Individual and Combined Effects of *CTLA4-CD28* Variants and Oxidant-Antioxidant Status on the Development of Colorectal Cancer

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Abstract. *Background: Colorectal cancer (CRC) is the third most frequent cancer worldwide. Research has revealed the contributions of the immune system and anti-inflammatory pathways in the development of cancer. The balance between cluster of differentiation 28 (CD28) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) signaling is important for the regulation of immune responses. The oxidant–antioxidant balance by sustaining redox control via several defense mechanisms is also an important factor for the progression of cancer. The aim of the present study was to determine the distribution of CTLA4/CD28 variants and oxidant-antioxidant status in patients with CRC. Materials and Methods: This study enrolled 80 patients with CRC and 115 healthy controls. We used a spectrophotometric assay to detect the levels of lipid peroxidation products malon dialdehyde (MDA) and lipid hydroperoxide (LHP), and measured the concentration of protein damage products, advanced oxidation protein products (AOPP) and protein carbonyl (PCO). Additionally, antioxidant levels were detected by measuring copper, zinc, superoxide dismutase (Zn-Cu SOD) and total thiol (T-SH) levels, and advanced glycation end-products (AGEs). The CTLA4 -318C>T, CTLA4 49A>G and CD28C>T genotypes were determined by using restriction enzymes. Results: AOPP and PCO levels were increased in patients with CRC as well as those of LHP, MDA and AGE, while the levels of antioxidants such as Cu-Zn SOD and T-SH were lower. Lower serum levels of CTLA4 and higher serum levels of CD28 were detected in patients and, an association of the CTLA4 -318C/T polymorphism was found in patients with CRC. Conclusion: Our oxidative stress was increased in patients with CRC, suggesting the contribution of this disturbed oxidative status to serum CTLA4 and CD28 levels, and to the pathogenesis of CRC.*

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Colorectal cancer (CRC), which has an incidence of 10% in men and 9.2% in women, is the third most frequent cancer worldwide. Environmental, lifestyle and genetic factors are known to be related to CRC progression (1). Research has shown that disruption of immune system responses and anti-inflammatory pathways play effective roles in the development of cancer (2).

It was shown that the balance between signaling of multiple co-stimulatory cell surface molecules such as cluster

of differentiation 28 (CD28) and cytotoxic T-lymphocyte antigen 4 (CTLA4; also known as CD152) is important for the regulation of immune responses. Indeed, the *CTLA4* gene is an important regulator of tumor immunity (3). Previous investigations revealed significant associations between polymorphisms of *CTLA4* and *CD28* genes, and susceptibility to various types of cancer, such as oral squamous cell carcinoma, breast or gastric cancer (4-6).

CTLA4, which is expressed by activated T-cells, is an inhibitory molecule that has an important role in self-antigen tolerance by providing a temporary delay in T-cell activation and proliferation *via* diminishing cytokine production, and cell-cycle arrest in T-cell proliferation. CTLA4 is related to CD28 which has 31% homology but with opposite effects. Although both cell surface molecules have ability to bind B7 family molecules, CD80 and CD86, on antigen-presenting cells (APC), CTLA4 has higher affinity (7-9).

The interaction between CD28 and B7 family molecules results in T-cell activation. However CTLA4 interaction on T-cells results in anergy of immune synapses, or immune tolerance, by reducing the contact period between T-cells and APCs (9, 10). CTLA4 binding on APCs results in down-regulation of B7 family molecules, thus promoting immune tolerance instead of immunoreactivity (11, 12). Previous studies have demonstrated that a temporary delay in CLTA4 appearance on T-cell surface in immune synapses enhances the immune response. Thus, it was accepted that CTLA4 blockade can enhance immunity to tumors and may therefore be a potentially promising immunotherapeutic approach to treat patients with various cancer types (13-20).

