

Effect of trail C1595T variant and gene expression on the pathogenesis of non-small cell lung cancer

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ABSTRACT

It is known that disorders in apoptosis function play an important role in the pathogenesis of many types of cancer, including lung cancer. Tumor necrosis factor related apoptosis inducing ligand (TRAIL), a type II transmembrane protein, is a death ligand capable of inducing apoptosis by activating distinctive death receptor. Our purpose in this study is to investigate the gene polymorphisms in TRAIL molecular pathway and TRAIL gene expression levels in non-small cell lung cancer (NSCLC) patients in terms of pathogenesis and prognosis of the disease. In this study, TRAIL C1595T polymorphism was genotyped using polymerase chain reaction-restriction fragment length polymorphism analysis in 158 patients with NSCLC and 98 healthy individuals. Surgically resected tissues were examined and classified histopathologically. In addition, TRAIL gene expression levels in tumor tissue and tumor surrounding tissue samples of 48 patients with NSCLC were determined using real-time polymerase chain reaction. TRAIL gene expression levels of NSCLC patients were detected significantly 28.8 fold decrease in the tumor tissue group compared to the control group ($p=0.026$). When patients were compared to tumor stage, expression of TRAIL gene in advanced tumor stage was found to be significantly 7.86 fold higher than early tumor stage [$p=0.028$]. No significant relationship was found between NSCLC predisposition and prognostic parameters of NSCLC with TRAIL genotypes, but the frequency of TRAIL gene 1595 CT genotype was observed to be lower in the patients compared to the other genotypes, and the difference was found to be very close to statistical significance ($p=0.07$). It can be suggested that TRAIL may play an important role in the development of NSCLC and may be an effective prognostic factor in tumor progression. It is known that disorders in apoptosis function play an important role in the pathogenesis of many types of cancer, including lung cancer. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a type II transmembrane protein, is a death ligand capable of inducing apoptosis by activating distinctive death receptor. Our purpose in this study is to investigate the gene polymorphisms in TRAIL molecular pathway and TRAIL gene expression levels in non-small cell lung cancer (NSCLC) patients in terms of pathogenesis and prognosis of the disease.

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1. Introduction

Lung cancer is the leading cause of cancer deaths in men and women all over the world [1]. Lung cancers can be divided into two major histological types, as non-small cell lung cancer (NSCLC) and small cell lung cancer. They behave differently in terms of biologically and sensitivity to chemotherapy and radiotherapy [2,3]. NSCLC is responsible for 80–85% of lung cancer patients and is divided into squamous cell carcinoma, adenocarcinoma, and large cell carcinoma subtypes based on histological features [4]. Lung cancer frequency is depended on many factors such as smoking, passive smoking, occupational and environmental factors,

genetic susceptibility, age, gender, and ethnicity [2]. Parameters effecting NSCLC prevalence include environmental and genetic factors, abnormalities in growth factor signaling pathways and tumor suppressor gene pathways, apoptosis escape and epigenetic modification mechanisms [5]. It has also been confirmed that genetic mechanisms such as gene mutation, deletion, and polymorphism determine the susceptibility of different individuals to lung cancer [6]. In recent years, studies have been carried out at the molecular level to reveal the pathogenesis of lung cancers [5]. Death ligands that can cause apoptosis through cell surface death receptors are also one of the molecules studied.

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is type II transmembrane protein that is a member of tumor necrosis factor family [7,8]. It selectively triggers apoptosis in cancer cells, while TRAIL does not affect normal cells [9–11]. Five TRAIL receptors have been identified: TRAIL-R1 (death receptor 4 – DR4), TRAIL-R2 (DR5/KILLER), TRAIL-R3 (decoy receptor 1 – DcR1/TRID), TRAIL-R4 (DcR2/TRUNDD), and osteoprotegerin (OPG). TRAIL may induce or inhibit apoptosis depending on the receptor type that is bounded. TRAIL-R1 and TRAIL-R2 contain cytoplasmic death domain and induce apoptosis after ligand binding. The death domain of TRAIL-R3, TRAIL-R4, and OPG is either truncated or lack and cannot induce apoptosis [8,12,13]. TRAIL has been shown to be expressed at the protein and mRNA level in different tissue such as liver, lung, placenta, kidney, spleen, heart, ovary, small and large intestine, and also in several cells, including immune cells [14–16].

