

Research Article

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The effect of CTLA-4 and CD28 gene variants and circulating protein levels in patients with gastric cancer

Mide Kanseri Hastalarda CTLA-4 ve CD28 Gen Varyantlarının ve Dolaşımdaki Düzeylerinin Değerlendirilmesi

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Abstract

Objective: Gastric cancer is one of the most common malignancies worldwide. The risk factors for gastric cancer include environmental and genetic factors. Inflammation and the immune system are known to contribute to the development of the gastric cancer. We examined the influence of critical polymorphisms of CTLA-4 and CD28 genes and circulating protein levels on the etiology of gastric cancer.

Methods: Genotyping of SNPs was performed in 55 gastric cancer patients and 105 healthy individuals using the PCR-RFLP method, and circulating levels of sCTLA-4 and sCD28 were measured.

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Results: There were no significant differences in the genotype and allele distributions of the evaluated SNPs [CTLA-4-318 C > T (rs5742909), CTLA-4 +49 A > G (rs231775), CD28 C > T (rs3116496)] between gastric cancer patients and controls ($p=0.36$, $p=0.78$, and $p=0.80$, respectively). The circulating levels of sCTLA-4 and sCD28 were significantly different between the gastric cancer group and the control group ($p<0.001$ and $p<0.001$, respectively).

Conclusion: The present results suggest that the CTLA-4 and CD28 gene polymorphisms that were evaluated do not play an important role in Turkish patients with gastric cancer. However, sCTLA4 and sCD28 levels were higher in cancer patients and may be useful as an auxiliary parameter in the diagnosis and monitoring of gastric cancer.

Keywords: Cluster of differentiation 28 (CD28); Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4); Gastric cancer; Polymerase chain reaction (PCR); Single nucleotide polymorphism (SNPs).

Özet

Amaç: Mide kanseri dünyadaki en yaygın kötü huylu tümörlerden biridir. Genetik ve çevresel etkenlere bağlı olarak hastalığın görülme riski artabilir. İnflamasyon ve bağışıklık sisteminin mide kanseri gelişimine katkıda bulunduğu bilinmektedir. Biz bu çalışmada CTLA-4 ve CD28 genlerindeki önemli polimorfizmleri ve bu genlerin ürünlerinin dolaşımdaki düzeylerinin mide kanseri etiolojisine etkisini araştırdık.

Yöntemler: 55 mide kanserli hasta ve 105 sağlıklı bireyin tek nükleotid polimorfizmlerinin (SNP) genotiplemesi

PCR-RFLP yöntemi kullanılarak gerçekleştirildi. Dolaşımdaki sCTLA-4 ve sCD28 düzeyleri ölçüldü.

Bulgular: Değerlendirilen SNP'lerin [CTLA-4 +49 A>G (Rs231775), CTLA-4 -318 C>T (Rs5742909), CD28 C>T (Rs3116496)] genotip ve alel dağılımlarında hasta ve sağlıklı bireyler arasında anlamlı bir fark yoktu ($p=0.36$, $p=0.78$, $p=0.80$). Ancak sCTLA-4 ve sCD28 düzeylerinin mide kanserli hastalarda anlamlı yüksek olduğu gözlemlendi ($p<0.001$, $p<0.001$).

Sonuç: Çalışmamızda değerlendirilen polimorfizmlerin mide kanserine yatkınlık oluşturmadıkları sonucuna ulaştık. Öte yandan sCTLA-4 ve sCD28'in mide kanserli hastaların tanısında ve izlenmesinde yardımcı parametreler olarak kullanılabileceğini düşünüyoruz.

Anahtar kelimeler: Farklılaşma kümesi 28 (CD28); Mide kanseri; Polimerize zincir reaksiyonu (PCR); Sitotoksik T-lenfosit ilişkili protein 4 (CTLA-4); Tek nükleotit polimorfizmi (SNPs).

