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Interleukin-6-Positive Immune Cells as a Possible New Immunologic Marker Associated With the Colorectal Cancer Prognosis

Maya Gulubova, MD, PhD,† Dimitur Chonov, MD,‡ Elina Aleksandrova, PhD,§ Koni Ivanova, MD, PhD,* M. Magdalena Ignatova, MD,* and Tatyana Vlaykova, PhD§‖*

Abstract: Chronic inflammation creates tumor microenvironment (TME) that facilitates colorectal cancer (CRC) cell proliferation, migration, metastasis, and tumor progression. Interleukin-6 (IL-6) is a proinflammatory cytokine with a pleiotropic effect on CRC development. We aimed to evaluate IL-6 expression in tumor cells and in immune cells in TME, to assess the serum level and IL6 -174 G/C genotype distribution and to correlate the results with selected morphologic and clinical parameters that may add useful information in understanding the mechanisms of human CRC progression. A total of 153 patients with CRC were recruited in the current study. We assessed the IL-6 serum concentration through the ELISA method, the expression of IL-6 in tumor and in immune cells by immunohistochemical and double immunofluorescence staining, the MSI status by immunohistochemistry for 4 mismatch repair (MMR) proteins, and the genotype distributions for IL6 -174G/C (rs1800795) single-nucleotide polymorphism through PCR-RFLP method. Our results showed that serum IL-6 level were increased in CRC patients as compared with healthy controls ($P < 0.0001$), and in patients with cancers with advanced histologic type (type IV). However, the higher concentration (above the median of 55.71 pg/mL) was with borderline association with longer survival of the patients after surgical therapy ($P = 0.055$, Log rank test). We also found that IL-6$^+$ immune cells prevailed in the invasive front (IF) of tumors compared with the tumor stroma (TS) ($P < 0.0001$). More IL-6$^+$ cells were recruited in the tumors with less advanced histologic type (I+II), with stronger inflammatory infiltrate in the IF, in early pTNM stages (I+II), without lymph node and distant metastases and the higher levels of IL-6$^+$ cells, especially in the IF, were associated with longer survival ($P = 0.012$). The results of our study suggest that although the serum levels of IL-6 are higher in CRC, the increased IL-6$^+$ cells in tumor microenvironment, both in the invasive front and in tumor stroma, as well as the higher serum levels are associated with good prognostic variables and longer survival of the patients mainly in the early stages of CRC.

Key Words: IL-6, Colorectal cancer, immunohistochemistry, polymorphism, immunofluorescence

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From the *Department of General and Clinical Pathology, Forensic Medicine and Deontology; ‡Department of Surgery; §Department of Medical Chemistry and Biochemistry, Medical Faculty, Trakia University, Stara Zagora, Bulgaria; †Department of Anatomy, Histology and Embriology, Pathology, Latin language, Forensic Medicine and Deontology, Faculty of Medicine, University Prof. Dr. Assen Zlatarov, Burgas; and ‖Department of Medical Biochemistry, Faculty of Pharmacy, Medical University of Plovdiv, Plovdiv, Bulgaria.

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Reprints: Elina Aleksandrova, PhD, Department of Medical Chemistry and Biochemistry, Medical Faculty, Stara Zagora 6000, Bulgaria (e-mail: elina.aleksandrova@trakia-uni.bg).

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Colorectal cancer (CRC) has increasing prevalence in the world. Chronic inflammation creates tumor microenvironment (TME) that facilitates tumor cell proliferation, migration, metastasis, and tumor progression.[1] Numerous inflammatory cytokines have been investigated in the diagnosis and prognosis of CRC.[2–7] Among these, interleukin-6 (IL-6) has important impact on CRC development.[8–11] It is a pleiotropic cytokine with distinguished protumor functions stimulating tumor cell proliferation, angiogenesis, metastasis, and tumor progression.[11–13] However, several studies have reported the associations of IL-6 with good prognosis of cancers with different histologic origin.[14,15] Similarly, the higher mRNA expression of IL-6 in malignant melanoma has been reported to associate with longer survival of the patients in comparison with lower mRNA levels, while no such associations have been observed for head and neck squamous cell carcinoma (HNSCC), ovarian and breast cancers.[16]

IL-6 is produced mainly by monocyte/macrophages in acute inflammation and by T lymphocytes in chronic inflammation.[17,18] IL-6 is also secreted by other immune

cell types, endothelial cells, stromal fibroblasts, and tumor cells themselves.[19,20] In a recent review, the recruited mast cells were shown to release a vast range of cytokines including IL-6, and were involved in the inflammation, leading to inhibition of tumor cell growth, and stimulating tumor cells' apoptosis.[21] The characterization of the immune status of the tumor is highly reliable in determining CRC prognosis[22] and lymphocytes and macrophages are important elements in the evaluation of the immunoscore, that is, the quantitative digital pathology assay for assessing the immune response in cancers.[23]

IL-6 binds its receptor, the IL-6 receptor (IL-6R) and signal-transducing membrane protein, glycoprotein 130 (gp 130) starting 2 signaling pathways, "classic" and "trans-signaling."[8,24] The "classic" signaling pathway is processed in the early immune responses[25] whereas the "trans-signaling" pathway takes part in the T-cell response[26] and in carcinogenesis.[27]

Increased serum levels of IL-6 in CRC patients, studied before surgery, has been associated with disease development and poor prognosis[3,28] and with certain clinical parameters such as tumor invasion, advanced tumor stage, the risk of development of CRC.[4,29] However, opposite results were reported for colorectal and breast cancers in early stages.[14,15]

IL-6 immunoreactivity has been investigated in the cytoplasm of tumor cells in a large group of CRC patients (n = 160).[10] The IL-6-positive (IL-6$^+$) tumor group has been associated with advanced tumor stage, lymph node metastasis, and vascular invasion and do not correlate with serum IL-6 levels.[10] Others reported that IL-6-positivity is higher in tumor cells than in normal mucosal cells, and expressed in metastasis in lymph nodes and in the invasive front of tumors.[30] IL-6 produced by murine CT26 CRC cell line exerts tumor promotion.[31]

IL-6$^+$ immune cells in CRC tissue have been described by a few authors.[2,10,25,32] As an indispensable regulatory signaling molecule, IL-6 mediates T-helper 17 (Th17) and T-regulatory (Treg) cell function in TME.[2,33] IL-6 has suppressive effects on dendritic cells (DC)-mediated Th1 cell responses,[34] and downregulates HLA class II and IL-12 production of human DCs.[19]

In addition to the role of IL-6 expression in CRC genetic polymorphisms in the *IL6* gene have also been studied. Functional studies suggest that the -174G/C polymorphism influences transcription, with the G-allele being associated with higher IL-6 expression (carriers of the GG/GC genotypes) compared with those of the CC genotype exhibiting lower IL-6 levels.[35] The *IL6* -174 G/C (rs1800795) SNP distribution among different populations has been studied but the results were inconsecutive and often conflicting.[36]

Given the above, we aimed to evaluate IL-6 expression in tumor cells and in immune cells in TME and to correlate the results with selected morphologic and clinical parameters that may add useful information in understanding the mechanisms of human CRC progression. However, the histologic evidence for IL-6 expression in CRC is not fully investigated. Since IL-6$^+$- macrophages and lymphocytes are abundant in CRC tissue we aimed to use their number in the immune score of CRC. Our study has also examined the association of serum IL-6 levels with clinicopathologic features of CRC and the association of *IL6* -174 A/G SNP with CRC susceptibility and severity.

