and indirectly facilitating tumor progression.

In patients with CRC, VEGFR expression was higher in Th1, Th2, CD8⁺ CTLs, compared to the control group. Specifically, our study assessed VEGFR-2 expression. VEGFR-2, also known as KDR, one of the three VEGF receptors, has been extensively studied as a target for the development of new anti-cancer agents. The KDR gene encodes VEGFR-2, which shows overexpression in endothelial cells. When bound to VEGF-A, VEGFR-2 is activated and initiates a phosphorylation process leading to increased endothelial cell proliferation and migration (11).

VEGFR-2 exhibits over-expression in neovascular tumor endothelial cells compared to normal endothelial cells (12). Over-expression of KDR has also been observed in various cancer types including breast cancer, CRC, non-small cell lung cancer, urothelial cancer, malignant melanoma, and B-cell lymphoma (13). It has been shown that VEGF-A reduces the cytotoxic activity of T cells. The addition of VEGF-A significantly reduced the number and

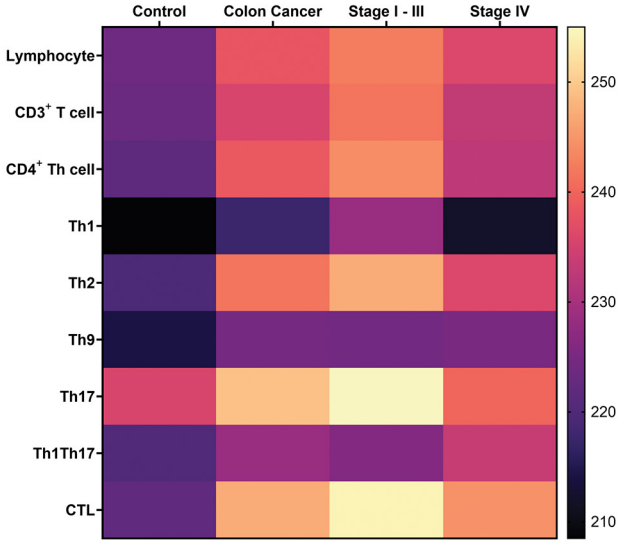

Figure 4. *Heatmap visualization of VEGFR surface expression [median fluorescence intensity (MFI)] in peripheral lymphocyte subsets according to disease stage in colorectal cancer. MFI values are presented for each lymphocyte subset (Lymphocytes, CD3⁺ T cells, CD4⁺ Th cells, Th1, Th2, Th9, Th17, Th1Th17, and CTL) across control subjects, all patients with CRC and subsets by stage (Stage I-III and Stage IV).*

proliferation rate of T cells in culture in a dose-dependent manner and CD3⁺ T cells expressed VEGFR-2 on their surface upon activation. Experiments with specific anti-

Table III. *Correlations between hemogram and biochemical parameters and T cell surface VEGFR expression (MFI) in patients with colorectal cancer.*