Introduction

Gastric cancer is a solid tumor in which malignant cells originate from the lining of the stomach, and most gastric cancers are adenocarcinomas. Gastric carcinoma (GC) is one of the most common malignancies worldwide. In Turkey, GC is the fifth most prevalent malignant tumor in adult males (6%) and the seventh most prevalent in females (2.8%) [1]. An increased risk for GC has been observed in people whose diets include large quantities of smoked foods, salted fish and meat, and pickled vegetables [2]. The initiation and progression of GC results from a complex interaction of inflammatory, genetic, and environmental factors. Nitrates and nitrites are substances commonly found in cured meats and can be converted by *Helicobacter pylori* into carcinogenic compounds. The rate of GC is approximately doubled in smokers [3].

GC is one of several cancers associated with infection, and epidemiological studies have shown that individuals infected with *Helicobacter pylori* have an increased risk for GC [4]. Inflammation and related cytokines play a crucial role during the epithelial transformation from chronic gastritis to GC. Gaps in the immune system are used successfully by malignant cells to maintain cancer progression, which involves tumor tissue rebuilding, angiogenesis, metastasis, and suppression of the innate anticancer immune response. Recent studies have shown that cluster of differentiation 28 (CD28) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) are immune mediators involved in malignant transformation [5, 6]. T lymphocytes are the

central immune regulator cells of the anti-tumor defense mechanisms [7]. CTLA-4 serves as an immunological checkpoint that plays important roles in downregulating T-cell activation and regulating peripheral tolerance [8]. The first step of T-cell stimulation is the binding of the T-cell receptor to the MHC receptor, which engages the cancer cell antigen on the antigen-presenting cell surface. CD28 mediates signals that promote T-lymphocyte differentiation and proliferation and amplify antibody production by B lymphocytes. Deficiencies in the CD28 pathway result in tolerance and anergy to tumorigenic antigens [9, 10]. The next step in T-cell stimulation is the binding of CD28 to the T cell by B7 on the antigen-presenting cell. This process activates PI3K/AKT, which upregulates the anti-apoptotic proteins Bcl-2 and Bcl-xL and induces T-cell proliferation and survival. Compared to the positive T-cell costimulatory molecule CD28, CTLA-4 has a much higher affinity for B7, leading to decreased T-cell activation when competing for their common B7 ligands [9, 11]. The CTLA-4 protein on activated T cells interacts with cell-surface molecules known as CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells [12]. CD28 is an important protein in immune regulation and is encoded by the same chromosomal loci as CTLA-4; thus, these proteins share 31% amino acid homology and interact closely [13]. The presence of CTLA-4 and CD28 in the circulation is reported in many inflammatory diseases, such as rheumatoid arthritis and graves multiple sclerosis, and in neoplasms, including colorectal cancer, breast cancer and multiple myeloma [14–20].

Cancer is thought to be triggered by environmental factors in genetically predisposed individuals. The observed genetic influence on cancer has prompted studies of gene polymorphisms involved in carcinogenesis. Polymorphisms in genes related to inflammation and the immune system have been studied in many of the common types of cancer. Single nucleotide polymorphisms (SNPs) are the most common genetic variations that affect the predisposition to gastric cancer. SNP association analysis has provided valuable information about the genetic susceptibility to GC. The genes encoding CD28 and CTLA-4 have similar structures, and both the human CD28 and CTLA-4 genes are located in the chromosome 2q33 region [13, 21]. At least three genetic polymorphisms have been reported in the human CTLA-4 gene: one in the promoter region at position -318, consisting of a C>T transition; a second in position +49 of exon 1 that lies in an A>G transition, resulting in a threonine (Thr) or alanine (Ala) dimorphism; and a third in the 3' untranslated region, with variable lengths of a dinucleotide (AT) repeat. Several studies have shown an association

between the A>G SNP in position +49 of exon 1 of the CTLA-4 gene and autoimmune diseases, such as type 1 diabetes, rheumatoid arthritis, and multiple sclerosis, and cancer, including colorectal cancer, cervical cancer, and malignant melanoma [22–26].