MATERIALS AND METHODS

Patients

The study group consisted of 153 CRC patients (age range: 55 to 88 y; 76 men, 77 women) that have undergone resection for localized CRC in the period of 1996 to 2019 at Department of Surgery, University Hospital "Prof St Kirkovich" Stara Zagora. An informed consent has been obtained from all patients before operation and the study have been approved by the Local Ethical Committee of the Medical Faculty, Trakia University-Stara Zagora, protocol №. 23/10.03.2023.

Clinical and demographic data of patients are presented in Table 1. Tumors are located in the cecum in 7 patients, ascending colon in 39 patients, transverse colon in 13 patients, descending colon in 11 patients, in sigmoid colon in 24 patients, and rectum in 59 patients. Classifications according the fifth and sixth edition of the TNM system were applied. Complete clinical and oncologic data including survival rates were available from the oncologic archives for 122 patients. The patients were followed up until October 2022. At the end of the follow-up period 69 patients survived. The median survival period for all patients was 32.20 months, ranging from 0.09 to 165.23 months. The follow-up data have been collected by a surgeon (D.C.), pathologist (K. I.), and oncologist (M.M.I.).

The control group consisted of 173 healthy individuals, unaffected by cancer-related conditions with mean age of 59.60 ± 0.87 (SEM) years (median of 60, range of 23-85 y). Eighty four (48.6%) of the controls were males and the rest of 89 (51.4%) were females from the same ethnic group and area of Bulgaria. Control individuals were volunteers or individuals attending prophylactic examinations.

Measurement of IL-6 Serum Concentration

The serum levels of IL-6 were assessed in 43 CRC patients and 64 control individuals with a commercial ELISA kit (cat. no. D6050; R&D Systems Inc.). ELISA method has been conducted by a molecular biologist (E. A.). The serum samples from 43 CRC patients and 64 control individuals, included in the prospective study were collected in the period of 2017 to 2019. They were included in the whole group of 153 patients. The serum samples were collected before operation, stored at -80$^{\text{âµ}}$ C until the measurement was performed when samples were thawed to room temperature and then IL-6 serum concentration of the cancer and the control group was determined on ELISA reader (ELISA reader BioTek ELx800). The IL-6 concentrations were calculated according to the standard curve and are presented in pg/mL serum.

TABLE 1. The Main Clinical and Histologic Parameters of the CRC Patients Group

Parameter	Number (%)
Clinical data	
Sex	n = 153
Male	76 (49.7)
Female	77 (50.3)
Age (y)	
Mean ± SEM	68.24 ± 9.81
Median (range)	70 (55-88)
Localization of the tumor	n = 153
Ascendant colon	39 (25.49)
Transverse colon	13(8.49)
Descendant colon	11 (7.19)
Sigmoid colon	24 (15.69)
Rectum	59 (38.56)
Cecum	7 (4.58)
pTMN stage (5th edition)	n = 153
I	46 (30.1)
II	67 (43.8)
III	31 (20.3)
IV	9 (5.9)
pTNM stage (6th edition)	n = 153
1	46 (30.1)
2A	65 (42.5)
2B	2 (1.3)
3A	5 (3.3)
3B	19 (12.4)
3C	7 (4.6)
4	9 (5.9)
Follow-up after surgery (mo)	n = 122
Mean ± SEM	41.57 ± 3.20
Median (range)	32.20 (0.09-165.23)
Status at the end of follow-up	n = 122
Alive	69 (56.6)
Deceased	53 (43.4)
Histologic data	
Differentiation grade of tumor	n = 153
High	6 (3.9)
Moderate	108 (70.6)
Low	27 (17.6)
Mucinous	12 (7.8)
Histologic type	n = 153
I	20 (13.1
II	33 (21.6)
III	78 (51.0)
IV	22 (14.4)
Inflammatory infiltrate in the invasive front	n = 153
No infiltration	21 (13.7)
Weak (+)	36 (23.5)
Moderate (++)	43 (28.1)
Strong (+++)	53 (34.6)
VELIPI	n = 107
No invasion	65 (60.7)
Yes invasion	42 (39.3)
MMR status	n = 144
MSS	81 (52.9)
MSI	63 (41.2)

MMR indicates mismatch repair; MMS, microsatellite stable; MSI, microsatellite instable; VELIPI, vascular/lymphatic/perineural invasion.

Tissue Samples

Tumor tissue samples have been collected from the invasive front (IF), from the tumor itself and from normal colon tissue (resection lines) measuring 10×15×15 mm and have been fixed in 10% neutral buffered formalin (Merck) for 24 hours and consecutively embedded in paraffin (paraffin dispenser Leica EG1150OH). Hematoxylin and eosin-stained tissue sections are examined (microtome Leica RM2235). Histologic grade, type, tumor site, lymphocyte infiltration, tumor invasion (venous, lymphatic, and perineural), lymph node status, pathologic stage, and survival time have been recorded.

Immunohistochemical Staining

Formalin-fixed paraffin-embedded 4 μm thick sections were dewaxed in xylene and dehydrated serially in graded ethanol. Endogenous peroxide activity has been quenched by incubation in 3% hydrogen peroxide. The avidin-biotin-peroxidase complex technique was applied. IL-6 antibody (NCL = L-IL-16) purchased by Leica and diluted 1:50 was used with the detection system is EnVisionTM FLEX+ System, HRP K8002. The reaction was visualized by a mixture of 3,3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO). The sections were counterstained by Mayer's hematoxylin. The method has been described previously.[32] We chose tumor tissue sections that do not contain necrosis. Negative controls are elaborated using PBS instead of primary antibodies.

Cell Counting

IL-6^+ immune cells were counted in the tumor stroma (TS) and in the invasive front, on 5 fields of vision in the areas with most intense cellular infiltrates (hot spots) at a magnification of 320 (0.74 mm^2 area).