On the other hand, an interesting report from Mougiakakos *et al*. suggested that CTLA4 leads to up-regulation of indoleamine 2,3-dioxygenase (IDO) which generates reactive oxygen species (ROS) by metabolizing tryptophan in APCs and, consequently, depletion of this important amino acid affects T-cell activation and proliferation, whereas the excessive and simultaneously produced ROS generates cytotoxic effects (11). In fact, it was shown that during an immune response or inflammation, activated T-cells and leukocytes release interferon-gamma (IFN-γ) which induces the expression and the activity of IDO to sustain degradation of tryptophan. Unfortunately, this depletion of tryptophan, in turn leads to antiproliferative and apoptotic effects on T-cells, resulting in immunosuppression *via* cell-cycle arrest, differentiation, adaptation or apoptosis of T-cells (21, 22). Moreover, several functionally and structurally different antioxidants have been also shown to inhibit IDO activity (23). During the degradation of tryptophan in the kynurenine pathway, it was shown that the presence of copper 3-hydroxyanthranilic acid, an intermediate molecule of the kynurenine pathway, generates free radicals such as superoxide and hydrogen peroxide (21, 24). However, the antioxidant role of this molecule was also shown, by its scavenging peroxyl radicals (21, 25). 3-Hydroxyanthranilic acid was associated with G_1/S arrest in T-cells (21, 26). Furthermore, tryptophan breakdown by IDO was also induced by low glutathione levels *via* inflammation (23).

Oxidative stress, defined as an imbalance between the levels of oxidants (free radical production) and antioxidants (free radical removal), is associated with carcinogenesis, affecting tumor formation, initiation, and cancer progression, and hence the incidence of cancer. During carcinogenesis, ROS levels in cancer cells increase, however, antioxidant levels are not correlated with this increase as they are attenuated (27-30).

Oxidative stress induced by ROS can alter cellular redox status and damage cellular macromolecules, leading to nucleic acid and protein modification, as well as lipid peroxidation and glycoxidation, which in turn results in impaired cell metabolism including cell signaling, proliferation and apoptosis and thus cancer in the long term (29, 31). These types of protein modifications mainly involve protein carbonyl groups (PCO), advanced oxidation protein products (AOPP) and various thiol fractions (total thiol groups, protein and non-protein thiol groups); for lipids, the oxidation can be sorted according to the alterations of lipid hydroperoxides (LHPs) and malon dialdehyde (MDA). Advanced glycation end products (AGEs) are also known as a biomarker of glycoxidation adducts. For cellular homeostasis, antioxidant defenses exist against, and can be described as the activity of major antioxidant enzymes such as catalase, copper and zinc-superoxide dismutase (Cu,Zn-SOD) and glutathione peroxidase (32, 33).

The aim of the present study was to investigate possible individual and common roles of *CTLA4* 49 A>G (rs231775), *CTLA4* -318 C>T (rs5742909) and *CD28* C>T (rs3116496) gene polymorphisms, the serum levels of CTLA4 and CD28, and oxidant-antioxidant status in patients with CRC to evaluate the risk and the progression of the disease, and to develop therapy strategies.

Materials and Methods

Patient selection and clinical investigation. A total of 80 patients diagnosed with CRC at the Istanbul Research and Education Hospital, Surgery Clinic, and 115 healthy volunteers as controls were included in the study. Patient groups were all newly-diagnosed with histopathologically confirmed primary CRC, and surgically treated before any radiotherapy or chemotherapy. All patients with previous chemotherapy, radiotherapy, and surgical history were excluded from the study. Cases of CRC diagnosed according to pathological staging information were confirmed by manual review of the pathology reports and clinical charts. Nodal status was categorized as no regional lymph nodes affected (N0), or the presence of at least one nodal metastasis. The control group included healthy individuals without any symptoms of cancer or any kind of cancer history in their families.

The study groups were age- and sex-matched and none of the participants had received antioxidant vitamin supplementation or antioxidant drugs within 12 months before the study entry. Fasting venous plasma samples were obtained from healthy volunteers and patients before operation.

All participants in the study provided their written consent prior to study. This study conformed with the Helsinki Declaration and blood samples were collected only with written informed consent. The study protocol was approved by both the Ethical Committee of the Istanbul Faculty of Medicine and the Research Fund of Istanbul University (Project No: 16205).

