Indian Journal of Experimental Biology
Vol. 62, September 2024, pp. 713-721
DOI: 10.56042/ijeb.v62i09.5682

Investigation of the role of CTLA-4 +49A/G (rs231775) polymorphism in non-small cell lung cancer and T cell immunity

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Received 19 September 2023; Revised 02 April 2024

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) was the first immune checkpoint molecule to be used as a drug target and led the way in the field of immunooncology. CTLA-4 increases the activation threshold of T-cells and reduces immune responses to weak antigens, such as self and tumour antigens. In our study, 56 patients were diagnosed with NSCLC, and a control group of 98 healthy volunteers was included. CTLA-4 +49A/G gene polymorphism and serum CTLA-4 levels were assessed. However, we found that CTLA-4 +49A/G gene polymorphism was associated with lymphovascular invasion (LVI) (*P*=0.049). The ratio of the heterozygous AG variant was 42.9% in patients with LVI, while it was 14.3% without LVI. This could indicate that the CTLA-4 +49A/G heterozygote AG variant increases the risk of LVI. In addition, we detected with the CTLA-4 +49A/G heterozygote AG variant had the worst mean overall survival at 56 weeks in the NSCLC patient group (X±SE=56.00±11.52, 95%CI 33.41-78.58, *P*=0.048). Furthermore, the patient group had significantly higher CTLA-4 serum levels (X±SE=121.57±11.89 pg/mL) compared with the control group (X±SE=79.09±3.09 pg/mL)(*P*=0.02). Our study data serve as a guide for future studies to elucidate the pathogenesis of NSCLC and evaluate the therapeutic significance of CTLA-4.

Keywords: CTLA-4, Lung cancer, NSCLC, T cell immunity

In the microenvironment of tumours, the role of T cells is crucial in determining the progression of the tumour[1-3]. In this context, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and CD28 play an essential role in regulating the activity of T cells by activating or deactivating them, respectively[4]. CTLA-4 is essential for controlling immune reactions and preserving self-tolerance[5]. As an immune checkpoint molecule, CTLA-4 serves as a negative regulator, reducing immune responses and T-cell activation[6]. Upon activation, cytotoxic T-lymphocytes (CTLs) identify and eliminate cancer cells from the same individual. The initial activation signal is transmitted via tumour-associated antigens (TAAs) presented by major histocompatibility complex (MHC) class I molecules on antigen-presenting cells (APCs) and confers specificity to the immune response[7,8]. The second signalling, known as "co-stimulatory signalling" facilitates interaction between T cells and APCs through molecules that engage with specific co-stimulatory receptors on T cells[9]. CTLA-4 is translocated to the surface of T cells where it competes with another immune checkpoint molecule, CD28 is a well-known co-stimulatory receptor that binds to two molecules, B7-1 (CD80) and B7-2 (CD86), which are expressed on APCs[10]. Interactions between B7 and CD28 promote T-cell proliferation, differentiation, and survival. By outcompeting CD28, CTLA-4 interrupts the stimulatory signals necessary for T-cell activation, thus curbing immune responses[11]. This mechanism of action makes CTLA-4 an attractive target for cancer immunotherapy, as blocking its function can enhance T-cell responses against tumour cells[12]. CTLA-4 is a homodimeric glycoprotein receptor found on CTLs and is a CD28 homolog CTLA-4 is a coinhibitory receptor that links to B7-1 and B7-2, similar to CD28[13]. However, CTLA-4 is expressed on T cells after they are activated, while CD28 is expressed before activation[8]. CTLA-4 inhibits interleukin-2 production and cell-cycle progression, leading to the establishment and maintenance of T cell tolerance[14]. Under normal conditions, it reduces the T cell response to both foreign antigens and autoantigens. However, in the tumour microenvironment, CTLA-4 expression on T cells is upregulated, primarily facilitated by TGF-β, a

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suppressive cytokine released by tumour cells CTLA-4 can act as a brake on T cell activation, making it more difficult for T cells to kill tumour cells. This can promote the risk of cancer development in the early stages of tumour growth[15,16]. CTLA-4 mRNA has been found to be expressed in a variety of non-T cells, including placental fibroblasts, cultured muscle cells, monocytes, and neoplastic cells, such as those derived from leukemia and solid tumours[17].