Quantitative Analysis of Immunoreactivity in Tumor Tissue (Tumor Glands)

The expression of IL-6 in tumor tissue was evaluated in deepest invasive sites. The percentage of positively stained tumor cells was assessed for each section after counting of 100 cells per high power field. Expression of IL-6 was classified as positive, when > 30% of tumor cells showed cytoplasmic staining.

Immunohistochemistry for Mismatch Repair (MMR) Proteins (MLH1, MSH2, MSH6, and PMS2)

MSI was evaluated as has been previously described.[31,32] Samples that lack nuclear staining of lymphocytes, some stromal cells, or the nuclei of adjacent normal epithelial cells are considered as uninformative.[37,38] Cancer biopsies were considered MSI when tumor cell nuclei were negative for one or more of the proteins MLH1, MSH2, MSH6, and PMS2. The MSS status was defined when tumor cellular nuclei were positive for all 4 analyzed missmatch repair markers.

Double immunofluorescence for Detection of IL6 and CD3

For double immunofluorescent staining, sections were incubated in primary antibody cocktail against IL-6 (Human Interleukin-6 IgG2a, NCL-L-IL-6, Novocastra; Leica) at a dilution of 1:50 and polyclonal rabbit antihuman CD3 (R5003; DAKO, Denmark) ready-to-use in a moist chamber for 1 hours at room temperature, the mixture solution was rinsed and the sections were washed

3 times in PBS, 5 minutes each wash. After that the sections were washed and incubated in a mixture of secondary antibodies: antihuman IgG (WHOLE MOLECULE) TRITC conjugate antibody (T5268; Sigma-Aldrich Inc.) and antirabbit IgG (WHOLE MOLECULE) FITC conjugate antibody (F 9006; Sigma-Aldrich Inc.) for 1 hour at room temperature in dark, the secondary antibody solution was then rinsed and slides were washed 3 times with PBS (5 min each wash) in dark, seal coverslip with PBS/glycerol was applied to prevent drying and movement under microscope. Control sections were processed as described above. Sections were examined through fluorescence microscopy (Leica DM2500; Germany). Images were analyzed using LAS Leica Microsystems CMS GmbH software.

DNA Isolation

Genomic DNA was extracted from fresh frozen tissues of subjects' tumor biopsies using a genomic DNA purification kit (ThermoFisher Scientific) and DNA from the controls was isolated from blood samples (ThermoFisher Scientific) according to manufacturer's protocol. Extracted DNA was stored at -20°C until further use. The concentration of resulting DNA was measured spectrophotometrically at 260 nm by NanoVue TM Spectrophotometer (Healthcare, Buckinghamshire, UK). The ratio of absorptions at 260 versus 280 nm was calculated to assess the purity of DNA samples.

Genotyping for IL6 -174G > C (rs1800795) Single-Nucleotide Polymorphism

Genotyping according to the *IL6* -174G > C (rs1800795) polymorphism was performed by PCR-RFLP-based methods. In brief, тhe PCR reaction was performed in a mix containing 30 to 50 ng genomic DNA, 0.8 pmol/μl of each primer (IL6-F: 5'-TTG TCA AGA CAT GCC AAG TGC T-3' and IL6-R: 5'-GCC TGA GAG ACA TCT CCA GTC C-3'), 1x Dream Taq Green PCR Master Mix with 2 mM $MgCl_2$ (2x; Fermentas Life Science) and bdH_2O up to the final volume of 12 μl. The temperature profile of the PCR reactions was as follows: denaturing at 95°C for 3 minutes, 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 62°C and polymerization for 30 seconds at 72°C, followed by final elongation at 72°C for 5 minutes.

A volume of 12 μl of the PCR products were digested with 3 U *Hin I* (*Nla III*) (Thermo Fisher Scientific Inc.) for 16 hours at 37°C in a mixture of 17 μl. The fragments were analyzed by electrophoresis in 3.5% agarose gel stained with ethidium bromide and detected by a UV transilluminator (Cleaver Scientific Ltd., Rugby, UK).

Statistical Analysis

Statistical analysis was performed on SPSS v16.0 (IBM, Chicago, IL). Continuous variables were presented as mean, SEM, median and ranges. These were assessed for normality of the distribution by Kolmogorov-Smirnov's test and Shapiro-Wilks' *W* test. The differences between the independent groups of the numerical variables with non-normal distribution were assessed by the Kruskal-Wallis and Mann-Whitney tests. The difference of the continuous variables between dependent groups was assessed by Wilcoxon-signed rank test. The correlations between the quantitative variables were evaluated by Pearson or Spearman test depending on the distribution (normal or non-normal, respectively).

The frequency of the genotypes in control and patient groups were assessed for deviation from the Hardy-Weinberg equilibrium (HWE) by χ^2 test. Binary Logistic regression analysis was applied for assessing the odds ratios and 95% CI when analyzing the effect of the genotypes on the risk for development of CRC.

The Log rank test was used for estimating the differences in the survival of the groups of patients and the cumulative survival curves were drawn with Kaplan-Mayer method. Factors with $P < 0.05$ were considered statistically significant.

RESULTS

IL-6 Serum Levels

The mean serum value of IL-6 in the control group [23.31 ± 4.43 (SEM) pg/mL, median of 10.15 pg/mL, range: 1.00 to 173.53 pg/mL] was significantly lower than in CRC patients [88.22 ± 14.60 (SEM) pg/mL, median of 55.71 pg/mL, range: 1.29 to 303.76 pg/mL] ($P < 0.0001$) (Table 2). The serum IL-6 level was statistically higher in patients with advanced histologic type cancers (type IV) in comparison to the other histologic types ($P = 0.041$, Kruskal-Wallis test), especially compared with the patients with type III cancers ($P = 0.026$, Mann-Whitney test) being with the lowest serum IL-6 concentration. These data suggest association between increased IL-6 serum level and presence of malignant tumor and with advanced tumor. The serum level most possibly is achieved by the secretion of the cytokine from IL-6^+ tumor epithelium, from tumor-associated fibroblasts and from tumor-infiltrating immune cells, proven by the immunohistochemistry of IL-6 and CD68 (Fig. 1) in serial cessions and by double immunofluorescence for of IL-6 and CD3 (Fig. 2).

When stratifying patients according to different clinicopathologic parameters of CRC we found significant difference in serum IL-6 levels according to histologic type of the cases. The group of patients with type I+II and IV had increased serum IL-6 with means of 117.88 and 159.49 pg/mL, respectively, compared with type III with 61.98 pg/mL ($P = 0.041$). No significant differences were detected according to other parameters (Table 2).

For the further analyses concerning possible effect of the serum IL-6 levels on the survival of the patients with CRC, the median value of 55.71 pg/mL was selected as a cutoff point.