Measurement of serum levels of CD28 and CTLA4. Fresh blood samples were immediately centrifuged at 3,000 rpm (1,361 × *g*) for 5 min to separate serum and samples were kept frozen at –20˚C until assayed. Serum CD28 and CTLA4 levels were determined with a commercially available sandwich ELISA kits (limit of detection: 0.78-50 ng/ml for CD28 and 0.16-10 ng/ml for CTLA4, sensitivity: 0.18 ng/ml for CD28 and 0.13 ng/ml for CTLA4 (Platinium ELISA, Bender MedSystems GmbH, Vienna, Austria).

Polymerase chain reaction (PCR)-based CTLA4 and CD28 genotyping. A 10-ml of sample of peripheral whole blood was collected from each participant into a test tube containing EDTA. Genomic DNA was isolated according to a salting-out technique (34). The DNA samples were analyzed for CTLA4 -318 C>T (rs5742909), CTLA4 49 A>G (rs231775) and CD28 C>T (rs3116496) polymorphisms by PCR with locus-specific primers, and restriction fragment length polymorphism (RFLP) analysis as previously reported (29,34,35).

Detection of lipid and protein oxidation products. Spectrophotometric assays were used to detect the levels of lipid peroxidation products PCO (36), MDA and LHP (37); and protein oxidation products, namely AOPP (38). Additionally, antioxidant levels were determined by measuring Zn,Cu-SOD (39), total thiol (T-SH) (40) and AGE levels.

Detection of plasma levels of Cu and Zn. As Cu and Zn elements are required for the activity of SOD, the levels of Cu and Zn were measured in plasma samples of patients and controls using atomic absorption spectroscopy (Thermo Electron Corporation AA Series, Thermo Scientific,Cambridge, United Kingdom) using Cu and Zn standard solutions (Spectro Econ, ChemLab, Belgium).

Statistical analysis. Statistical analysis was performed using SPSS software package (revision 11.5; SPSS Inc., Chicago, IL, USA). Mean values of clinical parameters were compared between patients and controls with unpaired Student's t-test and expressed as the mean±SD. Differences in the distribution of genotypes and alleles between patients and controls were tested using the Chi-square statistic. The Hardy–Weinberg equilibrium was tested for all polymorphisms. Allelic frequencies were estimated by gene counting methods. A univariate analysis was performed to compare the distribution of age and gender and the frequencies of alleles and genotypes. The statistical analyses of the non-normally distributed data of plasma oxidative stress parameters between patients and controls possessing the same genotypes were performed by using Mann-Whitney U-test. Linkage disequilibrium among *CTLA4* -318 C>T, *CTLA4* 49 A>G and *CD28* C>T polymorphisms were evaluated using D' and r2 values obtained through the Haploview 4.2 programme (http://www.broad.mit.edu/mpg/haploview/ documentation.php). Values of $p<0.05$ were considered statistically significant.

Results

All patients (n=80) and controls (n=115) had similar distribution of sex and age. A total of 80 patients were enrolled (33 women, 47 men; mean±SD age at diagnosis=62.94±12.27 years). A total of 13.1% of patients had left-sided CRC, and 23.0% had right-sided CRC; in 8.2% of patients, the tumor was localized in the cecum, 1.6% in the transverse colon, 36.1% in the sigmoidal colon, and 18.0% in the rectum. The other clinicopathological characteristics of patients are shown in Table I.

The genotypic and allelic frequencies of *CTLA4* -318 C>T, *CTLA4* 49 A>G, *CD28* C>T polymorphisms are shown in Table II. Significant associations were found in the distributions of CTLA4 -318 C>T genotypes between patients *vs*. controls ($p=0.021$). As seen in Table II, homozygous CC variant of *CTLA4* -318 C>T was more frequent in patients than in controls (92.5% *vs*. 77.9%, $p<0.05$). In addition, all TT genotype carriers were controls. For the *CTLA4* 49 A>G polymorphism, the genotype distributions were in agreement with the Hardy–Weinberg equilibrium in patients and controls ($p>0.05$). The frequency of homozygous CC genotype of *CD28* C>T polymorphism was higher in controls (12.4%) *vs*. CRC cases (6.3%), however, this difference was not statistically significant.

