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Investigation of Caspase 9 Gene Polymorphism in Patients With Non-small Cell Lung Cancer

SINA ERCAN[1], SIBEL ARINC[2], SEDA GULEC YILMAZ[3], CIGDEM ALTUNOK[4], FERIDE YAMAN[2] and TURGAY ISBIR[5]

[1]*Department of Thoracic Surgery Faculty of Medicine, Yeditepe University, Istanbul, Turkey;*
[2]*Süreyyapaşa Chest Disease and Thoracic Surgery Training and Research Hospital, Istanbul, Turkey;*
[3]*Department of Molecular Medicine, Institute of Health Sciences, Yeditepe University, Istanbul, Turkey;*
[4]*Department of Biostatistics and Medical Informatics, Faculty of Medicine, Yeditepe University, Istanbul, Turkey;*
[5]*Department of Medical Biology, Faculty of Medicine, Yeditepe University, Istanbul, Turkey*

Abstract. *Background/Aim: Non-small cell lung cancer (NSCLC) is one of the most common forms of lung cancer and the leading cause of cancer-related deaths in the world. Caspase 9 (CASP9) plays a central role in the intrinsic apoptotic pathway. The aim of the study was to investigate the role of caspase 9 gene polymorphism in patients with non-small cell lung cancer. Materials and Methods: The study included 96 NSCLC cases and 67 controls. CASP9 Ex5+32 G>A polymorphism was investigated by real-time polymerase chain reaction. Results: There was a significant difference between the groups in the frequency of CASP9 genotypes (p=0.008). The number of the carriers of the ancestral GG genotype, was significantly higher in the NSCLC group than in the control (p=0.009). The heterozygote GA genotype and mutant A allele frequency were significantly higher in the control group compared to the NSCLC group (p=0.005, p=0.009, respectively). Serum CASP9 levels were significantly lower in the patients group than in the control group (p<0.0001). Conclusion: CASP9 Ex5+32 GG genotype was a risk factor whereas the variant A allele could be a risk-reducing factor for NSCLC.*

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and is the one of the leading causes of death in the world (1). Approximately 85% of lung cancers are NSCLC and mostly detected at an advanced stage with poor treatment outcome and survival time (2). Classical chemotherapy is the major treatment for advanced NSCLC, but mortality rates remain high (3). Thus new therapeutic and preventive strategies based on molecular and genetic analyses are needed (3).

The term "caspase" defines 'cysteine-dependent aspartate-specific protease' activity which cleaves high-affinity cysteine residues from aspartate residues in target proteins (4). *CASP9*, a member of that caspase protein family, regulates programmed cell death named as intrinsic pathway (5). In this pathway, apoptosis is initiated by the release of cytochrome *c* from mitochondria in response to DNA damage or oxidative stress. Cytochrome c reacts with the apoptosome, that consists of activating factor 1 (Apaf-1), procaspase-9 and deoxyadenosine triphosphate (dATP). This multiprotein complex triggers a cascade of effector caspases. Activated *CASP9*, as an initiator caspase, regulates the effector caspases caspase 3 and caspase 7 and executes apoptosis (6, 7).

Single nucleotide polymorphisms (SNPs) are the most common variations in the human genome and it has been shown that some SNPs are associated with the pathogenesis of various cancers *via* regulating gene expression and protein synthesis (8). Apoptotic mechanisms may also be affected by caspase gene polymorphisms as a consequence of altered enzymatic functions (9). Determination of genetic variations of the caspase gene has shown that there could be an association between caspase deregulation and cancer susceptibility (10). However, while the direct functional effects of *CASP9* variants remain unclear, many studies have focused on *CASP9* variants and their role in apoptosis and cancer predisposition (11, 12). Therefore, this study aimed to investigate the possible role of *CASP9* variations in NSCLC in the Turkish population.

Materials and Methods

Study population. This hospital-based case-control study was conducted prospectively and all participants were recruited from Süreyyapaşa Chest Disease and Thoracic Surgery Training and Research Hospital and the Department of Thoracic Surgery in Yeditepe University Hospital, Istanbul, Turkey. Ninety-six patients

Correspondence to: Sina Ercan, MD, Department of Thoracic Surgery, Faculty of Medicine, Yeditepe University, Koşuyolu Mah. Koşuyolu Cad. No: 168, 34718 Kadıköy, Istanbul, Turkey. Tel: +90 2165785000, e-mail: sercan@yeditepe.edu.tr

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with NSCLC and 67 healthy controls were selected after clinical examination. Pathological investigations of patients were determined according to World Health Organization Classification of Lung Tumors (13). Approval of the study was obtained from the Medical Ethics Committee of Yeditepe University (Approval no:591, date:20.04.2016). Demographic and clinical characteristics of all patients were obtained from the medical records of the hospital and patients' clinical data were followed-up prospectively.