IL-6 Positivity in Tumor Glands

We have investigated 153 CRC patients immunohistochemically for IL-6 positivity in tumor glands

TABLE 2. The Serum IL-6 in Patients With CRC According to the Different Clinical and Histolohical Characteristics

	Serum IL-6 (pg/mL) (mean ± SEM)	*P* (serum)
Patients	88.22 ± 14.60	**< 0.0001**
Controls	23.31 ± 4.43	
Clinical characteristics		
Depth of invasion (pT)		0.924
pT1+pT2	89.09 ± 31.47	
pT3+pT4	92.36 ± 17.05	
Regional lymph node metastases		0.778
No mets	84.12 ± 18.99	
Yes mets	100.78 ± 23.73	
Distant metastases		0.680
No mets	94.71 ± 15.97	
Yes mets	63.85 ± 37.65	
TNM stages		0.778
Stage 1+2	84.12 ± 18.99	
Stage 3+4	100.78 ± 23.73	
3 y survivals		0.660
Less than 3 y	99.92 ± 19.75	
3 or more years	85.92 ± 24.26	
Histologic characteristics		
Differentiation		0.908
Low+mucinous	81.62 ± 25.53	
Moderate+high	94.12 ± 17.51	
Inflammatory infiltrate in the IF		0.356
None or weak (1+2)	100.78 ± 19.58	
Strong (3)	79.23 ± 22.93	
Histologic types		**0.041**
Types I+II	117.88 ± 33.50	a vs. b: *P = 0.086*
Type III	61.98 ± 15.00	a vs. c: *P* = 0.536
Type IV	159.49 ± 42.80	b vs. c: ***P* = 0.026**
VELIPI		0.717
No invasion	91.27 ± 21.56	
Yes invasion	92.04 ± 20.35	
Microsatellite instability		0.187
MSS	104.75 ± 22.99	
MSI	67.29 ± 16.92	

Bold values are statistical significant $P < 0.05$.
MMR indicates mismatch repair; MMS, microsatellite stable; MSI, microsatellite instable; VELIPI, vascular/lymphatic/perineural invasion.

and in tumor immune cells. The numbers of IL-6$^+$ immune cells in the IF were significantly higher [31.37 ± 2.57 (SEM) cells/mm^2, median of 22.79 cells/mm^2, range: 0.22 to 256.46 cells/mm^2] than in the TS [17.01 ± 1.66 (SEM) cells/mm^2, median of 12.13 cells/mm^2, range: 0.22 to 168.25 cells/mm^2, $P < 0.0001$, Wilcoxon-signed-rank test]. The mean percent of IL-6$^+$ tumor glands was 26.88 ± 1.83% (SEM) (median of 12.50%, range: 0.00% to 100%).

The percent of IL-6$^+$ tumor glands tended to be lower in low differentiated and mucinous cancers as compared with well and moderately differentiated ones ($P = 0.075$) (Table 3). The lower expression of IL-6 in tumor glands in CRC could be a sign of poor development of the cancer.

IL-6 Positivity in the Immune Cells in Tumor Microenvironment (TME)

IL-6$^+$ immune cells were significantly higher in the invasive front (IF) of CRC patients having histologic type I+II (36.29 ± 3.81 cell/mm^2) as compared with CRC patients with histologic type III+IV (29.05 ± 3.39 cells/mm^2) ($P = 0.026$) (Table 3). Similar difference was observed in the tumor stroma (TS), but without statistical significance ($P = 0.058$) (Table 3).

CRC patients with metastasis in regional lymph nodes have shown lower number of IL-6$^+$ immune cells in TS (15.27 ± 4.11 cells/mm^2), compared with patients without lymph node metastasis there (20.21 ± 2.60 cells/mm^2) ($P = 0.042$) (Table 3) Similarly, patients with distant metastases had tumors with lower number of IL-6$^+$ immune cells in TS than those without metastases ($P = 0.016$) (Table 3). This results confirm our previous investigations, where immune cells show higher density in earlier tumor stages.[39] As a consequence, patients with more advanced tumors (stage III/IV) have shown lower number of IL-6$^+$ immune cells in TS than those with earlier stages (stage I/II) ($P = 0.012$) (Table 3).

CRC patients with mucinous cancers and with low differentiated cancers show, although not significantly, lower numbers of IL-6$^+$ immune cells in the TS (12.91 ± 2.27 cells/mm^2) compared with more differentiated ones (20.57 ± 2.77 cells/mm^2) ($P = 0.140$) (Table 3).

However, the IL-6$^+$ immune cells in IF were significantly higher in cancers with less advanced histologic types (type I/II) in comparison with advanced (type III/IV) ($P = 0.026$), as well as in biopsies with strong inflammatory infiltrate in the invasive front ($P = 0.046$) (Table 3).

MSI patients have shown a tendency for higher numbers of IL-6$^+$ immune cells in the IF (33.28 ± 3.25 cell/mm^2) compared with patients with MSS cancers (29.86 ± 3.75 cells/mm^2) (Table 3). We suppose that the stronger infiltrate of IL-6$^+$ immune cells in MSI cancers is possibly due to M1-macrophages, which are mainly responsible for the immune surveillance against pathogenicity including transformed tumor cells and cells expressing aberrant proteins, which could be a consequence of microsatellite instability.

The number of IL-6$^+$ immune cells in IF and in TS did not differ between cancers with vascular/lymphatic/perineural invasion (VELIPI) and those without invasion (Table 3).

Survival Analysis

In the analysis of the patients' survival, the median values of IL-6$^+$ 22.79 cells/mm^2 in IF and 12.13 cells/mm^2 in TS, were used as cut-off values for dichotomizing of the patients population.

CRC patients with higher number of IL-6$^+$ immune cells in the IF showed longer survival (mean of 105.60 ± 10.10 mo) than those with lower number IL-6$^+$ immune cells in the IF (55.37 ± 7.12 mo) ($P = 0.012$) (Fig. 3A). This strong association however was significant only in the early stages of CRC ($P = 0.015$, 112.43 ± 11.32