The distributions of *CTLA4* -318 C>T, *CTLA4* 49 A>G, and *CD28* C>T genotypes according to gender, tumor stage, lymph node metastasis, and perineural invasion status in patients with CRC are summarized in Table III. It was found that the frequency of *CTLA4* -318 C>T variants in patients with advanced tumor stage (T3/T4) or nodal involvement (N1+N2+N3) was in the order of CC>CT>TT. On the other hand, no significant association was found between the *CTLA4* 49 A>G or CD28 C>T genotypes and clinical features of CRC.

The haplotype comparison analysis of *CD28* C>T, *CTLA4* -318 C>T and *CTLA4* 49 A>G variants in the study groups is shown in Table IV. The haplotype analysis revealed that the frequency of the combined genotype CTA (rs3116496, rs5742909, rs231775, respectively) was significantly higher in the control group than in patients with CRC ($\chi^2=4.927$, $p=0.0264$).

The protein oxidation markers, lipid peroxidation markers and antioxidant parameters were evaluated in the study groups. The concentrations of protein markers in the plasma of patients with CRC and controls are given in Table V. According to our results, AOPP and PCO levels were significantly higher in patients with CRC ($p=0.002$ and $p=0.001$, respectively). Likewise, the concentration of the

Table I. *The clinicopathological characteristics of patients with colorectal cancer patients(n=80). Chi-square test was used to compare characteristics of the study groups and only the percentages of the values were given.*

Characteristic	%
Tumor stage	
T1+T2	20
T3+T4	80
Lymph node status	
N+	51.7
N–	48.3
Metastasis	
Present	32.8
Absent	67.2
Perineural invasion	
Positive	31.7
Negative	68.3
Differentiation	
Well	72.1
Poor	27.9

Table II. *The distribution of cytotoxic T-lymphocyte-associated protein 4 (CTLA4) -318 C>T, CTLA4 -49 A>G and cluster of differentiation 28 (CD28) C>T genotypes in the study groups. Chi-square test was used to compare genotypes in the study group.*

Genotype	Patients with CRC, %	Controls, %
CTLA4 -318 C>T		
CC	92.5*	77.9
CT	7.5	20.4
TT	-	1.8
CTLA4 -49 A>G		
AA	47.5	46.9
AG	45	45.1
GG	7.5	8
CD28 C>T		
CC	6.3	12.4
CT	33.8	32.7
TT	60	54.9

*Statistically significant at $p<0.05$ by Chi-square test. CRC, Colorectal cancer.

lipid peroxidation markers in the plasma of patients with CRC and controls are given in Table V. The LHP, MDA and AGE levels were statistically significantly higher in patients with CRC ($p<0.001$). On the other hand, antioxidant parameters Cu-Zn SOD and T-SH levels, were significantly lower in the patient group ($p<0.001$ and $p=0.039$, respectively). Moreover, Zn levels were found to be significantly higher in the control group than in the CRC group ($p<0.001$). Likewise Cu levels were higher in the control group than patients, however, the difference was not statistically significant ($p>0.05$) (Table V).

The oxidant and antioxidant parameters according to *CTLA4* -318C>T, *CTLA4* 49A>G and *CD28* C>T variants were also investigated in the study groups (data not shown). When AOPP levels of those with *CTLA4* -318C>T genotypes in the CRC group were evaluated, it was found that the AOPP level in those with CT genotype was higher than in those with the CC genotype (1185.6±507.0 μmol/l *vs.* 125.1±49.8 μmol/l; $p=0.038$). In contrast, the AGE level of CT genotype carriers was lower than those with CC genotype (418.51±337.5 AU/mg protein *vs.* 1416.32±97.7 AU/mg protein; $p=0.022$). Moreover, CT genotype carriers were found to have a lower MDA level than patients with CC genotype (6.33±1.84 μmol/mg protein *vs.* 11.31±1.29 μmol/mg protein; $p=0.063$). When we evaluated MDA level according to *CD28* C>T genotype, we detected that the MDA level of patients with CRC with TT genotype were lower than of those with CC genotype (10.56±1.31 μmol/mg protein *vs.* 30.98±-μmol/mg protein; $p=0.091$), however, this relation only weakly approached statistical significance. No other significant differences were detected between other oxidant and antioxidant parameters by genotype. On the other hand, we found that the Cu level in patients with CRC with AG genotype of *CTLA4* 49 A>G and who had perineural invasion were lower than those without this kind of invasion (98.0±19.99 μg/dl *vs.* 155.87±14.9 μg/dl; $p=0.046$).