C.C *p*-Value	Ln Count	Nt Count	N/L Ratio	CRP	LDH	Alb.	Alkaline phosphatase	Alb./Alk. Phosph.	CEA	CA 19.9
Lymphocyte										
VEGFR (MFI)	**–0.28**	0.23	**0.35**	–0.05	0.17	0.02	**0.32**	–0.24	0.03	–0.09
	0.04	0.10	**0.01**	0.75	0.24	0.89	**0.02**	0.08	0.81	0.53
$CD3^+$ T Cell										
VEGFR (MFI)	**–0.30**	0.22	**0.35**	–0.09	0.14	0.14	**0.29**	–0.17	0.01	–0.10
	0.03	0.11	**0.01**	0.52	0.34	0.32	**0.04**	0.23	0.95	0.50
$CD4^+$ Th										
VEGFR (MFI)	–0.28	0.21	**0.35**	–0.09	0.13	0.14	0.28	0.12	–0.00	–0.12
	0.05	0.13	**0.01**	0.54	0.36	0.31	0.05	0.38	0.98	0.42
Th1										
VEGFR (MFI)	–0.16	0.15	0.18	–0.24	0.09	0.22	0.09	0.01	–0.17	–0.14
	0.27	0.29	0.19	0.09	0.52	0.12	0.53	0.96	0.25	0.34
Th2										
VEGFR (MFI)	**–0.29**	**0.31**	**0.41**	–0.01	0.16	0.12	**0.29**	–0.18	0.11	–0.06
	0.04	**0.03**	**0.00**	0.97	0.25	0.39	**0.04**	0.22	0.44	0.68
Th9										
VEGFR (MFI)	–0.12	**0.29**	0.25	–0.10	**0.36**	0.06	0.23	–0.18	0.03	–0.03
	0.39	**0.04**	0.07	0.51	**0.01**	0.66	0.11	0.21	0.82	0.82
Th17										
VEGFR (MFI)	–0.27	0.10	0.25	–0.17	0.08	0.16	**0.28**	–0.12	–0.10	–0.13
	0.05	0.47	0.07	0.23	0.59	0.27	**0.04**	0.41	0.51	0.36
Th1Th17										
VEGFR (MFI)	–0.14	0.28	0.26	–0.04	0.25	0.02	0.18	–0.11	–0.01	0.01
	0.32	0.05	0.06	0.78	0.07	0.90	0.21	0.43	0.96	0.94
$CD8^+$ TL										
VEGFR (MFI)	0.03	–0.04	0.03	0.14	–0.00	–0.15	0.02	–0.04	–0.22	–0.23
	0.85	0.78	0.85	0.32	0.98	0.29	0.92	0.76	0.12	0.11

Statistically significant *p*-values are shown in bold. C.C: Colorectal cancer; Ln Count: absolute lymphocyte count; Nt Count: absolute neutrophil count; N/L Ratio: neutrophil-to-lymphocyte ratio; CRP: C-reactive protein; LDH: lactate dehydrogenase; Alb: serum albumin; alkaline phosphatase: serum alkaline phosphatase; Alb./Alk. Phosph: albumin-to-alkaline phosphatase ratio; CEA: carcinoembryonic antigen; CA 19-9: carbohydrate antigen 19-9. Regarding immune cell subsets: $CD3^+$ T Cell: total T lymphocytes; $CD4^+$ Th: helper T lymphocytes; Th1, Th2, Th9, Th17, and Th1Th17: helper T-cell subsets; $CD8^+$ TL: T lymphocytes; VEGFR: vascular endothelial growth factor receptor; MFI: median fluorescence intensity represents the quantitative level of surface expression measured by flow cytometry.

VEGFR-2 antibodies revealed that the direct suppressive effect of VEGF on T-cell proliferation is mediated by VEGFR-2 (14).

In a melanoma mouse model, blocking VEGF signaling with sunitinib led to an increase in CXCL10 and CXCL11 levels in tumor vessels, and an 18-fold increase in $CD3^+$ T-lymphocyte infiltration in tumors (15). VEGF signaling directly affects T-cell development, homing, and cytotoxic functions. Additionally, VEGF promotes the formation of abnormal tumor vessels, adversely affecting T-cell migration from lymph nodes to the tumor bed (16). The VEGF/VEGFR-2 pathway also suppresses $CD3^+$ T cells and their cytotoxic effects. Manzoni and his colleagues examined the effects of bevacizumab on T-cell and B-cell levels in a group of 51 patients with metastatic colorectal cancer. The baseline levels of T-cells and B-cells were lower than those of a group of healthy participants. Treatment with bevacizumab led to a significant increase in T-cell and B-cell levels, perhaps due to neutralization of VEGF inhibitory effect of dendritic cell (DC) maturation. However, the correlation between increased levels of T-cells and B-cells and a better clinical outcome did not reach statistical significance (17). Our study indicates that the increase in VEGFR expression in T lymphocyte subsets