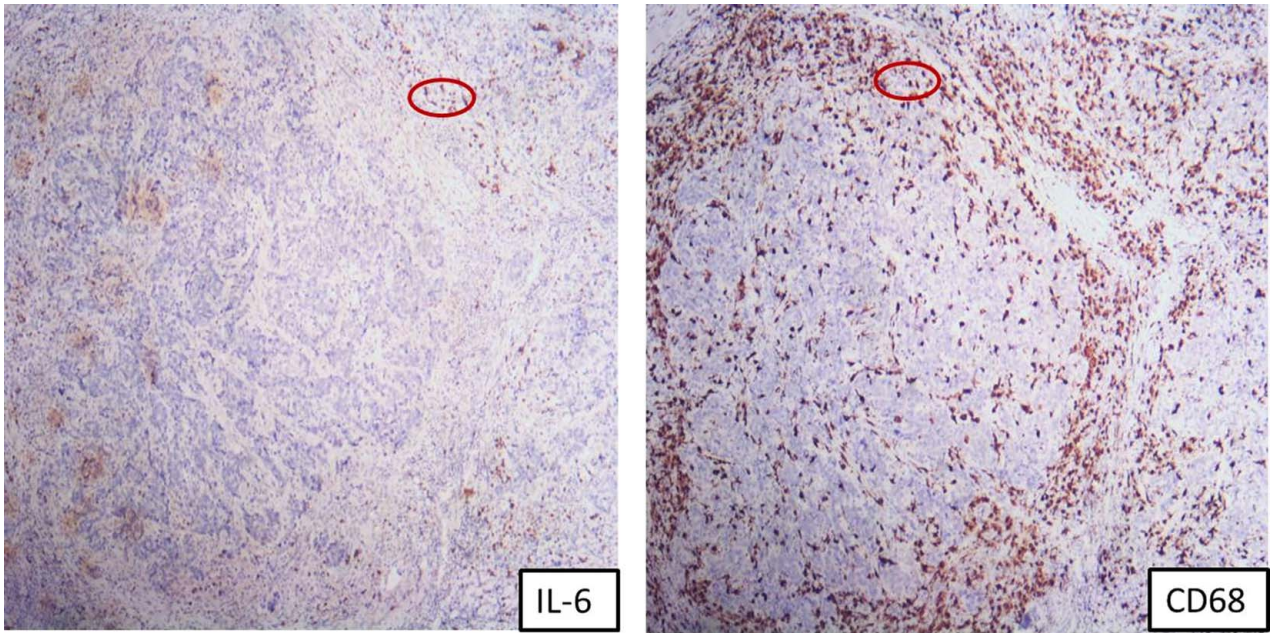

FIGURE 1. IL-6 positive immune cells and CD68 positive macrophages around tumor nests determined by immunohistochemistry. Magnification x200.

vs. 58.41 ± 8.89 mo) (Fig. 3B), but not in the advanced ($P = 0.943$, 41.42 ± 10.44 vs. 46.41 ± 12.04 mo).

When analyzing the effect of IL-6+ cell in TS, we found analogous observation, but without significance: CRC patients with higher numbers of IL-6^+ immune cells in TS (mean of 97.18 ± 11.20 mo) showed tendency for longer survival than those with lower number of IL-6+ immune cells in TS (63.76 ± 7.65 mo) ($P = 0.089$) (Fig. 3C).

A tendency for longer survival was also observed for the patients with higher serum level of IL-6 (mean of 30.05 ± 3.51 mo) in comparison to the patients with lower serum IL-6 levels (19.54 ± 4.54 mo, $P = 0.090$, Log rank test) (Fig. 3D). This association increased its significance after adjustment for the pTNM stages (I+II vs. III+IV) ($P = 0.024$), being significant for early stages ($P = 0.048$, 32.18 ± 4.32 mo vs. 13.83 ± 6.22 mo) and nonsignificant for advance stages ($P = 0.225$, 25.17 ± 4.69 mo vs. 11.55 ± 4.04 mo).

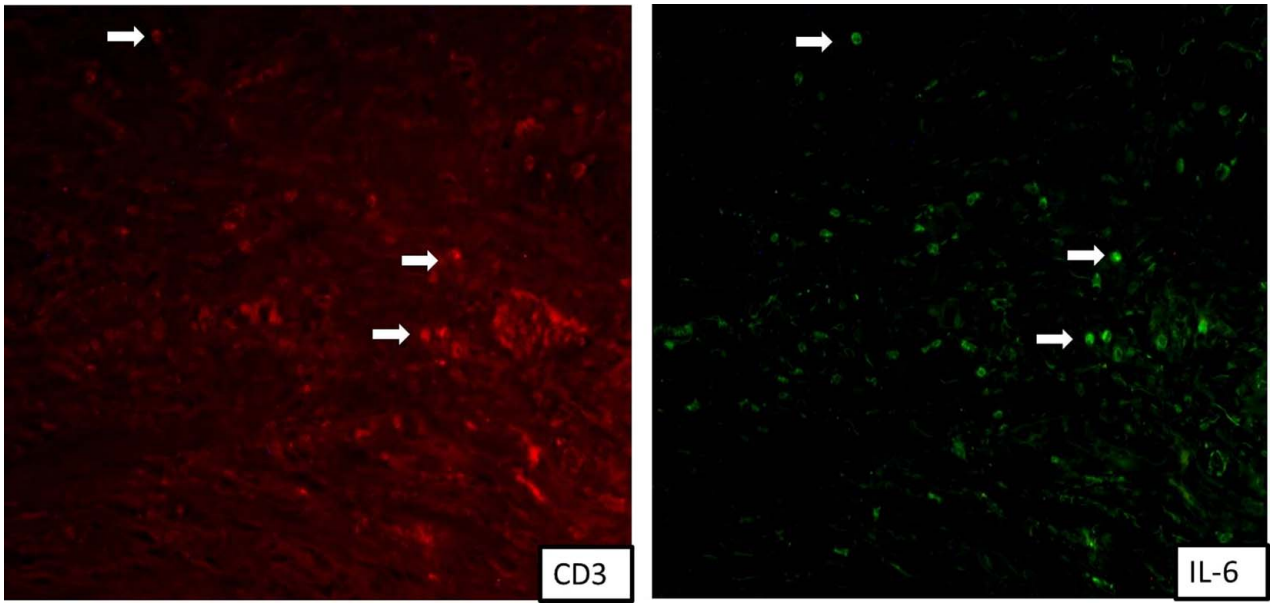

FIGURE 2. Double immunofluorescence staining for CD3 and IL-6 positive immune cells. Magnification x200. full color online

TABLE 3. Associations of IL-6 Positivity in the Tumor Microenvironment With Clinical and Histological Characteristics of CRC Patients