As shown in Table VI, serum levels of CTLA4 and CD28 were measured in patients and healthy controls by ELISA, and it was found that the serum CTLA4 level in patients was significantly lower than that in healthy individuals ($p<0.001$). In contrast, the serum CD28 level in healthy individuals was significantly higher than that in patients with CRC ($p<0.001$).

The serum CTLA4 and CD28 levels were also evaluated according to *CTLA4* -318C>T, *CTLA4* 49A>G and *CD28* C>T variants, however, only *CTLA4* 49 A>G polymorphism was statistically associated with serum CTLA4 and CD28 levels. The serum CD28 level in patients with CRC with AG genotype for *CTLA4* 49 A>G and who had angiolymphatic invasion was lower than that of those without this kind of invasion (2.55±0.18 ng/ml *vs.* 3.05±0.33 ng/ml; $p<0.09$). No other association was found between serum CTLA4 and CD28 levels and the clinical parameters of patients or *CTLA4* 49 A>G and, *CTLA4* -318C>T and *CD28* C>T genotypes (data not shown).

Discussion

Like all other cancer types, the risk factors for CRC includes accumulation of genetic, epigenetic and lifestyle or environmentally-induced defects, resulting in sustained proliferative potential, inflammation, induction of angiogenesis,

Table III. *Distribution of genotype by clinicopathological features in patients with colorectal cancer. Chi-square test was used to compare alleles and clinicopathological characteristics in the study group.*

Feature	Genotype, %								
	CTLA4 0318 C>T (rs5742909)			*CTLA4* 49 A>G (rs231775)			*CD28* C>T (rs3116496)		
	CC	CT	TT	AA	AG	GG	CC	CT	TT
Gender									
Female	93.9	6.1	0	51.5	45.5	3	9.1	27.3	63.6
Male	91.5	8.5	0	44.7	44.7	10.6	4.3	38.3	57.4
T0Stage									
T3+T4	95.8	4.2	0	41.7	45.8	12.5	8.3	33.3	58.3
T1+T2	100.0	0	0	58.3	41.7	0	8.3	25.0	66.7
Lymph node status									
N1+N2+N3	93.3	6.7	0	36.7	46.7	16.7	3.3	36.7	60.0
N0	100.0	0	0	50.0	46.4	3.6	14.3	25.0	60.7
Metastasis									
Yes	94.7	5.3	0	31.6	47.4	21.1	0	42.1	57.9
No	97.4	2.6	0	51.3	43.6	5.1	12.8	33.3	53.8
Perineural invasion									
Yes	100.0	0	0	47.4	47.4	5.3	0	42.1	57.9
No	95.1	4.9	0	41.5	46.3	12.2	12.2	29.3	58.5
Tumor differentiation									
Well	91.7	8.3		41.7	58.3	0	8.3	8.3	83.3
Poor	96.8	3.2		45.2	48.4	6.5	3.2	45.2	51.6

%, Percentage of individuals.