We assessed the potential effects of CTLA4 and CD28 gene polymorphisms on the susceptibility to GC. We evaluated CTLA-4 and CD28 genotype and allele distributions [CTLA-4 +49 A>G (+49 A>G) (rs231775), CTLA-4 –318 C>T (–318 C>T) (rs5742909), CD28 C>T (rs3116496)] and serum levels of CTLA-4 (sCTLA-4) and CD28 (sCD28) in patients and controls and performed statistical analysis to determine their association with GC.

Materials and methods

Patient characteristics

This study was approved by the Ethics Committee of Istanbul University and conducted in Turkey according to the Helsinki Charter. All participants provided written informed consent. Fifty-five GC patients (41 males and 14 females, mean age: 55.8 ± 11.1) and 105 healthy individuals (40 males and 65 females, mean age: 52 ± 12.8) volunteered for this study. All of the participants were questioned in person regarding age, gender, time of diagnosis, history

of smoking and the presence of other systemic diseases. The clinicopathological characteristics of GC patients are reported in Table 1.

Sample collection and polymorphism genotyping

Genomic DNA isolation from all participants was performed using the salting out technique, in which whole-blood samples were collected in EDTA tubes [27]. Gene polymorphisms [+49 A>G (rs231775), –318 C>T (rs5742909), and CD28 C>T (rs3116496)] were analyzed by PCR using locus-specific primers and restriction fragment length polymorphism (RFLP) as previously described [28]. The primers used for amplification were as follows: CTLA-4: F: 5'-AAA TGA ATT GGA CTG GAT GGT-3', R: 5'-TTA CGA GAA AGG AAG CCG TG-3', and F: 5'-GCT CTA CTT CCT GAA GAC CT-3', R: 5'-AGT CTC ACT CAC CTT TGC AG-3', and for CD28: F: 5'-TTT TCT GGG TAA GAG AAG CAG CGC-3', R: 5'-GAA CCT ACT CAA GCA TGG GG-3'. PCR reaction mixtures contained 150 ng of DNA template, 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, 200 μM of each dNTP, and Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania). The enzymes BbVI, Tru1, and Eco47III were used for the detection of corresponding genotypes (+49 A>G, –318 C>T, and CD28 C>T, respectively) using RFLP. Visualization of the digestion products after the agarose gel electrophoresis was performed using UV light. The +49 A>G PCR conditions were as follows: an initial melting step of 45 s at 95°C; then 35 cycles of 45 s at 94°C, 45 s at 59.2°C and 45 s at 72°C; and a final elongation step of 5 min at 72°C. The PCR products (162 bp) were digested with the BbVI restriction enzyme for 3 h at 65°C. The digested G allele formed 88-bp and 74-bp fragments, and the undigested A allele was a 162-bp fragment. The –318 C>T PCR conditions were as follows: an initial step of 45 s at 95°C; 35 cycles of 45 s at 94°C, 45 s at 58°C, and 45 s at 72°C; and a final elongation step of 5 min at 72°C. For –318 C>T genotype analyses, the Tru1I (MseI) restriction enzyme was used to digest the PCR products. After enzymatic digestion, the TT genotype displayed 21- and 226-bp fragments. The CC genotype was determined by the presence of 21-, 96-, 130-bp fragments. CD28 C>T PCR conditions were as follows: an initial melting step of 45 s at 95°C; then 35 cycles of 45 s at 94°C, 45 s at 56.1°C and 45 s at 72°C; and a final elongation step of 5 min at 72°C. The 148-bp PCR-product digestion was performed overnight using Eco47 III at 37°C. After enzyme digestion, individuals homozygous for the C allele were identified by a 148-bp fragment, and those homozygous for the T allele were identified by 126- and 22-bp fragments.

Table 1: Clinicopathological characteristics of gastric cancer patients.