	IL-6$^+$ immune cells/ mm^2 in IF (mean ± SEM)	IL-6$^+$ immune cells/ mm^2 in TS (mean ± SEM)	IL-6$^+$ tumor glands (%) (mean ± SEM)	*P* (IL6$^+$ in IF)	*P* (IL6$^+$ in TS)	*P* (IL6$^+$ tumor glands)
Clinical characteristics						
Depth of invasion (pT)				0.982	**0.006**	0.374
pT1+pT2	31.32 ± 5.11	12.21 ± 2.61	32.22 ± 4.82			
pT3+pT4	37.76 ± 6.33	21.33 ± 2.82	27.78 ± 3.16			
Regional lymph node metastases				0.851	**0.042**	*0.099*
No mets	33.61 ± 6.21	20.21 ± 2.60	31.59 ± 3.08			
Yes mets	34.31 ± 6.60	15.27 ± 4.11	23.16 ± 4.90			
Distant metastases				0.131	**0.016**	0.401
No mets	39.52 ± 5.82	20.81 ± 2.67	30.20 ± 2.96			
Yes mets	22.05 ± 3.47	10.70 ± 1.56	21.06 ± 5.84			
TNM stages				0.518	**0.012**	0.153
Stage 1+2	39.09 ± 7.06	21.42 ± 2.93	31.84 ± 3.33			
Stage 3+4	31.09 ± 5.32	14.61 ± 3.20	24.80 ± 4.25			
3 y survivals				**0.004**	0.423	*0.085*
Less than 3 y	29.30 ± 4.77	19.13 ± 3.23	22.81 ± 2.44			
3 or more years	35.64 ± 3.17	17.50 ± 1.88	31.03 ± 2.92			
5 y survivals				**< 0.0001**	0.452	0.706
Less than 5 y	28.22 ± 3.68	18.16 ± 32.49	25.71 ± 2.20			
5 or more years	42.45 ± 4.39	18.98 ± 2.74	28.75 ± 3.85			
Histologic characteristics						
Differentiation				0.153	0.140	*0.075*
Low+mucinous	38.36 ± 13.01	12.91 ± 2.27	20.50 ± 4.50			
Moderate+high	33.79 ± 2.82	20.57 ± 2.77	29.60 ± 2.42			
Inflammatory infiltrate in the IF				**0.046**	0.851	0.823
None or weak (1+2)	29.66 ± 3.41	16.92 ± 2.16	26.93 ± 2.22			
Strong (3)	36.05 ± 3.99	17.16 ± 2.84	27.36 ± 3.53			
Histologic types				**0.026**	*0.058*	0.367
Types I+II	36.29 ± 3.81	23.00 ± 4.19	27.72 ± 3.07			
Type III+IV	29.05 ± 3.39	14.04 ± 1.32	26.14 ± 2.33			
VELIPI				0.189	0.404	0.305
No invasion	29.76 ± 2.85	16.35 ± 2.24	25.67 ± 2.80			
Yes invasion	26.31 ± 3.93	15.19 ± 2.65	31.26 ± 3.85			
Microsatellite instability				0.157	0.998	0.878
MSS	29.86 ± 3.75	17.41 ± 2.53	25.97 ± 2.24			
MSI	33.28 ± 3.25	16.49 ± 1.98	28.02 ± 3.05			
IL6 -174G > C (rs1800795) genotypes				**0.036** (Kruskal-Wallis)	0.660 (Kruskal-Wallis)	0.554 (Kruskal-Wallis)
GG	40.12 ± 5.36	20.76 ± 5.33	29.03 ± 3.72	0.248 (a vs. b)	0.885 (a vs. b)	0.959 (a vs. b)
GC	35.98 ± 6.63	16.65 ± 2.21	29.76 ± 3.30	**0.017 (a vs. c)**	0.421 (a vs. c)	0.382(a vs. c)
CC	15.67 ± 2.04	14.14 ± 4.80	20.00 ± 6.55	**0.034 (b vs. c)**	0.397 (b vs. c)	0.372 (b vs. c)
IL6 -174G > C (rs1800795) genotypes				**0.020**	0.367	0.278
GG+GC	37.74 ± 4.42	18.39 ± 2.59	29.44 ± 2.45			
CC	15.67 ± 2.04	14.14 ± 4.80	20.00 ± 6.55			

MMR indicates mismatch repair; MMS, microsatellite stable; MSI, microsatellite instable; VELIPI, vascular/lymphatic/perineural invasion.

Genotyping for the IL6-174G/C SNP

The PCR-RFLP method was applied for genotyping of the studied individuals. The PCR product contained 198 bp and after the digestion with *Hin I* (*Nla III*) restriction enzyme the following DNA fragments were produced: 2 fragments of 167 bp and 31 bp when the G/G genotype was present; 4 fragments of 167, 125, 45, and 31 bp for the C/C genotypes and 4 fragments of 167, 125, 45, and 31 bp for carriers of the heterozygous genotype-G/C (Fig. 4).

For this SNP, 110 patients with CcRC and 173 control individuals were successfully genotyped. There was no deviation of the genotype distribution from the Hardy-Weinberg equilibrium (HWE) in the patients' ($P = 0.471$) and in the controls' ($P = 0.763$) groups. There was no statistically significant difference between the genotype distributions of the patient and control groups ($P = 0.814$) (Table 4).

The genotype frequencies did not differ between sex in the patients' group ($P = 0.938$). In controls, there was a