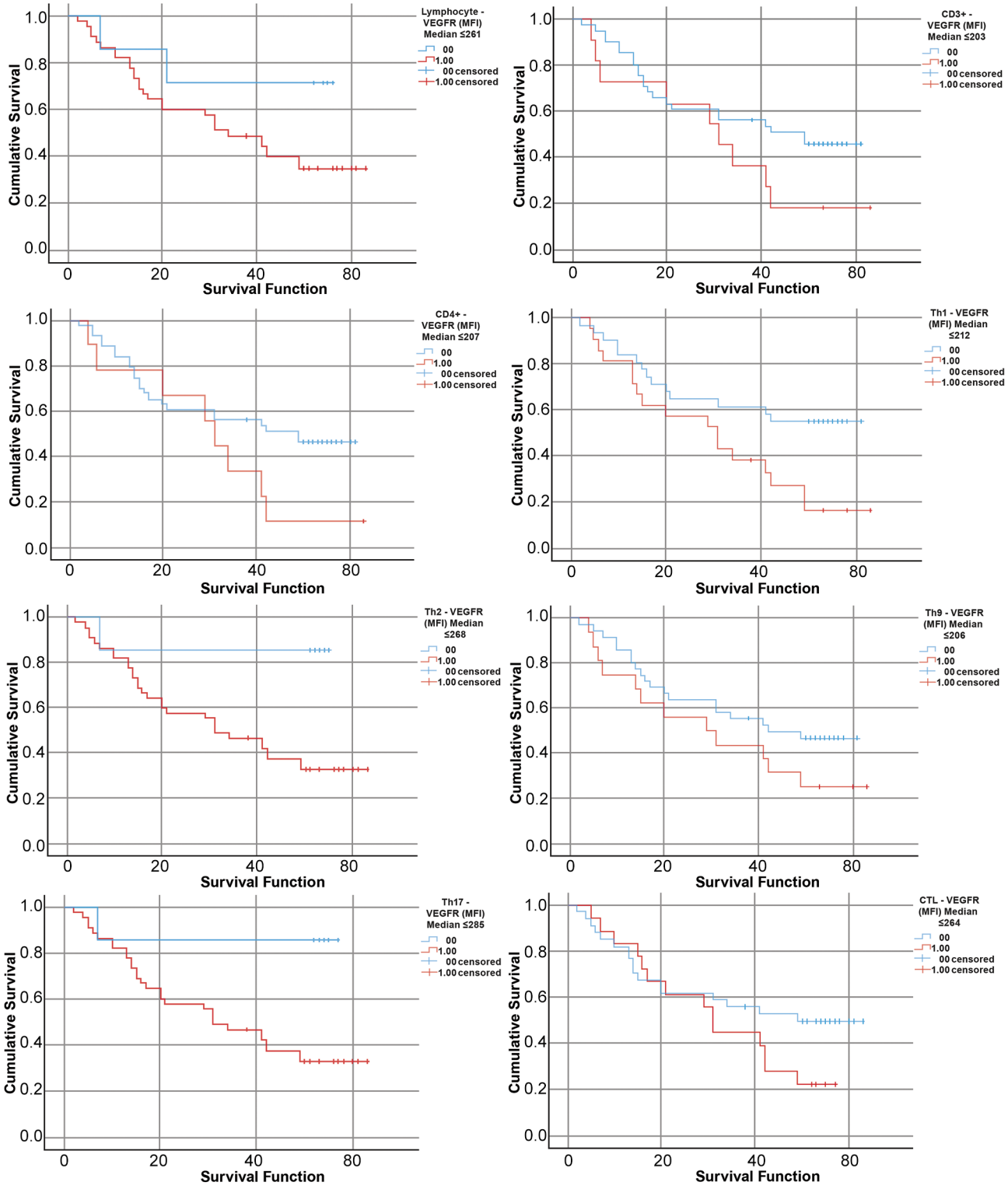

Figure 5. *Analysis of median overall survival according to VEGFR expression in patients with colorectal cancer.*