Genetic analysis. After obtaining an informed consent from the partipiciants, peripheral blood samples were collected into ethylenediaminetetraacetic acid (EDTA) coated polystyrene tubes. DNA extraction was performed by iPrep Purification Instrument (Invitrogen, Life Technologies, Carlsbad, CA, USA) by using 350 μl of whole blood and Invitrogen iPrep PureLink gDNA blood isolation kit (Invitrogen, Life Technologies). The concentration of the isolated DNA samples was determined spectrophotometrically with the NanoDrop 2000 (Thermoscientific, Waltham, MA, USA), 1.7-1.9 optical density range were taken for genotyping. *CASP9* gene Ex5+32 G>A (rs1052576) polymorphism was analyzed by Applied Biosystems 7500 Fast Real Time PCR instrument (Applied Biosystems, Foster City, CA, USA) by using TaqMan Genotyping Assay and TaqMan Genotyping Master Mix (TaqMan Reagents, Applied Biosystems). Reaction mixture and conditions were carried out according to the manufacturer's instructions.

Determination of serum CASP9 Levels. Peripheral blood samples collected into sterile 8 ml vacuum gel blood collection tubes and allowed to clot for 15 min at room temperature. Then serum was separated by centrifugation. The serum samples were put into eppendorf tubes and stored at −80˚C until testing. Serum *CASP9* levels were determined by enzyme-linked immunoassay (ELISA) (Poweam Medical Co. Nanjing City, Jiangsu Province, China).

Statistical analysis. Statistical analyses were performed using SPSS Ver. 23 software (SPSS Inc, Chicago, IL, USA). Data are reported as mean±standard deviation (SD) or number and percentage. The significance of the differences between groups were examined by Student's *t*-test, in case of variables that were not normally distributed, and the Mann-Whitney *U*-test was used to compare the groups. Chi square and Fisher's exact tests were used to evaluate the difference in the existence of the *CASP9* Gene Ex5+32 G>A (rs1052576)) polymorphism in the patient and control groups. Power of analysis was evaluated with *post-hoc* power analysis. Risk estimations were examined with odds ratio (OR) at 95% confidence interval (CI), $p<0.05$ denoted as statistically significant.

Results

The demographic characteristics of NSCLC and control groups are given in Table I. The analysis included 96 patients with NSCLC and 67 healthy control subjects aged 61.25±10.62, and 60.79±8.27 years, respectively. No significant difference was found between NSCLC and control groups in terms of median age ($p=0.755$). Moreover there was no significant difference with regards to gender between study groups ($p=0.555$). There was significant difference between active smokers and non-smokers, between the groups ($p<0.0001$).

Table I. *Demographic characteristics of the study population*.

	Control		NSCLC		*p*-Value
	n	%	n	%	
Total	67	100	96	100	
Age (years), mean±SD	61.25±10.62		60.79±8.27		0.755
Gender (Male/Female)	9/58	13.4/86.6	10/86	10.4/89.6	0.555
Age					
<60	36	53.7	45	46.9	0.389
≥60	31	46.3	51	53.1	
Smoking Status					
Active smoker	32	47.8	17	17.7	<0.0001*
Non-smoker	35	52.2	79	82.3	

NSCLC: Non-small cell lung cancer; n: number of individuals; SD: standart deviation, *p-values less than 0.05 denoted statistical significance.

The genotype and allele frequencies for *CASP9* Ex5+32 G>A polymorphism in NSCLC patients and controls are given in Table II. *CASP9* Ex5+32 G>A genotype frequencies between NSCLC group and control group were significantly different ($\chi^2=9.665$; $p=0.008$).

The frequency of the GG genotype was significantly higher in the NSCLC group than in the controls ($\chi^2=4.450$, $p=0.009$, OR=2.929, 95%CI=1.285-6.679). Also, there were statistically significant correlations between the groups regarding GA genotype ($\chi^2=7.816$, $p=0.005$, OR=0.405, 95%CI=0.214-0.768). The AA homozygote genotype did not differ significantly between the groups ($\chi^2=0.365$, $p=0.721$, OR=1.133, 95%CI=0.620-2.470). Ancestral G allele frequency was not significantly different between the groups ($\chi^2=0.365$, $p=0.546$, OR=0.808, 95%CI=0.405-1.614), yet mutant A allele frequency was significantly higher in the control group ($\chi^2=4.489$, $p=0.009$, OR=0.341, 95%CI=1.051-0.778). These results indicated that carrying variant A allele decreased the NSCLC risk by 2.9 fold (OR=0.341, 95%CI=0.150-0.778). Determination of *CASP9* Ex5+32 G>A polymorphism and histological types in patients with NSCLC showed that neither genotype variants nor allele frequencies were significantly different between groups (Table II).

As shown in Figure 1, *CASP9* serum levels were significantly lower in the patient group than in the control ($p<0.0001$). However, there was no statistically significant difference between the groups regarding *CASP9* serum levels in *CASP9* Ex5+32 G>A genotype carriers (p>0.05).