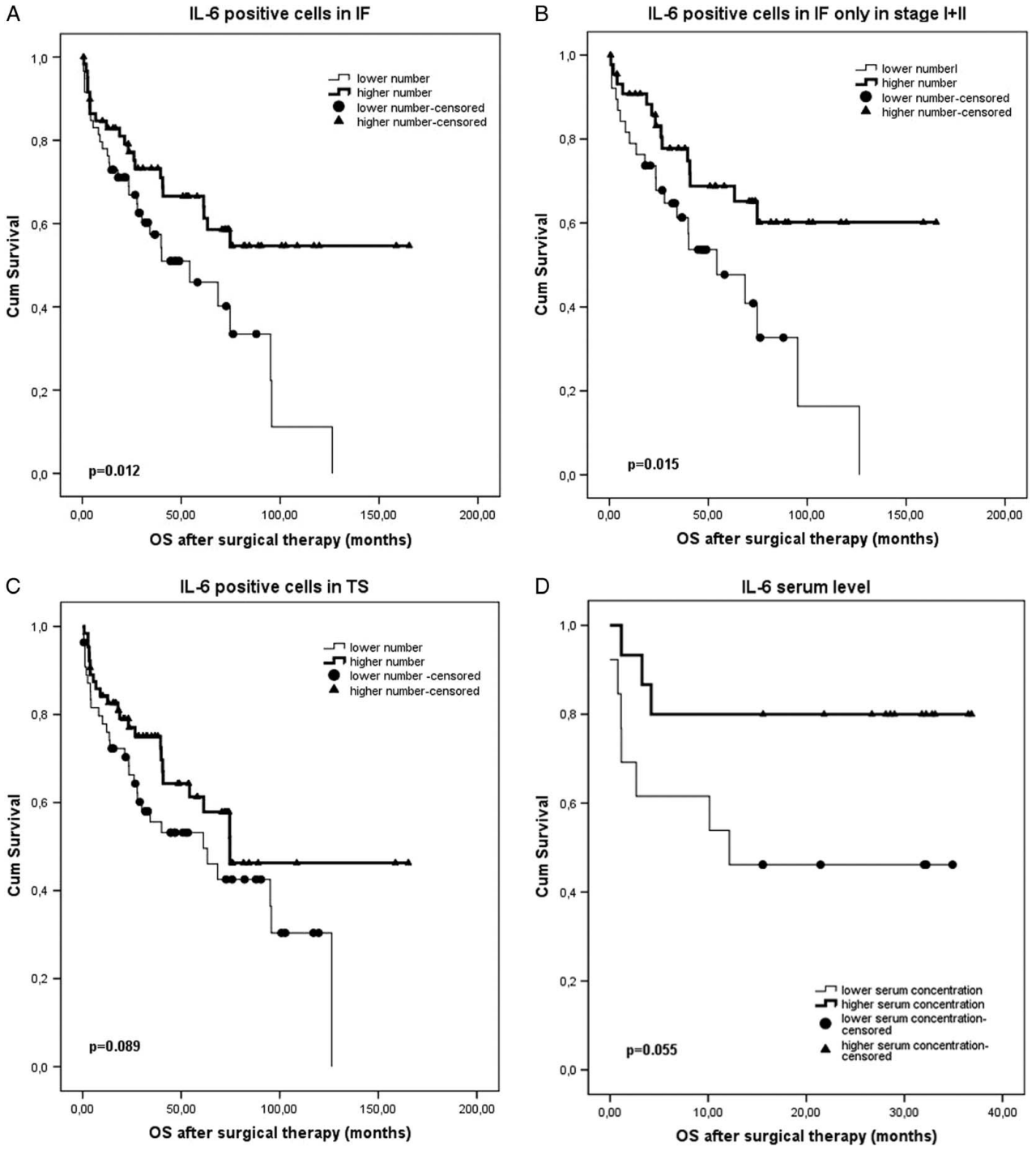

FIGURE 3. Survival analysis of CRC patients according to the number of IL-6 positive cells in the invasive front (IF) (3A, 3B), in tumor stroma (TS) (3C) and IL-6serum concentration (3D), Log rank test, OS- overall survival.

tendency for more common G/G genotype in males (50%) than in females (36%) ($P = 0.062$).

Complete clinical data were available for 66 of the patients which were successfully genotyped. The carriers of different genotypes did not differ in the pTNM staging ($P = 0.511$), presence of lymph ($P = 0.902$) or distant metastases ($P = 0.307$), grade of differentiation of the tumors ($P = 0.404$) or presence of inflammatory infiltrates ($P = 0.965$).

We found that patients carrying the C/C genotype had more frequently (75%) vascular infiltration and lymphatic vessels and perineuronal infiltration (VELIPI) than the patients with G-allele genotypes (GC+GG, 18.9%).

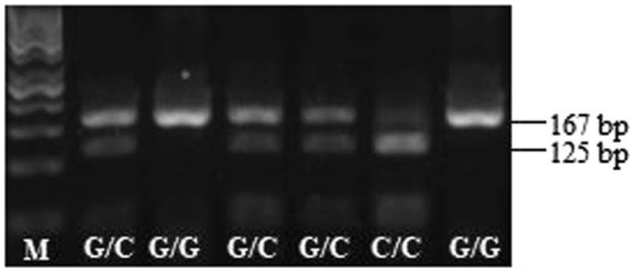

FIGURE 4. Gel electrophoresis image of PCR-RFLP fragments for the IL6-174G/C SNP. M- 50 bp DNA marker.

When compared the number of IL-6$^+$ cells between patients with different genotypes we found that the number of IL-6$^+$ cells in the tumor front (margin) of the patients with G-containing genotypes (G/G+G/C) was significantly higher [mean of 37.74 ± 4.42 (SEM) cells/mm^2] than that of the patients with C/C genotype [mean of 15.67 ± 2.03 (SEM) cells/mm^2, $P = 0.020$, Mann-Whitney U test] (Table 3). Similar observation was also found about the number of CD8$^+$ cells in the tumor front [(margin):49.72 ± 7.28 (SEM) cell/mm^2 vs. 19.07 ± 4.50 (SEM) cells/mm^2, $P = 0.026$, Mann-Whitney U test]. No significant differences were found for the same type of cells in the tumor stroma ($P = 0.367$ for IL-6$^+$ and $P = 0.406$ for CD8$^+$ cells).

We also found that cells expressing STAT3 were more prevalent in tumor stroma and in the tumor front of patients with C- containing genotypes [G/C+C/C, 16.57 ± 2.49 (SEM) cells/mm^2, for TS and of 22.60 ± 2.41 (SEM) cells/mm^2 for IF] than those of patients with GG genotypes [9.85 ± 2.52 (SEM) cells/mm^2, for TS and of 20.39 ± 6.75 (SEM) cells/mm^2 for IF, $P = 0.021$ for TS and $P = 0.069$ for IF].

When studying the survival of patients with different *IL6* -174G/C (rs1800795) genotypes we did not find any difference ($P = 0.562$, Log rank test) independently on the stage and sex (Fig. 5).

TABLE 4. Allele and Genotype Frequencies of the *IL6* -174G/C Gene Polymorphism in Patients With CRC and Controls

	Patients	Controls	
	n (Frequency)	n (Frequency)	
IL6 -174Gl C	**n = 110**	**n = 173**	**OR (95% CI), *P***
Genotype frequency ($P = 0.814$)			
GG	44 (0.400)	74 (0.428)	1.0 (referent)
GC	57 (0.518)	83 (0.480)	1.155 (0.699-1.910), $P = 0.574$
CC	9 (0.082)	16 (0.092)	0.946 (0.385-2.322), $P = 0.904$
GC+CC	66 (0.492)	99 (0.572)	1.121 (0.691-1.820), $P = 0.644$
Allele frequency ($P = 0.833$)			
−174 G	145 (0.659)	231 (0.668)	1.0 (referent)
−174 C	75 (0.341)	115 (0.332)	1.039 (0.727-1.484), $P = 0.834$

DISCUSSION

IL-6 exerts tumor-promoting activities by activation of growth and survival of tumor cells.[24] It has been shown that IL-6 is secreted by T lymphocytes and macrophages in tumor stroma and through trans-signaling in tumor epithelial cells, tumor progression has been promoted.[40] The tumor-promoting effect of IL-6 can be blocked with anti-IL-6R antibodies.[24] Recent studies have shown correlation between IL-6 serum level in CRC (measured before operation) and poor 5-year overall survival, tumor invasion, metastasis and advanced tumor stage.[4,5,39,41–45] Our results show that serum IL-6 level is increased in CRC patients as compared with healthy controls and is of diagnostic value like in previous studies.[3] Moreover, for the first time we have found that the increased serum IL-6 level is significantly associated with advanced histologic type (type IV) that is usually observed in the advanced tumor stages.[32] The histologic types from I to IV have been excellently defined by Kioshima et al[44] and we successfully used them in all our CRC patients to determine the histologic differentiation and infiltration of CRC.