CTLA-4 is located on chromosome 2q33. Its functionality can be affected by several genetic variations, including single nucleotide polymorphisms (SNPs), which can contribute to disease phenotypes[18]. Numerous CTLA-4 polymorphisms that can affect gene expression, cause amino acid substitutions, and alter mRNA splicing have been identified. These polymorphisms have been associated with a predisposition to autoimmune diseases[19-25]. In conclusion, genetic polymorphisms within the CTLA-4 gene may also influence antitumour responses, including proliferation and activation of CTLs in cancer patients[26,27]. However, despite extensive research on CTLA-4 SNPs in autoimmune diseases, published data on their role in human cancers are limited[26,28,29]. The most recent meta-analysis, which included reports on lung cancer, established a link between CTLA-4 SNPs and the risk of multiple cancer types[30].

Limited studies investigate the relationship between the CTLA4 +49AG gene and lung cancer. Our study is important in investigating serum CTLA4 levels in healthy individuals and NSCLCs, detailed clinicopathology connections of CTLA4 +49AG gene variants, and especially their relationship with survival. This study investigated the possible relationship between a selected CTLA-4 polymorphism that affects the leader sequence (+49A/G) of the soluble CTLA-4 protein and non-small cell lung cancer. The selected CTLA-4 polymorphism, which affects the leader sequence of the CTLA-4 protein (+ 49A/G), is determined within the scope of this study. We also aimed to examine whether CTLA-4 levels in an NSCLC patient's serum poses a risk for the development of NSCLC.

Materials and Methods

Collection of samples

The study included 56 patients diagnosed with NSCLC stage IA-IIIB and 98 healthy volunteers. CTLA4- polymorphisms of the patients and healthy volunteers were studied. The patients were informed about the study, and written consent was obtained. DNA was isolated from the blood. The purity and concentrations of the DNA samples were calculated. Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism, and Agarose Gel Electrophoresis were used for these DNA samples.

DNA isolation by salt precipitation method

Blood samples were placed in 50 mL Falcon tubes and three volumes of Lysis Buffer (RBL) Erythrocyte Lysing Solution were added. 15-20 min at +4°C. Blood was maintained for hemolysis. The mixture was then centrifuged at 1500 rpm for 10 min at +4°C. The supernatant was discarded, and the pellet was homogenized. Lysis Buffer (RBL) Erythrocyte lysing solution was added to the pellet, twice the initial volume, and after it was kept at +4°C for 15-20 min, it was centrifuged at 1500 rpm for 10 min. The supernatant was discarded, and the pellet was homogenised. Ten millilitres of Leukocyte Breakdown Solution (WBL), 75 µL of proteinase K, and 500 µL of 10% sodium dodecyl sulfate (SDS) were added and incubated in a 65°C water bath for 1h. Then 3.7mL of 9 M ammonium acetate (NH4Ac) was added and mixed. The material was centrifuged for 20-30 min at 4500 rpm at +4°C. The upper part containing the DNA was taken into a clean falcon tube, and the precipitate was discarded. 2× volume of 99% ethanol was added to DNA. The precipitated DNA was transferred to 70% ethanol in a 1.5mL microtube and then centrifuged at 14000 rpm for 1 min. The supernatant was removed and pellet was dried at 56°C for 20-30 min. The DNA was dissolved in distilled water and maintained at 56°C for 20-30 min. OD measurements were performed using a spectrophotometer to determine the purity and amount of DNA was obtained. DNA was stored in a refrigerator at +4°C[31].