The TRAIL gene located on chromosome 3q26 is approximately 20 kb in length [17,18], and this gene which encodes an mRNA of 1.77 kb is composed of 5 exons and 4 introns [12,13]. Despite the fact that polymorphisms of TRAIL have been reported in various cancers such as lung cancer [6], colon cancer [12], breast cancer [13,19], gastric cancer [20], bladder cancer [21], prostate cancer [22], and renal cancer [23], in some diseases such as type 2 diabetes mellitus [24], intervertebral disc disorder [25], multiple sclerosis [26], ulcerative colitis [27], and fatty liver disease [28]. However, the role of TRAIL polymorphism in lung cancer is yet to be elucidated [29].

In a study of 592 patients with NSCLC, Luo et al. found that the CT + TT mutant genotype of the TRAIL C1595T variant was lower in the NSCLC group than in the control group, but this result was not statistically related. T mutant allele frequency was found to be significantly higher in patients with NSCLC [6]. The expression of apoptosis-inducing ligands such as TRAIL has been suggested to play an important role in cell regulation and may provide an immunological advantage for tumor cells [4]. TRAIL have determined is expressed by 91% of patients with NSCLC in immunohistochemical-based studies [3,4]. Spierings et al. have determined immunohistochemically low TRAIL expression in 9% of NSCLC samples and high TRAIL expression in 59% of NSCLC samples in 87 stage III NSCLC patients [4]. As also stated in the article of Luo et al., in an immunohistochemical study performed in 60 NSCLC tissue, the positive staining rate of TRAIL protein was found to be significantly lower in the tumor tissue compared to the adjacent normal tissue [6].

The influence of the 3'UTR region on gene regulation is a key mechanism and it is expressed that the polymorphism in position 1595 of the TRAIL gene may be a factor that can regulate the protein by altering the microRNA binding sequence of TRAIL [28].

In our study, it was aimed to determine genotype and allele frequencies by examining the gene polymorphisms on TRAIL, one of the important molecules in apoptosis pathway in patients with NSCLC and to investigate TRAIL gene expression levels. It was also aimed to determine whether these genotype and allele frequencies and TRAIL gene expression levels were correlated with the histopathological parameters of the disease. As a result of the obtained data, it will be evaluate whether a possible association between gene polymorphisms of the TRAIL molecular pathway and TRAIL gene expression levels in patients with NSCLC is effective on the pathogenesis and prognosis of the disease.

2. Methods

2.1. Subjects

In our study, peripheral blood samples of 158 patients, 27 female and 131 male, who were diagnosed as NSCLC at the Department of Thoracic Surgery, Cerrahpaşa Medical Faculty, Istanbul University were taken and tumor tissue and tumor surrounding tissue samples of 48 patients ($n = 11$ (%22.2) female, $n = 37$ (%77.8) male) were resected. Our work was carried out by the Ethics Committee of Istanbul University Cerrahpaşa Medical School with the permission number 219543.

2.2. Genotyping

DNA isolation was performed by salting out peripheral blood samples from NSCLC patients and healthy subjects [30]. For the TRAIL C1595T polymorphism the forward primer 5'-TGA GCA CTA CAG CAA ACA TGA-3' and reverse primer 5'-GCA CCA CTA AAA GAT CGC AGT-3' were used at a concentration of 10 pmol/μl for each primer. The PCR reaction mixture contained 150 ng DNA template, 1.75 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl(pH 8.4), 600 μM dNTP (iNtRON Biotechnology Co., Korea) and 0.06 unit Taq DNA polymerase (iNtRON Biotechnology Co., Korea). The PCR conditions were determined as first denaturation at 95°C for 5 min, 35 cycles at 94°C for 45 s, 58°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 5 min. PCR product was digested by RsaI(MBI Fermentas, CA) at 37°C for 2.5 h. After enzymatic restriction, two fragments having 59 and 332 bp(C allele) or three fragments having 59, 146, and 186 bp(T allele) were identified.

2.3. Gene expression

Tumor tissue and the tumor surrounding tissue samples were surgically dissected. Samples were stored in liquid nitrogen until use. Total RNA was isolated from tissues using the TRIzol method. cDNA synthesis from total RNA was performed by using the High-Capacity cDNA Reverse

Table 1. Genotype and allele frequencies of TRAIL C1595T variant in patients with NSCLC and controls.