Previously, 2 quantitative data base analyses that explore the potential diagnostic and prognostic value of serum IL-6 in CRC have been conducted.[4,5] Wang and colleagues have studied 14 articles (1245 CRC patients) and have found that the increased IL-6 levels have been associated with tumor invasion, distant metastasis, and advanced tumor stage. Authors have not found correlation between serum IL-6 level and regional lymph node metastases. Xu and colleagues investigated 16 articles conclude that increased IL-6 serum levels are diagnostic and prognostic for CRC development. These manuscripts confirm the function of IL-6 as promoter of CRC tumorigenesis exerted through activation of oncogenic transcription factors NF-κB and STAT3.[5] Because of its low molecular weight IL-6 can freely transfuse through intracellular junctions and enter microcirculation rapidly. Therefore, it can promote tumor cell proliferation through IL-6-JAK-STAT3 pathway.[14,38] In our cohort of 153 CRC patients serum level of IL-6 does not show any correlation with other clinicopathologic parameters.

In a previous study[32] we have revealed in a cohort of 104 CRC patients by immunohistochemistry that IL-6$^+$ immune cells are mainly macrophages (CD68$^+$) and lymphocytes (CD3$^+$). Together with other immune cells such as FoxP3$^+$, IL-17$^+$, and STAT3$^+$ the IL-6$^+$ cells can be a prognostic characteristic for patients. In addition, we have found that IL-6$^+$ immune cells prevailed in the IF of tumors and IL-6$^+$ cells are recruited in the tumor in its early stages (I+II) compared with advanced stages. It is known that in chronic inflammation IL-6 is required for the development of Th17$^+$ cells and for the inhibition of Tregs.[24] From one hand, IL-6 impairs the adaptive immune response and enables tumor cells to evade the immune surveillance.[46] In contrast, IL-6 suppresses the major histocompatibility complex (MHC) II in dendritic cells.[19,33,47]

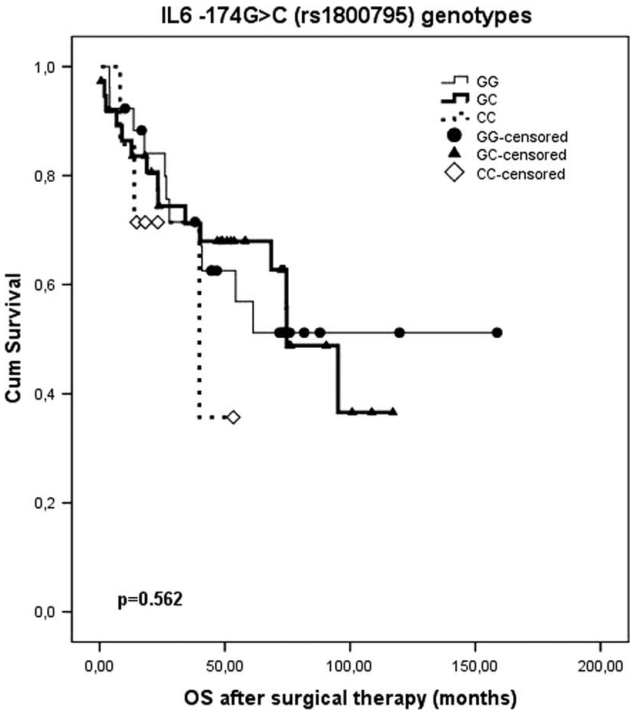

FIGURE 5. Survival graph of CRC patients acoording to the rs1800795 SNP. OS-overall survival.

Our present study shows for the first time IL-6 expression in the immune cells (lymphocytes and macrophages) in different compartments of TME in a group of 153 CRC patients. We have found correlation between number of IL-6$^+$ immune cells and histologic type, tumor stage, differentiation grade, and MSS/MSI status. It has been demonstrated earlier that the expression level of IL-6 in tumors is increased in CRC patients and that reflects the status and prognosis of CRC.[9,10,30,48] In this study we confirm that the immune cells in TME have higher density in the early stages of the disease and lower density in the advanced stages and this was also true for the histologic type, proven in our previous investigation.[32] In addition we have shown that decreased number of IL-6$^+$ immune cells in the primary tumor is associated with presence of lymph node (LN) metastases. The association between increased IL-6 immunoreactivity in tumor epithelium (tumor glands) and presence of regional LN metastases and of vascular invasion has been already reported.[10,30] In our opinion, the advanced tumor stages and histologic types showed suppression of effective cellular immunity and prevailing of immunosuppression. We, like others, show high IL-6 intensity in tumor epithelium with low grade of differentiation.[30] Another study[1] has shown that IL-6 mRNA increase in advanced tumor stages (T3/T4) in CRC but do not correlate with TNM stages and with LN metastases. This may be explained by the fact that not only immune cells but also stromal fibroblasts and tumor cells themselves secrete IL-6. Using immunohistochemistry authors reveal increased number of IL-6$^+$ immune cells in CRC microenvironment compared with control mucosa, but in a small number of patients (n = 40). Regarding our study IL-6$^+$ immune cells may be a marker for cancer development.

Until now, to the best of our knowledge, there have been no relevant studies, analyzing the IL-6$^+$ immune cell number and MSI/MSS status in CRC. In the present study, we have found decreased IL-6$^+$ immune cell numbers in the IF of MSS tumors. It is possible that the protumor functions of IL-6 are poorly presented in MSS status that is known to be less immunogenic and associated with poor prognosis.[45,49]

In the present study survival has been longer in CRC patients with increased IL-6$^+$ immune cells number in the IF as compared with lower IL-6$^+$ immune cell number there. Concerning survival it has been shown previously that IL-6 overexpression in tumor glands is associated with poor survival.[10] Nevertheless, our study is focused on IL-6$^+$ immune cells in a large cohort of CRC patients (n = 153) with many clinicopathologic parameters and followed up for a long period of time. The increased IL-6 serum levels also show poor survival.[4,5] That discrepancy with our results is due to the fact that there are many other sources except immune cells that increase IL-6 production in the serum, that is, tumor epithelium, cancer-associated fibroblasts, etc. Therefore, the enumeration of IL-6$^+$ immune cells might be a new way to assess the impact of IL-6 on CRC development.

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