Polymerase chain reaction and Restriction fragment length polymorphism

The PCR sequence analysis applied in the study was performed according to the appropriate melting temperatures of the primers suitable for the gene region to be analysed. To amplify the gene, we utilised the following primers: the forward primer with the sequence 5'-GCT CTA CTT CCT GAA GAC CT-3' and the reverse primer with the sequence 5'-AGT CTC ACT CAC CTT TGC AG-3' For the detection of gene polymorphisms, 10pmol primer

□

pairs for CTLA-4 +49 A/G (rs231775) gene polymorphism analysis, locus specific forward and reverse primers, restriction fragment length, and PCR were performed. The 1.5mM dNTP mixture contains 10× reaction solution and 2mM $MgCl_2$. An appropriate PCR reaction was carried out under the optimum conditions for the PCR mixture to be prepared at the appropriate concentration of DNA samples. The PCR reaction was carried out at 94°C for 3 min, 94°C for 15 s, 58°C for 30 s, 72°C for 1 min (33 cycles), and 72°C for 7 min. To determine the related gene polymorphisms after PCR, the PCR products were cut by incubation with *Bbv*I and *Tru*1I (*Mse*I) restriction enzymes at the optimum temperature and time to be determined for each enzyme. PCR products were loaded on an agarose gel at 3% concentration and electrophoresed at 100 volts for 20-30 mins. With this process, we aimed to display PCR products obtained using restriction enzymes. The products that were placed in the electrophoresis device, loaded into the gel, and separated from each other in the gel, were examined under ultraviolet (UV) light[32].

Determination of serum CTLA-4 level by ELISA method

The human Soluble Cytotoxic T-lymphocyte-associated protein-4 (sCTLA-4) ELISA test kit developed by Abbkine (Abbkine Biotechnology Co., Ltd, Wuhan, China) was used in this study. The samples were reconstituted using the sample diluent provided in the kit. Forty microlitres of sample diluent and 10μL of serum (sample) were added to each sample well. The mixture was then incubated at 37°C for 45 min. After incubation, 250μL of wash buffer was added, and the cells were washed five times. After drying, 50μL of HRP-conjugate was added, and the plate was covered and incubated for 30 min at room temperature. Again, 250μL of washing buffer was added, and the cells were washed five times. Then, 50μL of chromogens A and B were added to the wells that were thoroughly washed. The plate was incubated at room temperature for 15 min in the dark, and 100μL of stop solution was added to each well and read in the ELISA device without waiting. The results were calculated in pg/mL after reading at 450 nm wavelength using an ELISA reader. The sensitivity of the kit was determined to be less than 3.0pg/mL[33].

Statistical analysis

Statistical analysis was performed using SPSS software package (version 21.0.0.0 SPSS Inc., Chicago, IL, USA). We performed the Shapiro-Wilk test to check the normality assumption on the patient and control group. An independent t-test and Chi-square were conducted for each group to analyse the demographic data. CTLA-4 +49 A/G genotypes between groups analysis performed Chi-square analysis. We used the Chi-square statistic to compare the distribution of genotypes and alleles between the patients and controls. Kruskal-Wallis test was performed to compare genotypes and sCTLA4 levels. We conducted a Kaplan-Meier survival analysis test to compare genotypes and overall survival (OS) time. Allelic frequencies were estimated by gene counting methods. A univariate analysis was performed to compare the distribution of pathological parameters and age and gender and the frequencies of alleles and genotypes.

Results

To determine the 49 A/G variants of the CTLA-4 gene, a total of 56 NSCLC patients (5 females and 51 males) diagnosed with NSCLC and 98 healthy volunteers (57 females and 41 males) were studied as controls. The mean ages of the patients and control group were 60.7±11.25 and 56.5±12.46 years respectively, (P=0.050). There were statistically significant differences between the NSCLC patients and control groups in terms of gender and smoking status ($P<0.001$) (Table 1). The patient group had higher serum CTLA-4 levels (121.57±11.89pg/mL) compared with the control group (79.09±3.09pg/mL). This difference was statistically significant (P<0.05) (Table 2). The scatter plot of serum CTLA-4 levels of

Table 1 — Demographic data of the non-small cell lung cancer patients and the healthy controls