Genotypes and alleles	Patients n (%)	Controls n (%)	p Value
Genotype			
CC	99 (62.7)	55 (56.1)	0.24
CT	43 (27.2)	36 (36.7)	
TT	16 (10.1)	7 (7.1)	
Allele			
C Allele	241 (76.3)	146 (74.5)	0.65
T Allele	75 (23.7)	50 (25.5)	

n: number of individuals

Transcription Kit (LifeTech, Applied Biosystem, USA). As a result of our literature researches and pilot studies, it was determined that GAPDH gene is suitable as housekeeping gene. PCR primers and probes for the TRAIL and GAPDH gene were selected using the Single Tube TaqMan Gene Expression Assay (LifeTech, Applied Biosystem, USA). The real-time PCR reaction mixture contained 100 ng cDNA, 2X PZR Master Mix (LifeTech, Applied Biosystem, USA) and 20X Primer and Probe ready mix (LifeTech, Applied Biosystem, USA) for both primers. Real-time PCR conditions were determined as UDG incubation at 50°C for 2 min, AmpliTaq Gold UP enzyme activation at 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. Real-time PCR analysis was performed using Stratagene Mx3005p instrument (Agilent Technologies, CA, USA) with TaqMan Gene Expression Master Mix (LifeTech, Applied Biosystem, USA).

2.4. Statistical analysis

In our study, Chi square and further tests were applied by using SPSS 7 package program for statistical analysis of TRAIL C1595T variant, TRAIL gene expression values, demographic data, and histopathological parameters. The variables of statistically significant difference in the univariate analysis were then introduced into the multivariate analysis. Mann–Whitney *U* test, which is one of the non-parametric tests, was applied by using GraphPad Prism 5 program for the statistical analysis of gene expression levels of tumors and control tissues. The data were verified with both statistical programs. *p* values that are lower than 0.05 were regarded as statistically significant.

3. Results

3.1. TRAIL C1595T polymorphism genotype and allele distributions

In our study, we examined TRAIL C1595T polymorphism in 158 NSCLC patients and 98 control groups. The genotype and allele frequencies of the TRAIL C1595T variant in NSCLC patients and controls are shown in Table 1. In our study, when the heterozygous CT genotype of the TRAIL C1595T variant was compared to the homozygous CC and TT genotypes, the

frequency of CT genotype carriers in the patient group was found to be lower than that of the control group and this difference was detected to be close to statistically significance ($p = 0.07$). Although TRAIL C1595T homozygous CC and TT genotype ratios were higher in patients than control and heterozygous CT genotype ratios were lower, there was no statistically significant difference between patient and control groups in terms of genotype distribution of C1595T variant ($p = 0.24$). There was no statistically significant difference between patient and control groups in terms of C allele carrier of the TRAIL gene C1595T variant ($p = 0.28$). Although the T allele frequency was lower in patients with NSCLC than in the control group, the difference between patient and control group was not statistically significant ($p = 0.15$). In addition, it can be stated that the T allele carrier may lead to about nearly twice the risk increase in terms of mutation presence (Fisher's Exact test, $p = 0.297$). The distribution of TRAIL C1595T genotypes according to clinical parameters and tumor characteristics of patients with NSCLC is shown in Table 2. When the genotype and allele distributions of TRAIL C1595T variant were examined according to the histopathological findings of NSCLC patients, perinural invasion, lymphatic invasion, vascular invasion, lymph node metastasis, tumor stage, and metastasis of distant organs were not associated with genotype. We found no statistically significant difference between genotype and allelic distribution and gender with respect to multivariate analysis ($p > 0.05$).

3.2. TRAIL gene expression

The gene expression levels of TRAIL were determined using the $\Delta\Delta Ct$ method. A calibrator was established to normalize the obtained $\Delta\Delta Ct$ value. Separate calibrators were used for each group because of the heterogeneous distribution of tumor tissue group and tumor surrounding tissue group values of NSCLC patients. Calibrator selection was done take notice of data such as geometric and arithmetic

Table 2. Genotypic distribution of TRAIL C1595T variant according to histopathological findings of NSCLC patients.