Characteristic	%
Tumor stage	
T1 + T2	6.6
T3 + T4	93.4
Lymph node metastasis	
N1 + N2 + N3	91.7
N0	8.3
Presence of distance metastasis	
Present	64.5
Absent	35.5
Tumor type	
Adenoma	68.8
Other	31.2
Differentiation	
Low grade	10
Moderate grade	60
High grade	30
Staging	
Stage 1	3.8
Stage 2	30.8
Stage 3	7.7
Stage 4	57.7

A commercial human sandwich ELISA kit (Platinum ELISA, Bender MedSystems GmbH, Vienna) was used to analyze circulating levels of sCD28 and sCTLA4. The within-run and between-run coefficients of variation (CVs) were 4.8% and 5.9%, respectively. Venous blood samples were collected in serum-separating tubes without anticoagulant. The samples were centrifuged at $1000 \times g$ for 10 min within 30 min of collection. The eluted sera were aliquoted into portions and preserved at -80°C until analysis under laboratory conditions.

Statistical analyses

The data were evaluated using the SPSS (Statistical Package for Social Sciences) 21 program package (IBM, New York, NY, USA). Descriptive statistics were calculated, including the mean, standard deviation (SD), minimum (min), maximum (max) and percentages. The results were evaluated using a 95% confidence interval, and the probability value (p) was set at 0.05. The mean values of the clinical parameters between patients and controls were compared using the unpaired Student's t -test and expressed as the mean $\pm$ SD. The chi-square (χ^2) test was performed to compare categorical distributions and to detect differences in the distribution of genotypes and alleles between patients and controls. Spearman correlation analysis was applied to evaluate the relationship between sCTLA-4 and sCD28 parameters. Deviation from the Hardy-Weinberg Equilibrium (HWE) was examined by the χ^2 test. Independent sample t -tests were used to compare group means. Calculations of +49 A>G, -318 C>T, and CD28 C>T haplotype frequencies were evaluated by using the log of the odds (LOD) and r^2 in Haploview Software, version 4.2 [29].

Results

A total of 3 SNPs were successfully genotyped in 55 GC patients and 105 healthy controls. There was no significant difference in age between GC cases and controls ($p=0.45$). In addition, there was no significant difference in smoking status between patients and controls ($p=0.51$). The clinicopathological characteristics of GC patients are summarized in Table 1.

The serum levels of sCTLA-4 and sCD28 are presented in Figure 1A,B. Significant differences were detected between GC patients and controls ($p<0.001$ and $p<0.001$ for sCTLA-4 and sCD28, respectively). We detected a