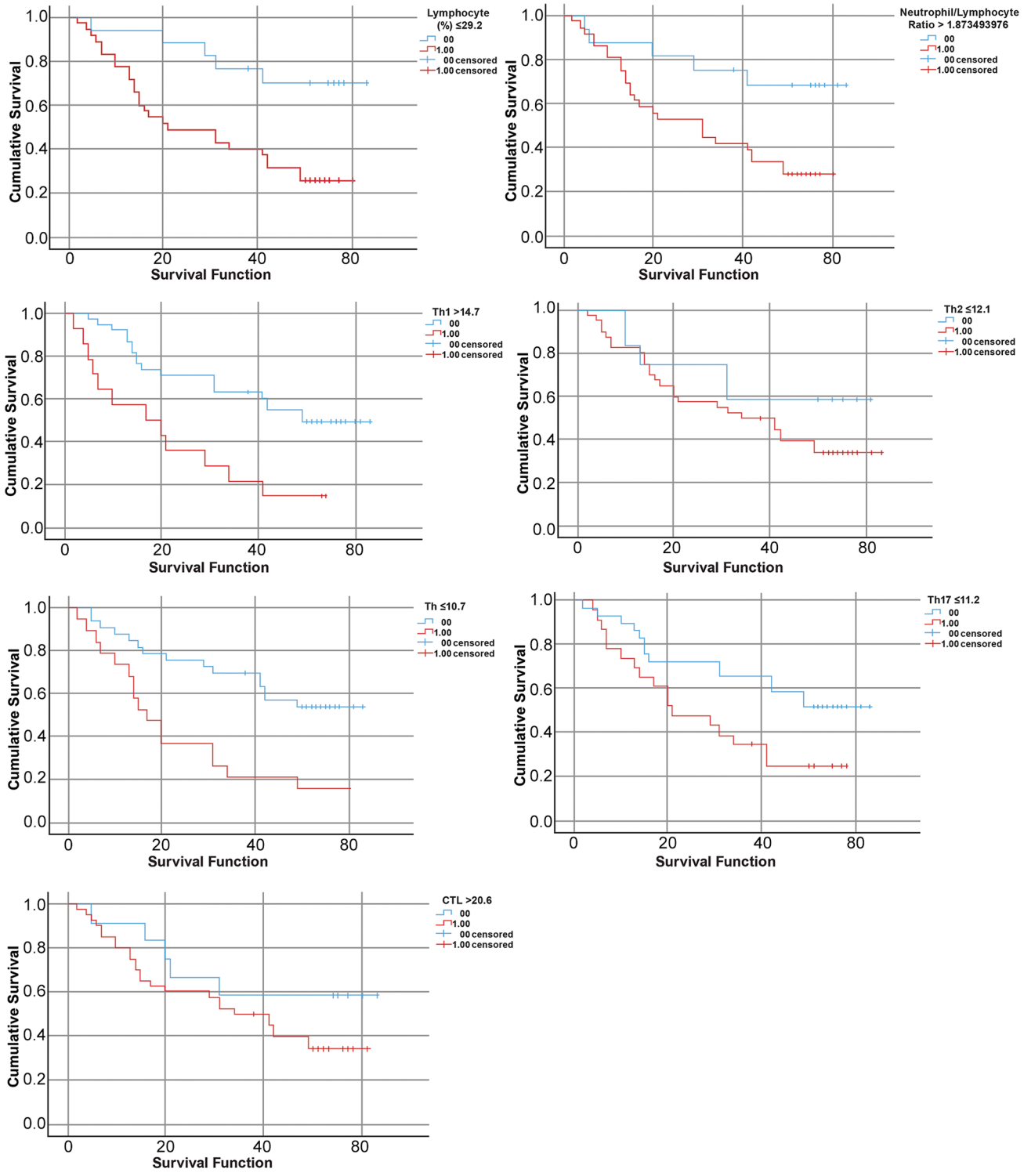

Figure 6. *The effect of lymphoid cell subsets on median overall survival in patients with colorectal cancer.*

in patients with CRC negatively impacts overall survival with decreasing T cell migration and cytotoxic functions.

In this study, the VEGFR expression levels of Th1 and Th17 in early-stage patients were higher compared to both advanced-stage patients and the control group. These findings suggest that T-cell migration is adversely affected at the very onset of the disease during the early stages. The VEGFR expression levels of $CD8^+$ CTLs in patients with early-stage CRC were lower than those in patients with advanced-stage disease. This may indirectly indicate that $CD8^+$ CTLs have a higher potential for migration to the tumor tissue in advanced-stage disease compared to early-stage. An increase in VEGFR expression was observed in $CD8^+$ CTLs. These results suggest that, compared to healthy individuals. immune cell migration in patients with advanced-stage CRC is likely impaired. It has been previously shown that the expression levels of VEGF were found to be associated with decreased $CD8^+$ TH1 cell response on colorectal tumors (18).