Histopathological parameters	CC n(%)	CT n(%)	TT n(%)	p Value
Lymph node metastasis				
N123	30 (65.2)	11 (23.9)	5 (10.9)	0.638
N0	36 (58.1)	20 (32.3)	6 (9.7)	
Tumor stage				
T3 + T4	21 (61.8)	11 (32.4)	2 (5.9)	0.567
T1 + T2	45 (60.8)	20 (27.0)	9 (12.2)	
Metastasis				
No	65 (62.5)	28 (26.9)	11 (10.6)	0.110
Yes	1 (25.0)	3 (75.0)	0 (0.0)	

n: number of individuals.

Some histopathological data obtained from our patients could not be reached.

mean and lowest Ct value except for cases with excessive gene expression values. The value of the calibrator sample is fixed at 1. The value selected as the calibrator was used for normalization only, not including statistical analysis. Relative quantitation levels were obtained from normalized Ct values and the tumor tissue group and the tumor surrounding tissue group were evaluated separately and compared with each other. In our study, TRAIL gene expression levels were determined from a total of 48 tumor tissues and tumor surrounding tissue samples. We were revealed that the TRAIL gene expression levels expressed 28,8 times lower in the tumor tissue group than that of the control tissue group and this difference was statistically significant ($p = 0.026$) (Figure 1). According to the fold change values of TRAIL gene expression levels as regards the control tissue of tumor tissues of our cases, while high TRAIL gene expression levels were observed in 16.6% of cases, low TRAIL gene expression levels were detected in 83.3% of cases. When TRAIL gene expression levels were compared with tumor stage, one of the histopathological parameters, we were revealed that the TRAIL gene expression levels were 7.86 times higher in the advanced tumor stage (T3/T4) than in the early tumor stage (T1/T2) ($p = 0.028$). The data related to the comparison of demographic characteristics and histopathological parameters of TRAIL gene expression levels are given in Table 3. In our study, no significant difference was found between TRAIL gene expression levels and genotype and allele distribution of TRAIL C1595T variant ($p > 0.05$).

4. Discussion

Lung cancer is a major health problem all over the world, with more than 1.8 million new cases being diagnosed each year and loss of about 1.6 million of these cases [31–33]. With the NSCLC being a multifactorial disease with associated to genetic risk, lifestyle and environmental exposure risk [34], no

Table 3. Comparison of TRAIL gene expression levels with demographic and histopathological data.

Demographic and histopathological parameters	TRAIL Gene expression level
Sex	
Male	7.64 ± 5.19
Female	2.34 ± 1.06
Smoking status (Package/Year)	
Age ≤ 50 years	2.24 ± 0.5
Age > 50 years	12.02 ± 9.28
Alcohol intake	
Yes	8.53 ± 6.76
No	3.21 ± 0.62
Tumor subtype	
Squamous cell carcinoma	17.54 ± 15.73
Adenocarcinoma	2.53 ± 0.71
Lymph node metastasis	
Yes	7.38 ± 5.23
No	3.93 ± 0.91
Tumor stage	
T1–T2	1.92 ± 0.52*
T3–T4	15.10 ± 11.20*
Metastasis	
Yes	0
No	6.32 ± 3.90

n: number of individuals; the values in the table are given as $X \pm SD$; * $p = 0.028$.

mechanism alone can fully explain all aspects of NSCLC. Tumorigenesis in NSCLC is related to especially the p53, k-ras, and EGFR genes, which are seen in different forms of mutation and different frequencies among non-smokers and smokers [6].

Studies have shown that the 3'-UTR region of exon 5 of TRAIL plays an important role in regulation of the TRAIL gene [6,24,35]. The 3'-UTR, which functions as a regulatory region at the level of protein expression, binds not only to mRNA but also to mRNA coated with RNA proteins [RBPs] and microRNAs (miRNAs), which play important roles in the regulation of translation [24,36]. Studies have suggested that an allelic variation which changes the level of expression of the protein in the genome of patients, particularly in the 3'-UTR, plays an important role in the outcome of the disease [36]. Therefore, in recent studies, the effect of TRAIL gene SNPs on NSCLC sensitivity is being investigated because of the complex