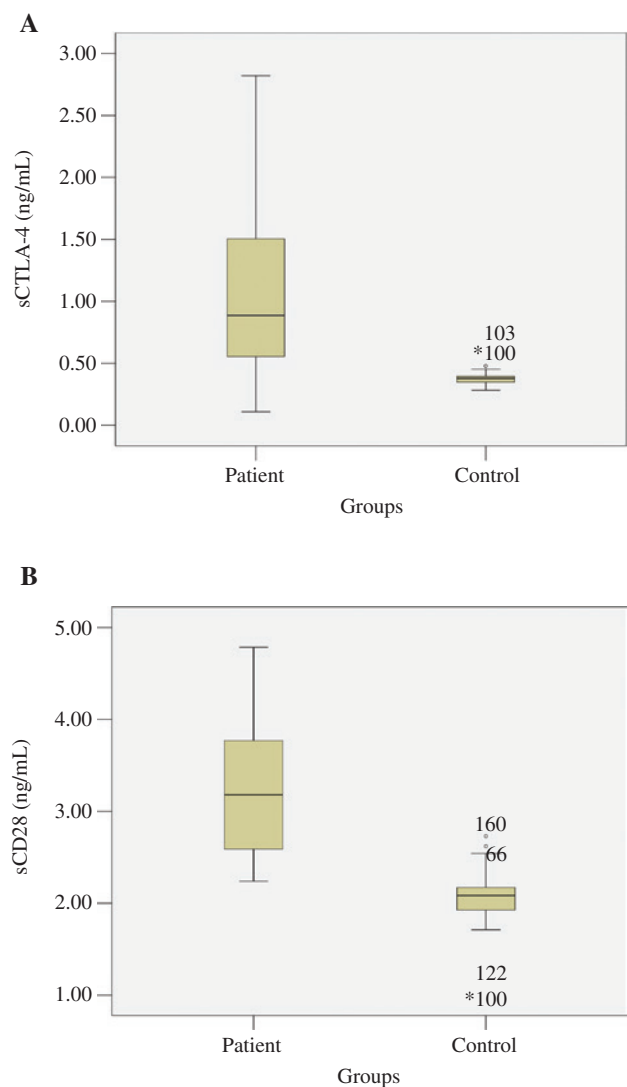


Figure 1: The distribution of the sCTLA-4 levels (A) and sCD28 levels (B) are depicted graphically by Box-plot graphic according to the groups. The median values, quartiles and outlier samples are demonstrated in the graphics.

significant positive relationship between sCTLA-4 and sCD28 levels ($r=0.571$, $p>0.001$).

The genotype distribution and allele frequencies of all three SNPs are shown in Table 2. The frequencies and odds ratios for the HWE proportions of the SNPs are also shown in Table 2. For all three SNPs, the genotypic distribution conformed to HWE. There was no significant difference in the genotype and allele distributions of the evaluated SNPs between GC patients and controls ($p=0.36$, $p=0.78$, and $p=0.80$ for the three SNPs, respectively). The genotype distribution of the SNPs according to the pathological findings in GC patients is demonstrated in Table 3. We applied stratified analyses by tumor stage, lymph node

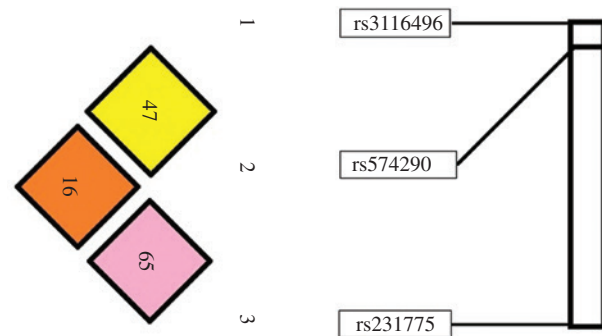
Table 2: The genotype and allele frequencies of CTLA4 –318 C>T, CTLA4 +49 A>G, CD28 C>T polymorphisms in gastric cancer patients and controls.

	Patient group (%)	Control group (%)
CTLA-4 –318 C>T		
CC	80	78.1
CT	14.5	20
TT	5.5	1.9
T allele	12.7	11.9
C allele	87.3	88.1
CTLA-4 +49 A>G		
AA	41.8	47.6
AG	49.1	43.8
GG	9.1	8.6
A allele	66.4	69.5
G allele	33.6	30.5
CD28 C>T		
CC	7.3	10.5
CT	34.5	33.3
TT	58.2	56.2
C allele	24.5	27.1
T allele	75.5	72.9

invasion and metastatic status of the GC patients. We did not detect a significant difference in relation to these pathologic findings. Despite the lack of a significant difference, there were remarkable differences in the frequency distribution. For CTLA-4 +49 polymorphisms, all patients in the lower tumor stage had the homogenous AA genotype, whereas the prevalent genotype in patients with distant

metastases was the homogenous GG genotype (65%). We observed that the CC genotype is common in patients without lymph node involvement (100%). For CTLA-4 –318 polymorphisms, the T allele seems to increase the risk of lymph node involvement and distant metastasis.