Parameters		NSCLC Patients		Control Group		*P* value
Age (X±SD)		60.70±11.25		56.15±12.46		0.050
		n	%	n	%	
Smoking status	Yes	52	92.9	7	7.1	
	No	4	7.1	91	92.9	<0.001*
Gender	Male	51	91.1	41	41.8	
	Female	5	8.9	57	58.2	<0.001*

[*<0.001, n: number of individuals, NSCLC: non-small cell lung cancer, X:mean, SD: standard deviation]

Table 2 — Serum CTLA-4 levels in patients and control groups

Parametres	Patients Group X±SE	Control Group X±SE	*P* value
Serum sCTLA-4 (pg/mL)	121.57±11.89	79.07±3.09	0.002*

[*P<0.05 X: mean, SE: standard error, NSCLC: non-small cell lung cancer]

the groups is shown in Fig. 1. The genotype and allele distribution of the 49 A/G variants of CTLA-4 between in patients and control groups are shown in Table 3. Homozygous AA and GG genotypes in CTLA-4 +49 A/G polymorphisms ratios were detected higher in patients (57.1% and 10.7% respectively) than in controls (50.7% and 7.5% respectively). Heterozygous AG genotype ratios were lower in patients (32.1%) than in controls (42%). But these differences were not statistically significant. (*P*=0.327) (Table 3) The carrier status of the A allele (AA+AG) for the CTLA-4 +49 A/G polymorphism in the same patient group was found to be slightly higher (73.2%) than that in the control group (71.5%), but the difference was not statistically significant

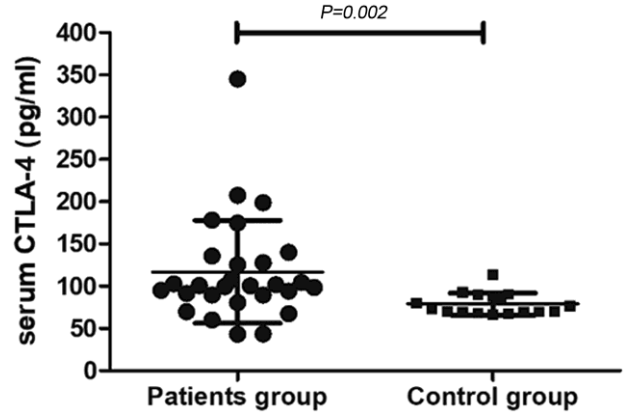

Fig. 1 — The scatter plots of serum CTLA-4 levels.

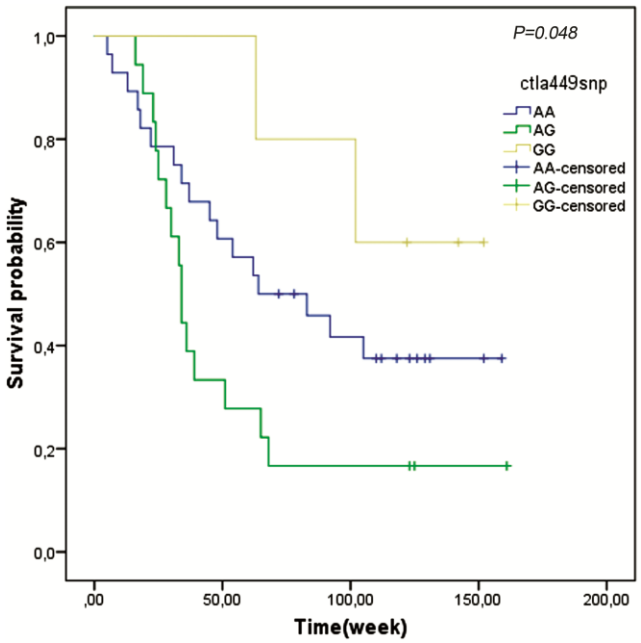

Fig. 2 — Kaplan-Meier curves of CTLA-4 +49 A/G genotypes (AA, AG, and GG) with non-small cell lung cancer patients.