Table IV. *Haplotype comparison analysis of cluster of differentiation 28 (CD28), cytotoxic T-lymphocyte-associated protein 4 (CTLA4) -318 C>T and CTLA4 -49 A>G and variants between patients with colorectal cancer and controls.*

Haplotypes of CD28 C>T, CTLA4 -318 C>T and CTLA4 -49 A>G, respectively	Case:control ratio	χ^2	*p*-Value
CTA	4.5:155.5 18.6:207.4	4.927	0.0264
CT	4.6:155.4 18.8:207.2	4.928	0.0264
TA	6.0:154.0 27.0:199.02	8.051	0.0045

The statistical analyses of the non-normally distributed data of plasma oxidative stress parameters between patients and controls share the same genotypes were performed by using Mann-Whitney U-test. Linkage disequilibrium among CD28 C>T, CTLA4 -318 C>T and CTLA4 -49 A>G and polymorphisms were evaluated using D' and r2 values obtained through the Haploview 4.2 program. A value of $p<0.05$ indicates statistical significance.

and escape from apoptosis. The role of immune system in tumor development and progression by recognition and suppression of malignant cell growth is well-known (41, 42). An effective immune response for significant antitumor effect requires increased immune system activation and reduced inhibitory and suppressive molecules belonging to the immune system (43, 44). In light of this, recent studies focused on the development of immunotherapeutic strategies to improve antitumor capacity of the immune system (45). It is well-known that tumor cells use inhibitory pathways critical for physiological homeostasis to silence the host immune system (46). CTLA4/CD28 co-stimulatory signaling is an immunoregulatory checkpoint which regulates the generation and maintenance of the immune response (47).

CTLA4 protein, expressed by activated T-cells, has 223 amino acids and this predicted sequence was homologous with that of CD28 protein. Both are also functionally related as they both bind to the same B7 family members, CD80 and

Table V. *Oxidant and antioxidant levels in patients with colorectal cancer patients and controls.*

Analysis	Controls	Patients with CRC	*p*-Value
PCO (nmol/mg protein)	1.09±0.26	1.61±0.61	0.001
AOPP (μmol/l chloramine-T equivalent)	38.54±18.71	57.14±24.27	0.002
LHP (μmol/mg protein)	1.25±0.27	1.75±0.83	<0.001
MDA (μmol/mg protein)	7.51±1.50	11.44±6.14	<0.001
AGEs (AU/mg protein)	992.78±436.5	1472.7±482.6	<0.001
T-SH (nmol/mg protein)	1.41±0.73	1.08±0.37	0.039
Cu-Zn SOD (U/mg protein)	5.43±0.88	3.82±0.79	<0.001
Cu (μg/dl)	158.9±5.70	144.4±6.91	>0.05
Zn (μg/dl)	111.5±4.15	63.46±2.25	<0.001

Values of $p<0.05$ indicate statistical significance. PCO, Protein carbonyl; AOPP, advanced oxidation protein products; LHP: lipid hydroperoxides; MDA: malon dialdehyde; AGEs, advanced glycation end-products; T-SH, total thiol; Cu-Zn SOD, copper,zinc-superoxide dismutase.

CD86, on APCs. However, while CTLA4 has much more affinity to these counter receptors, CD28 and CTLA4 have opposing effects on T-cell activation. CTLA4 has an inhibitory effect on T-cell responses, leading to augmentation of cytokine production and T-cell proliferation (8).

The molecular mechanism of how CTLA4/CD28 signaling contributes to various autoimmune diseases and types of cancer are still not well known. It was shown that *Ctla4* knockout mice undergo a massive CD28-dependent expansion of autoreactive T-cells in lymph nodes, spleen and peripheral organs, and due to the generation of lymphoproliferative disease, these mice die less than 4 weeks after birth (45, 48, 49). On the other hand, it was shown that by exogenous CTLA4 application, mice were rescued from lethality (45, 50).

Preclinical research has shown that blockade of CTLA4, which was thought to inhibit T-cell activities, actually augments endogenous responses to cancer cells, which in turn results in cancer cell death. Thus, clinical development of several CTLA4 antibodies has been improved for the treatment of advanced or metastatic malignancies. In murine models, utilization of anti-CTLA4, led to complete regression or deceleration in tumor growth in several types of cancer including of the bladder, brain, fibrosarcoma and ovary. However, it was revealed that in melanoma, lymphoma, lung and some types of breast cancer, anti-CTLA4 treatment was not effective. Furthermore, the sensitivity to anti-CTLA4 treatment was not completely efficient in CRC (45).