The haplotype frequencies, haplotypic associations and linkage disequilibrium (LD) among CTLA-4 +49 (rs231775), CTLA-4 –318 (rs5742909) and CD28 (rs3116496) polymorphisms were evaluated using HaploView software. LD analysis between these polymorphisms

**Figure 2:** Linkage disequilibrium (LD) analysis of CTLA-4 +49 (rs231775), CTLA-4 –318 (rs5742909) and CD28 (rs3116496). The LD plot was generated by HaploView software version 4.2 using r^2 and LOD values (LOD is the log of the likelihood odds ratio, r^2 is the correlation coefficient between the two loci and the log of the likelihood odds ratio). The pairwise LD values ($D1=0$ to 100%) of all single nucleotide polymorphisms are shown in the colored squares. A value of 100 ($D1=1$) represents the maximum possible LD.**Table 3:** The genotype distribution of CTLA-4 –318 C>T (rs5742909), CTLA-4 +49 A>G (rs231775) and CD28 C>T (rs3116496) according to pathological findings in gastric cancer patients.

SNP	CTLA-4 –318 C>T			CTLA-4 +49 A>G			CD28 C>T		
	CC (%)	CT (%)	TT (%)	AA (%)	AG (%)	GG (%)	CC (%)	CT (%)	TT (%)
Tumor stages									
T3 + T4	75	14.3	10.7	53.6	39.3	7.1	7.1	25	67.9
T1 + T2	50	50	–	100	–	–	–	50	50
p Value			0.406			0.441			0.715
A. Lymph nodes									
N1 + N2 + N3	76.2	19.0	4.8	61.9	33.3	4.8	4.8	33.3	61.9
N0	100	–	–	66.7	33.3	–	33.3	–	66.7
p Value			0.835			0.569			0.653
Distant metastasis									
Positive	80	15	5	65	30	5	5	20	75
Negative	63.6	18.2	18.2	5.5	45.5	9.1	9.1	36.4	54.5
p Value			0.455			0.569			0.507
Stage									
S3 + S4	87.5	6.3	6.3	43.8	50	6.2	75	25	–
S1 + S2	55.6	22.2	22.2	44.4	44.4	11.1	44.4	44.4	11.1
p Value			0.2			0.901			0.192

Clinicopathological characteristics in the study group. %, percentage of individuals.

demonstrated very low LDs, D1, LOD, and r^2 values. The LDs, D1, LOD, and r^2 values were 0.47, 3.48, 0.086, and 0.26, respectively, between rs3116496 and rs5742909; 0.16, 0.6, 0.02, and 0.02 between rs3116496 and rs231775; and 0.65, 1.15, 0.027, and 0.18 between rs5742909 and rs231775 (Figure 2).

Discussion

The development of GC is a complex, multistep process involving multiple genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, and signaling molecules [30]. The immune system uses signaling pathways to limit the severity and duration of T-cell activation. These pathways are required for normal homeostasis because their defect or inadequacy often causes negative immune regulation. CTLA-4 is a mediator that downregulates T-cell-mediated immune responses and is expressed on both regulatory and activated T cells. CTLA-4 and CD28 share gene homology and have common B7 co-stimulatory ligands. CTLA-4 can downregulate T cells via the B7/CTLA-4 pathway by blocking the positive co-stimulatory signal conducted by the B7/CD28 pathway. In the present study, we observed that the serum levels of sCTLA-4 and sCD28 were significantly higher in the GC patients than in the healthy controls ($p < 0.001$, $p < 0.001$, respectively). We also calculated the correlation between the sCTLA-4 and sCD28 levels measured in our study. We confirmed that sCTLA-4 and sCD28 are co-expressed. Studies have reported elevated sCTLA4 levels in patients with various types of cancer, including mesothelioma, acute lymphoblastic leukemia and breast cancer [31–33]. Similarly, the expression of CD28 is increased in patients with colorectal carcinoma, myeloma and melanoma [17, 34, 35]. Conversely, there are studies reporting decreased sCTLA-4 and sCD28 levels in clear cell renal cell carcinoma and breast cancer [36, 37]. This observation indicates that the efficacy of immune checkpoint inhibition is variable across different types of cancer.