(*P*=0.740) (Table 3). The association between the CTLA-4 +49 A/G gene variants with OS was presented in Table 4. The patients with the AG variant had the worst mean OS with 56 weeks (X±SE=56.00±11.52 weeks, 95% CI 33.41-78.58, *P*=0.048). The patients with the AA variant was 87.61 weeks of mean OS (X±SE=87.61±11.47 weeks, 95% CI 65.12-110.10). The patients with the GG variant was 124.20 weeks of mean OS (X±SE=124.20 ±16.19 weeks, 95% CI 92.45-155.94). The association of CTLA4 +49 A/G genotypes (AA, AG, and GG) and OS time Kaplan-Meier curves were presented in Fig. 2. The association of CTLA4 +49 A/G genotypes (AA, AG, and GG) and sCTLA-4 levels was analysed. No statistically significant difference was found in the analysis comparing the mean sCTLA4 levels of the AA, AG, and GG variants (*P*=0.061).

Clinical and pathological characteristics of the NSCLC patients as shown in Table 5. In the patient's group; tumour grade, lymph node metastasis, histological type, presence of lymphovascular invasion (LVI), presence of perineural invasion, and presence of distant metastasis parameters distribution were shown. Three patients were excluded from the statistical analysis as their histological type parameters were unknown. Pathological parameters, serum CTLA-4 levels, and CTLA-4 +49 A/G genotypes were compared in Table 6. CTLA-4 +49 A/G gene polymorphism was associated with LVI (*P*=0.49) (Table 6). The ratio of the heterozygous AG variant was 42.9% in NSCLC patients with LVI, while it was 14.3% in patients without LVI. This could indicate that the CTLA-4 +49 A/G heterozygote AG variant increases the risk of LVI. There was no

Table 3 — CTLA-4 +49 A/G genotypes in patients and control groups

Genotypes CTLA4 +49 A/G	NSCLC Patients		Control Group		*P* value
	n	%	n	%	
AA	32	57.1	54	50.4	0.327
AG	18	32.1	45	42.1	
GG	6	10.7	8	7.5	
G Allele	30	26.8	61	28.5	0.740
A Allele	82	73.2	153	71.5	

[NSCLC : Non-small cell lung cancer, n: number of individuals]

Table 4 — Overall survival of CTLA-4 +49 A/G genotypes

Genotypes CTLA4 +49 A/G	Overall survival time (week) X±SE	95% CI	*P* value
AA	87.61±11.47	65.12-110.10	0.048
AG	56.00±11.52	33.41-78.58	
GG	124.20±16.19	92.45-155.94	

[X:mean, SE: standard error]

statistical significance between and CTLA-4 49 allele types with gender and tumour grade, histological type, lymph node metastasis, perineural invasion, distant metastasis, and (Table 6). The other compared parameters in Table 6: serum CTLA-4 levels and gender, tumour grade, histological type, lymph node metastasis, LVI, perineural invasion, and distant metastasis were not statistically significant.

Table 5 — Clinical and pathological characteristics of the NSCLC patients

Parameters	n	%
Tumour grade		
T1	22	39.3
T2	18	32.1
T3	13	23.2
T4	3	5.4
Lymph node metastasis		
N0	36	64.9
N1,2,3	20	35.1
Histological type		
Squamous Cell	24	45.3
Adenocarcinoma	23	43.4
Other	6	11.3
Perineural invasion		
Yes	17	30.4
No	39	69.6
Lymphovascular invasion		
Yes	35	62.5
No	21	37.5
Presence of distance metastasis		
+Yes	2	3.6
No	54	96.4

[n: number of individuals]