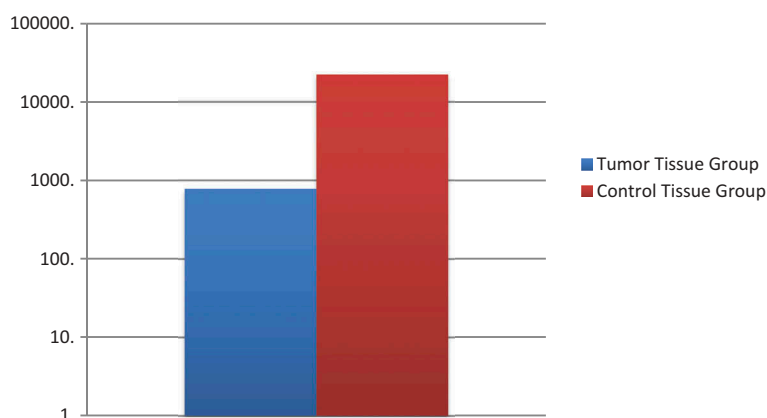


Figure 1. Comparison of expression levels of TRAIL gene in the tumor tissue group and the control tissue group.

mechanism of TRAIL-induced apoptosis and downstream factors [6].

Luo et al. found that the CT + TT mutant genotype of the TRAIL C1595T variant was lower in group with NSCLC than the control group, but is not statistically significant, and that the frequency of T mutant allele was significantly higher in patients with NSCLC. In conclusion, Luo et al. reported that T allele presence may play a role in NSCLC susceptibility [6]. Our results which are statistically insignificant are consistent in terms of the CT + TT mutant genotype with this result reported by Luo et al. In this study, the CT genotype frequency of the C1595T variant was lower than the CC + TT genotypes in patients with NSCLC compared to controls, and this data was found to be very close to statistical significance ($p = 0.07$). This result suggests that in CT genotype of C1595T variant for NSCLC patients seems to be a protective factor for lung cancer risk. According to the results of previous studies, homozygous or heterozygous genotypes of TRAIL C1595T variant in different cancer types appear to have different frequencies between patients and controls. Wang et al. found that the T allele frequency of the TRAIL C1595T variant was significantly lower in patients with gastric cancer than controls [20]. Timirci-Kahraman et al. found that the frequency of TRAIL 1595 TT genotype was lower in bladder cancer patients than in healthy controls, and the frequency of CT genotype was significantly higher [21]. Yaylim et al. in patients with colorectal cancer [12] and Yildiz et al. in patients with breast cancer [13] did not find any significant difference in TRAIL C1595T genotype distribution. In our study, we have considered the frequency of this genotype and alleles in Turkish cases.

In our study, we did not detect any statistical significance between TRAIL C1595T polymorphism and the clinical features of NSCLC patients. When we conducted a comprehensive literature review to examine the effect of TRAIL gene polymorphisms on demographic characteristics and histopathologic parameters of patients, we found that TRAIL polymorphisms in general were not a significant effect on these variables. However, in some studies it was predicated that TRAIL gene variants have an effect on the stage, grade or severity of the disease. Luo et al. found that the frequencies of CT+ TT genotype and T allele of the C1595T variant in Han Chinese cases was significantly higher in stage III and IV NSCLC than in stage I and II patients and Investigators have suggested that at least the C1595T variant of these results may be associated with the lesion severity and prognosis of NSCLC [6]. In our study, although not statistically significant, the C1595T CT + TT genotype and T allele frequency were lower in stage III and IV NSCLC patients than that of found in stage I and II patients (frequencies of CT+ TT genotype: 29.4% and 39.2%, T

allele: 26.5% and 39.2%, respectively), and our results are inconsistent with the results of Luo et al. When we approach this issue as general carcinogenesis, Wang et al. found that the frequency of TRAIL C1595T variant T carriage in patients with poorly differentiated gastric adenocarcinoma was significantly lower [20]. Yaylim et al. found that TRAIL C1595T CC and homozygous CC + TT genotypes frequency were significantly higher in patients with advanced stage tumors of colorectal cancer than in patients with early stage tumors [12]. Yildiz et al. detected TRAIL C1595T CT genotype at a significantly lower frequency in patients with advanced stage tumor than those with early stage tumor in breast cancer [13]. The formation of a codominant phenotype as both dominant and recessive with the contribution of both alleles is relevant for many genes. In this context, we think that the heterozygous carrier of the relevant variant of TRAIL may be important in Turkish Population.