We investigated gene polymorphisms that might influence GC susceptibility. We selected functional polymorphisms that were previously described as risk factors for the development of GC. Polymorphisms in the CD28 gene (rs3116496) and in the CTLA4 gene, including one in exon 1 (+49A > G; rs231775) and one in the promoter region (–318 C > T; rs5742909), have been reported to increase the susceptibility to some cancer types, including GC [38–41].

Many studies have examined the association between the CTLA4 gene (+49A > G; rs231775) polymorphism and cancer incidence. It was previously reported that the presence of this polymorphism in the CTLA4 gene increases the risk of cervical cancer, non-small-cell lung cancer and renal cell carcinoma [42, 43]. However, negative results have also been reported. No significant associations were found between the CTLA-4 +49A > G polymorphism and the risk of breast cancer or osteosarcoma [18, 44]. We did not observe a significant association between the CTLA4 +49A > G polymorphism and GC. Similarly, Hadinia et al. [45] reported no difference in the genotype distribution between GC patients and controls. We observed that the CC genotype was prevalent in our study population. In addition, we did not find a significant difference in the allele and haplotype frequencies between patients and healthy individuals. Remarkably, all patients with stage T1 and T2 cancer were AA homozygous, whereas patients with the AG or GG genotype were in an advanced tumor stage. In addition, the prevalent genotype in patients with distant metastases was GG (65%). This finding suggests that the G allele increases the risk of negative disease symptoms. The CTLA4 rs231775 G > A polymorphism (+49 guanine to adenine mutation), a SNP in exon 1, encodes an alanine (Ala) to threonine (Thr) substitution in an amino acid residue in the leading sequence of CTLA4 [46]. The G allele may weaken the efficiency of messenger RNA and decrease the expression of CTLA4 [47].

Previous studies have reported an association between the CTLA-4 – 318C > T polymorphism and malignant tumor risk. Associations of T-cell regulatory gene polymorphisms with the susceptibility to gastric mucosa-associated lymphoid tissue lymphoma, cervical cancer, and non-small-cell lung cancer have been previously reported [37, 48–50]. However, negative results have been reported for renal cell carcinoma and gastric and colorectal cancers [38–40]. In our study, there was no significant difference in the genotype and allele distributions of the CTLA-4 – 318C > T polymorphism between GC patients and controls. All of the patients who had no lymph node involvement had the CC genotype. Having the T allele increased the risk of lymph node involvement and distant metastasis. The CTLA-4 – 318C > T polymorphism is localized in the promoter region, and the TT genotype may be associated with this condition. The T allele of the CTLA-4 – 318C > T polymorphism has been associated with greater promoter activity than the C allele, and with significantly increased expression of both CTLA-4 mRNA in unstimulated T cells and cell-surface CTLA-4 on activated T cells [51].

Polymorphisms in the genes encoding CD28 have been demonstrated to be associated with susceptibility to non-small-cell lung cancer and cervical cancer [35, 52]. Conversely, negative results were reported for colorectal cancer [26]. The present results suggest that CD28 gene polymorphisms do not play an important role in the susceptibility to GC. We did not observe a remarkable allele and genotype frequency distribution.

HaploView was used to analyze and visualize patterns of linkage disequilibrium (LD) in the genetic data. These findings indicated that the three single nucleotide polymorphisms were inherited separately (Figure 2).

The relatively small group of patients is one limitation of this study. Furthermore, the gender distribution was not equivalent among the groups.

According to this study, CTLA-4 and CD28 gene polymorphisms did not play an important role in Turkish patients with gastric cancer. However, sCTLA4 and sCD28 levels were elevated in patients with gastric cancer and may be useful as an auxiliary parameter in its diagnosis.

This study is one of only a few studies addressing the differences in gene polymorphisms and related gene products in the susceptibility to gastric cancer. These data contribute novel findings to the literature.

Conflict of interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

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