Discussion

This study investigated the possible relationship between a selected CTLA-4 polymorphism that affects the leader sequence (+49A/G) of the soluble CTLA-4 protein and non-small cell lung cancer. The selected CTLA-4 polymorphism, which affects the leader sequence of the CTLA-4 protein (+ 49A/G), is determined within the scope of this study. We also aimed to examine serum CTLA-4 levels in NSCLC. The patient group had higher serum CTLA-4 levels compared to the control group, and the difference was statistically significant. Polymorphism studies have been frequently conducted to investigate the etiology of cancer and evaluate cancer risk[34-37]. Song *et al*. reported that the CTLA-4 + 49A/G polymorphism may be a prognostic factor for advanced NSCLC[38]. Supporting this result we detected that the AG variant had the worst mean overall survival at 56 weeks in the patient group (X±SE=56.00±11.52, 95% CI 33.41-78.58, *P*=0.048). Many studies have sought the possible association between CTLA-4 gene (+49A/G; rs231775) polymorphism and the incidence of cancer[39-44]. It has been previously reported that the

Table 6 — Pathological parameters, serum sCTLA-4 levels and CTLA-4 +49 A/G genotypes of patients group

Parameters	sCTLA-4 (pg/mL)		CTLA-4 +49 AG n (%)			
	X±SE	P *value*	AA	AG	GG	*P value*
Gender						
Female	87.75±20.15	0.554	3 (60)	2 (40)	0 (0)	0.704
Male	124.40±12.67		29 (56.9)	16 (31.4)	6 (11.8)	
Tumour grade						
T1+T2	124.23±16.17	0.849	22 (61.1)	11 (30.6)	3 (8.3)	0.706
T3+T4	115.61±14.30		10 (62.5)	5 (31.2)	1 (6.3)	
Histological type						
AC			14 (60.9)	7 (30.4)	2 (8.7)	0.849
SCC			12 (50)	9 (37.5)	3 (12.5)	
Other			4 (66.7)	1 (16.7)	1 (16.7)	
Lymph node metastasis						
N0	123.29±15.60	0.935	22 (61.1)	11 (30.6)	3 (8.3)	0,641
N1, N2, N3	117.73±17.80		10 (50)	7 (35)	3 (15)	
Lymphovascular invasion						
Yes	111.07±71.51	0.330	18 (51.4)	15 (42.9)	2 (5.7)	0.049*
No	202.13±8.80		14 (66.7)	3 (14.3)	4 (19)	
Perineural invasion						
Yes	111.12±12.71	0,287	8 (47.1)	8 (47.1)	1 (5.9)	0.265
No	132.00±20.24		24 (61.5)	10 (25.6)	5 (12.8)	
Distance metastasis						
Yes	98.35	0.769	2 (100)	0 (0)	0 (0)	0,459
No	122.51±12.34		30 (55.6)	18 (33.3)	6 (11.1)	

[**P*<0,05, X:mean, SE: standard error, n: number of individuals, NSCLC: non-small cell lung cancer, AC: adenocarcinoma, SCC: squamous cell carcinoma]