According to correlation tests, lymphocyte count was negatively correlated with Th17. In our study, there was no difference in Th17 cell count compared to the control group. These results once again suggest that immune tolerance, rather than inflammation, is more prominent in CRC. The NLR was negatively correlated with $CD3^+$ T cells (p=0.01) and positively correlated with Th17. The NLR, calculated as a simple ratio between neutrophil and lymphocyte counts measured in peripheral blood, serves as a biomarker that combines two aspects of the immune system. It is primarily used to compare the innate immune response, driven by neutrophils, with the adaptive immune response, supported by lymphocytes (19). An increased NLR has been shown to be a poor prognostic factor in CRC, as in many other cancers (20). In our study, a positive correlation was found between Th17 and NLR. Unlike Th1 and Th2 cells, Th17 cells produce IL-17. Recent research has shown that IL-17 signaling promotes tumor growth (21).

The survival analysis demonstrated that VEGFR-mediated regulation within T-cell subsets and systemic immune balance have significant prognostic importance in colorectal cancer. A low NLR and high lymphocyte percentage were associated with longer overall survival. indicating that adequate immune competence plays a decisive role in disease control. It is noteworthy that lower Th1 levels were associated with prolonged overall survival, suggesting that excessive Th1 activation may reflect an exhausted or dysfunctional immune phenotype rather than an effective antitumor response. Conversely, elevated Th9 and Th17 levels were associated with superior overall survival. This finding suggests that proinflammatory subsets may enhance antitumor immunity by promoting T-cell infiltration into the tumor microenvironment and facilitating effector T-cell activation. The secretion of IL-9 from Th9 cells, which supports dendritic cell maturation and $CD8^+$ T-cell activation, and the production of IL-17 from Th17 cells, which enhances effector cell migration in specific immune contexts. further support this observation. The hypothesis that prolonged survival in patients with lower $CD8^+$ cytotoxic T lymphocyte levels is associated with impaired migration capacity and reduced toxicity due to chronic stimulation merits further investigation. The correlation between elevated VEGFR expression in Th1, Th2, and Th17 subsets and diminished survival indicates that VEGFR signaling contributes to reduced antitumor activity and immune dysfunction. Taking these findings together, it can be concluded that VEGFR expression may influence prognosis not only through its effects on angiogenesis but also *via* immune-mediated mechanisms.

Conclusion

This study is the first to comprehensively evaluate the distribution of peripheral T lymphocyte subgroups and the expression of VEGFR on these cells in patients with CRC. Elevated Th1 levels and increased expression of VEGFR in multiple T cell subsets were observed, particularly in early-stage patients. suggesting early immune dysregulation. Notably, increased VEGFR expression in $CD3^+$ and $CD4^+$ T cells was significantly associated with reduced overall survival, highlighting the prognostic importance of

VEGFR-mediated immune suppression. Furthermore, correlations between immunophenotypic markers and hematologic/biochemical parameters, including NLR and carcinoembryonic antigen (CEA). support the role of T cell exhaustion and impaired migration in poor prognosis. By demonstrating the clinical relevance of VEGFR expression on peripheral lymphocyte subsets, this study provides novel insights into tumor-induced immune modulation and underscores the need for further research on their potential as prognostic biomarkers and therapeutic targets in CRC.

Conflicts of Interest

The Authors declare no competing interests in relation to this study.

Authors' Contributions

Concept: MA, AÇ, HA, ME. Design: MA, OY. Supervision: all Authors. Data collection and/or processing: all Authors. Analysis and/or interpretation: OY, FS, MA. Literature search: MA, AÇ, HA, ME. Writing: OY, MA. Critical reviews: all Authors.

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Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine learning software, were used in the preparation, analysis, or presentation of this manuscript.

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