Redox biology has been shown to provide non-invasive markers for assessing ROS production *in vivo*. High levels of oxidative species induce formation of free radicals. On the other hand, IFN-γ is known to be secreted from activated T-cells during inflammation or immune response, which in turn activates IDO for tryptophan breakdown. During this degradation, in addition to ROS generation, activation and proliferation of T-cells were reduced, which results in immunosuppression (11, 21-26, 51). It was also shown that IDO activity was inhibited by several functionally and structurally different antioxidants (23). Therefore, in the present study, the parameters of redox status, oxidant and antioxidant balance, which can trigger inflammation were investigated and analyzed together in respect to serum levels of CTLA4 and CD28 variants.

Table VI. *The serum levels of cluster of differentiation 28 (CD28) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) in patients with colorectal cancer and controls.*

	Control	Patients	*p*-Value
CTLA4 (ng/ml)	0.372±0.039	0.326±0.034	<0.001
CD28 (ng/ml)	2.103±0.220	2.630±0.473	<0.001

Mean values of clinical parameters between patients and controls were compared with unpaired Student's *t*-test. The results are shown as the mean±SD. Values of $p<0.05$ indicate statistical significance.

Chandramathi *et al*. reported high levels of AOPP, H_2O_2 and MDA in patients with CRC (52). Avinash *et al*. observed very significantly high increase in AOPP, and decrease in total thiols and antioxidant capacity among patients with CRC (53). Yang *et al*. concluded from their research that in proteins containing cysteine residues, oxidation is paralleled by CRC-related changes in biological function or loss of enzyme activity (54). Wei *et al*. revealed that the serum total antioxidant capacity and SOD levels were lower in patients with CRC, and in contrast, the MDA level was higher (55). Leufkens *et al*. investigated the association between reactive oxygen metabolites (ROM) and ferric-reducing ability of plasma (FRAP) and CRC risk in a cohort-nested case–control study. They found that while FRAP was not

related to CRC risk, higher ROM levels were associated with the increased risk of CRC. However, they reported that this association was only seen in patients with a relatively short follow-up, suggesting that the association may be due to the production of ROS by preclinical tumors (56).

Vecchia *et al*. (57) and Santiago-Aeteche *et al*. (58) reported an inverse association between total antioxidant capacity and CRC risk. Janssen *et al*. investigated Cu, Zn-SOD in relation to the overall survival of patients with CRC and found no difference between normal mucosa and carcinomas. They also evaluated the effects of Cu-Zn SOD content of the tissues on clinicopathological features such as gender, age, tumor localization and differentiation grade, however, with the exception of the latter, they reported no association (59). However, Skrzydlewska *et al*. showed a positive association between the activity of SOD and risk of CRC, as well as lipid peroxidation in respect to MDA level (60). In addition to this study, Skrzydlewska *et al*. reported a significant increase in MDA and Cu-Zn SOD levels in CRC patients (61). In contrast, Upadhya *et al*. reported a decrease in MDA levels of patients with CRC compared with controls (62).

In the present study, protein and lipid oxidation parameters, AOPP, PCO, LHP, MDA and AGE, were significantly increased in patients with CRC. In contrast and as expected, the antioxidant parameters (Cu-Zn SOD and T-SH) were significantly lower in the patient group. In addition, lower serum CTLA4 and higher serum CD28 levels were detected in patients with CRC. Our results are in accordance with those of previous studies. However, different results were also reported in literature, which suggests the importance of dietary behavior or sustaining physiological conditions over CTLA4 such as the indirect generation of ROS by degradation of tryptophan *via* IDO. As explained, blockade of CTLA4 could be a potential immunotherapeutic approach to several cancer types by enhancing T-cell immonoreactivity as an antitumoral effect (9-20). On the other hand, our results show high oxidant and low antioxidant status may increase inflammation as a defense mechanism. In fact, high expression of serum CTLA4 was shown in several autoimmune diseases (63-65) and in certain types of cancer, such as breast (66), and lung (67) cancer. In addition, some researchers showed that the function of T-cells was inhibited by high levels of soluble CTLA4 and the role of soluble CTLA4 is not as well known as that of membrane-bound CTLA4 (68-70). Soluble CD28 is one of the major co-stimulatory molecules playing a role in T-cell activation and proliferation. It has been reported that soluble CTLA4 plays a suppressive role in tumor immunoregulation by inhibiting the interaction between CD28 and B7 molecules (66, 70), ultimately leading to antiproliferative and apoptotic effect on T-cells; as CTLA4 is expressed by T-cells, this could explain the lower level of serum CTLA4 found in patients compared to controls. On the other hand, the metabolites generated during tryptophan degradation have both either oxidant or antioxidant function. Thus, several different antioxidant mechanisms can also be activated against IDO activity in order to maintain cellular redox homeostasis (23). For co-stimulatory CD28, we suggest that because of a low level of CTLA4, the CD28 level remains higher so as to maintain physiological status. We hypothesize that our results are in accordance with this physiological compensation mechanism as a defense.