presence of a polymorphism in the CTLA4 gene (+49A/G; rs231775) increases the risk of cervical cancer, non-small cell lung cancer, and renal cell carcinoma[45,46]. Located on the CTLA-4 gene; +49 A>G; rs231775, -318 C>T; rs5742909, -1722 T>C; rs733618, -1661 A>G; rs4553808 and +6230 G>A; The rs3087243 polymorphic regions have been studied in certain diseases and it has been stated that different alleles and genotypes of this gene may be associated with genetic susceptibility for various cancer types[36]. Threonine is substituted for alanine (CTLA-4+) by a single nucleotide polymorphism (SNP) at position 49 (rs231775) in exon 1 of the CTLA-4 gene[47,48]. According to studies, the +49G allele of CTLA-4, which encodes for the threonine residue, is linked to lower levels of protein expression compared to the +49A allele, which encodes for the alanine[49,50]. Because CTLA-4 is so important for preserving immune tolerance, decreased CTLA-4 expression has been linked to increased T cell activation and an increased risk of developing autoimmune diseases[51-53]. On the other hand, it has been discovered that the +49G allele is linked to a higher risk of developing a number of autoimmune diseases, including type 1 diabetes, Graves' disease, and autoimmune thyroiditis[54]. It has been reported that, the +49G allele can change CTLA-4's affinity for its ligands CD80 and CD86, altering immune reactions[55]. Additionally, the +49G allele has been linked to decreased CTLA-4 inhibitory function, which leads to increased T cell activation and may increase the risk of autoimmunity[52]. While the A allele belonging to the CTLA-4 49 A>G polymorphism shows different frequencies depending on ethnicity; L. Karabon *et al*. and Sun *et al*. reported that the A allele belonging to the CTLA-4 +49 A>G polymorphism was more common among NSCLC patients, while[26,34]. Similarly, our data also supported those findings. We have found that the frequency of the A allele was found to be higher in the NSCLC patients than in the G allele. In our previous study, we found that CTLA-4 49 A/G genotypes in patients with colorectal cancer did not show a significant difference when compared with the hematological parameters of the patients[56]. In the same study, it was emphasised that carrying the A allele may be associated with higher CTLA-4 expression compared to G allele. The results of this study suggest that carrying the A allele may be associated with inhibition of T cell proliferation and activation[56]. Liu *et al.* reported significant differences in genotype and allele frequencies of CTLA-4 +49A>G polymorphism among NSCLC cases and control[57]. Chen *et al.* reported no differences in CTLA-4 +49A>G polymorphism in cases and controls in Chinese population[58]. In another study, we investigated the possible relationship between CTLA-4 gene variants that function in T cell immunity and glial tumour. While there was no significant relationship between CTLA-4 49 A>G genotype distribution in glial tumour groups we found that patients with homozygous AA or GG (71.2%) had a higher frequency than that of the healthy individuals (53.4%), (OR: 1.33; 95%CI: 1.065-1.667) (P=0.015)[59]. In this study, there was no statistical significant difference between the control and patient groups in terms of both CTLA-4 gene 49 A/G genotype and allele distribution. However, it was observed that significantly more patients had the AG genotype in the presence of LVI. In addition, we detected NSCLC patients were higher serum CTLA-4 levels than controls. Studies to date have shown that CTLA-4 protein functional changes can be induced by genetic variations[60]. Among all CTLA-4 gene variants, +49 A>G, which causes changes in all amino acids (threonine to alanine), is the most extensively studied polymorphism[52,53,56,59-63]. Isitmangil *et al.* found that CTLA-4 +49 A/G genotypes were associated with breast cancer[61]. AA and GG genotypes homozygous for CTLA-4 +49 A/G were found to be more common in patients than those in controls. They found that these findings were mainly associated with presence of the AA genotype[61].

Arıkan *et al*. observed that the serum levels of CTLA-4 and sCD28 were significantly higher in patients with gastric cancer compared to those in healthy controls[62]. CTLA-4 levels were found to be elevated in patients with mesothelioma, acute lymphoblastic leukemia, and breast cancer[64-67]. However, on the other hand no significant association was found between the CTLA-4 +49A/G polymorphism and breast cancer, osteosarcoma, and gastric cancer[68,69]. In addition, all patients with stage T1 and T2 were homozygous for AA, and patients with AG and GG genotypes had more commonly advanced staged tumours. In addition, the prevalence of the GG genotype was GG (65%) in patients with distant metastases. This suggests that the G allele might be associated with more aggressive tumour phenotype[61].

Our study has limitations, including its retrospective nature, selection bias, and sample size. Also, smokers were the majority in the patient group,

which was the limitation in smoking status statistical calculations.

Conclusion

In conclusion, we did not find a different CTLA-4 +49A/G genotype and allele frequencies in lung cancer patients compared to healthy individuals. However, CTLA-4, +49 A/G heterozygous AG variant was associated with LVI and that may be associated with aggressive tumour behavior. In addition, we detected with the AG variant had the worst mean overall survival. NSCLC patients were higher serum CTLA-4 levels. Our study may serve as a guide for future studies to elucidate the pathogenesis of NSCLC or evaluate CTLA-4 in terms of its therapeutic value. Given the limited size of the study, it is necessary to analyse observations in larger sample groups.

Conflict of interest

Authors declare no competing interests.

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