In our study, it was found that TRAIL gene expression levels were 28.8 times lower in the tumor tissue group than in the control tissue group ($p = 0.026$). According to the control tissue of the tumor tissue, 16.6% of the cases were detected high TRAIL gene expression levels, while 83.3% of cases were observed low TRAIL gene expression levels. This conclusion supports the idea that the apoptosis mechanism of patients with NSCLC may impair functioning of the TRAIL molecular pathway. When TRAIL gene expression levels were examined in terms of tumor stage, which is one of the prognostic parameters, TRAIL gene expression level was 7.86-fold higher in advanced tumor stage (T3/T4) than early tumor stage (T1/T2) ($p = 0.028$). This result suggests that TRAIL may be important for the prognosis and survival of NSCLC. However, we did not analyze the survival in terms of TRAIL genotypes.

Spierings et al. have found immunohistochemically low TRAIL expression in 9% of NSCLC samples and high TRAIL expression in 59% of NSCLC samples in 87 stage III NSCLC patients and that poorly differentiated tumor areas of NSCLC patients exhibit a strong staining pattern for TRAIL [4]. Luo et al. indicated that the genetic polymorphisms and haplotypes of TRAIL gene correlated significantly with the NSCLC susceptibility in the group of Chinese patients [6]. Although these results and the results of our study are supportive of each other, it is necessary to confirm these results with another molecular biologically supported work because it is an immunohistochemical-based study. Macher-Goeppinger et al. found that TRAIL immunohistochemically expressed at high levels in 7% and low levels in 93% of renal cell carcinoma patients and when performing quantitative PCR analysis to patients at group with high TRAIL immunohistochemistry score, have confirmed the result of immunohistochemical analysis by detecting high TRAIL mRNA

expression level [23]. Piras-Straub et al. found that TRAIL mRNA expression was significantly lower in 66% of all hepatocellular carcinoma tissues than tumor surrounding tissues, 11% showed equivalent TRAIL expression, and 23% showed higher TRAIL expression levels. They have also shown that TRAIL expression is significantly associated with high-grade or advanced stage tumors. In conclusion, they declared that TRAIL is an important factor in the development and growth of hepatocellular carcinoma, and at the same time it is a marker of recurrence and survival of the disease [37].

Recent studies have expressed that the variant of TRAIL at site 1595 could affect TRAIL gene expression by changing a potential miRNA binding sequence, due to 3'-UTR of TRAIL gene may bind both naked mRNA and mRNA covered RNA proteins and miRNAs by involved in gene regulation [24,36]. In order to assess the significance of our results in this way of thinking, when we analyzed the TRAIL C1595T polymorphisms and TRAIL gene expression in NSCLC patients together, we could not find a statistically significant association between C1595T variants and TRAIL gene expression levels. In a study examining fatty liver disease, it was noted that there was no effect on gene expression levels of TRAIL gene polymorphism in position 1595 [28]. This conclusion on gene expression of the TRAIL gene polymorphisms of Yan et al. supports our result.

It has been published that the TRAIL C1595T variant changes in different points of multistage carcinogenesis, different cancer types and different ethnic groups in multistage carcinogenesis [21,38]. According to the results of our study, the lower frequency of TRAIL C1595T variant CT genotype in patients with NSCLC, although not statistically significant, suggests that this genotype appears to be a protective factor in terms of risk of lung cancer. However, low TRAIL gene expression may be an important factor in the prognosis and survival of NSCLC. When we review all our results together, in revealing the pathogenesis of NSCLC and evaluating the in terms of prognostic of the disease, it can be suggested that the TRAIL gene polymorphism and gene expression levels of our study contain a specific value. Although TRAIL C1595T variant was not associated with disease pathogenesis, TRAIL gene expression was associated with advanced tumor progression in our study. This relationship may have influenced other variants except the TRAIL C1595T variant or this effect may be regulated by epigenetic mechanisms, such as mRNAs, independent of TRAIL.

This data will shed light on the future work of TRAIL to reveal the pathogenesis of NSCLC or to address with regard to its therapeutic target. Our study was the first study in which TRAIL C1595T polymorphism was evaluated together with TRAIL gene

expression levels in patients with NSCLC and at the same time TRAIL gene variant carrier was studied for the first time in Turkish NSCLC cases. Also, we did not analyze the survival of the patients with regard to TRAIL polymorphism and expression levels. In order to be able to evaluate more objectively the data we have revealed, it is necessary to continue to work by expanding the number of patient samples. However, in the future, as well as examining other SNPs belonging to TRAIL gene, it is aimed to determine TRAIL gene expression levels by immunohistochemical and cytogenetic tests in different cell lines and to reveal its effects.

Disclosure statement

None of the authors have any conflicts of interest or financial disclosure related to this study.

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