As Cu and Zn are required for the activity of SOD as cofactors, we measured the levels of Cu and Zn and found them to be lower in the patient group. This suggests that the SOD activity is insufficient due to low levels of Cu and Zn. Our results were in accordance with previous reports. Christudoss *et al*. reported a significant decrease in plasma Zn levels in cancerous tissues in the colon of dimethyl hydrazine-treated rats thus, the authors concluded that the decrease in plasma Zn and their related enzymes are associated with the development and progression of preneoplastic lessions in colonic carcinomas (71).

Polymorphism studies provide important information about cancer development and progression, as well as helping to improve anticancer drugs and the efficiency of cancer treatment (72). Therefore, for individualized therapy strategies and assignment of mechanistic effects, gene polymorphisms cannot be ignored. Accordingly, polymorphisms of *CTLA4*, which plays an important role in the down-regulation of T-cell activation, and its co-stimulator *CD28* were investigated in this study. Our results showed that the presence of T allele of CTLA4 -318 C>T was associated with CRC development which may be related to suppressing the antitumor immune response of T-cells by increasing transcriptional activity, however as a limitation of our study further activation studies will required to improve this hypothesis. In addition, *CTLA4* -318 C>T substitution was found to be associated with cervical cancer in that T allele has stronger negative regulation of T-cell proliferation and function (73). However, interestingly, our results show that the frequency of the homozygous CC variant of *CTLA4* -318 C>T was higher in patients with CRC than controls, which suggests immunosuppression rather than immunoreactivity due to low levels of CTLA4 as the patients with CRC with CC genotype were found to have a poor prognosis, with higher frequencies of invasion, metastasis or poor differentiation.

On the other hand, it has been claimed that individuals having A allele of *CTLA4* 49 A>G polymorphism have higher CTLA4 expression than those having the G allele. Therefore, it was suggested that the A allele is associated with inhibition of T-cell proliferation and activation, and previous studies revealed an association between CRC and *CTLA4* 49 G>A polymorphism. For example, in Chinese

population CTLA4 49 G>A polymorphism was found to be correlated with CRC risk, whereas there was no relation found in a Turkish population, and our results are consistent with those of Dilmec *et al*. (9, 35, 74).

In conclusion, our data show that oxidative stress was increased in patients with CRC. We suggest that disturbed oxidative stress status and trace element levels may contribute to the pathogenesis of CRC. In addition, when we look at previous studies of cancer, the immune system plays an effective role in cancer progression. From this point of view, it can be suggested that the risk of CRC may be evaluated with soluble CTLA4/CD28 levels or associated with polymorphisms. The present study was a preliminary study to establish the link between oxidative stress and *CTLA4* -318 C>T, *CTLA4* -49 A>G and *CD28* C>T polymorphisms, soluble CTLA4 and CD28 levels, and pathogenesis of CRC among a Turkish population. However, the main limitations of the present study were the lack of measurements of IDO activity and IFN-γ, therefore this preliminary study should be extended with such assays in large study populations to obtain more reliable results.

Conflicts of Interests

The Authors declare that no competing interests exist in regard to this study.

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