Discussion

Apoptosis, also defined as programmed cell death, is a crucial physiological mechanism that controls cell proliferation homeostasis. Dysregulation of apoptosis is an important hallmark in carcinogenesis (14). There are two main apoptotic

Table II. *The distribution of Caspase 9 genotype and allele frequencies in patient and control groups.*

	NSCLC (n=96)	Control (n=67)	*p*-Value	Odds Ratio	95%CI
Genotype			X^2=9,665; p=0.008*		
GG	30 (31.3%)	9 (13.4%)	0.009*	2.929	1.285-6.679
GA	36 (37.4%)	40 (59.7%)	0.005*	0.405	0.214-0.768
AA	30 (31.3%)	18 (26.9%)	0.721	1.133	0.620-2.470
Allele			Allelic Count		
G	96 (50%)	58 (43.3%)	0.546	0.808	0.405-1.614
A	96 (50%)	76 (56.7%)	0.009*	0.341	0.150-0.778

n: Number of individuals; OR: odds ratio; CI: confidence interval; X^2: Chi square used for comparison of patients with NSCLC and control group; *p<0.05 denotedstatistically significant differences.

pathways the extrinsic (receptor mediated) pathway and the intrinsic (mitochondrial) pathway, which utilize a cascade of enzymes called caspases. Initiator caspases, such as caspase 8 and caspase 10, trigger the extrinsic pathway, whereas *CASP9* is involved in the intrinsic pathway. The instrinsic pathway is initiated with the release of cytochrome *c* from the intermembrane space of mitochondria. It forms a multiprotein complex named apoptosome, which consists of cytochrome *c*, apoptotic peptidase activating factor 1 (APAF1), deoxyadenosine triphosphate (dATP) and procaspase 9. *CASP9* is subsequently activated and triggers effector caspases resulting in apoptosis (15, 16).

Several studies have evaluated the carcinogenesis risk in *CASP9* gene variations, whose functional effects and clinical implications are still unclear. New studies are needed to determine the functional effects of *CASP9* variations and cancer predisposition (17). *CASP9* Ex5+32 G>A polymorphism has been reported as a key genetic variation inducing changes in amino acid sequence and altering protein functions (18). Therefore, the present study aimed to investigate the role of *CASP9* polymorphism on NSCLC risk.

It has been shown that *CASP9* variants change the enzyme's activity, suggesting a relationship between *CASP9* polymorphism and lung cancer risk. Also, a possible correlation between *CASP9* variants and tumor histology has been investigated to determine the genetic etiology of lung cancer. However, no evidence was found regarding the relationship between histological tumor type and *CASP9* polymorphisms, however, it has been reported that *CASP9* promoter variations are associated with the risk of lung cancer owing to altered *CASP9* enzyme activity (19). In another study, *CASP9* variants have been suggested to have essential roles in apoptosis inhibition and lung cancer susceptibility (20). Combined analysis of *CASP9* gene polymorphisms and epxression has revealed the importance of CASP9 polymorphism on the risk of lung cancer. *CASP9* allele variation carriers had lower *CASP9* levels and apoptotic capacity, therefore had a higher risk of lung cancer

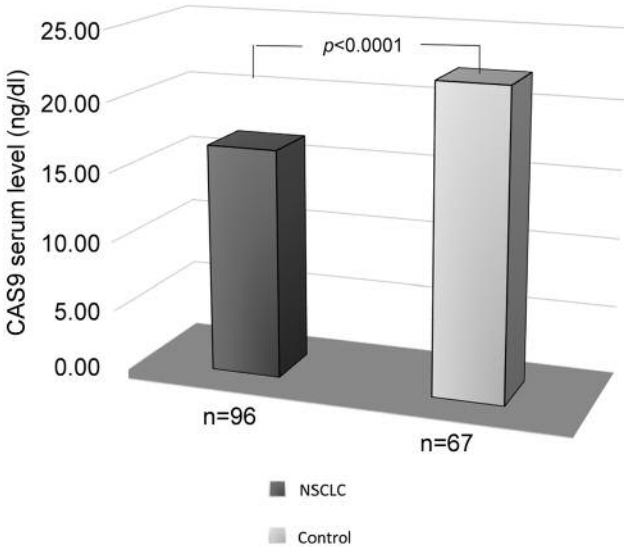

Figure 1. *Serum caspase 9 levels of the study populations.*

(21). Thus, *CASP9* Ex5+32 G>A polymorphism emerges as a remarkable SNP to determine association between gene variations and cancer susceptibility. In the present study, the *CASP9* variant A allele was identified as a risk-reducing factor; it caused a 2.9-fold decrease in the NSCLC risk. These results are consistent with a meta-analysis performed by *CASP9* promoter and exon sequence examination on the susceptibility to multiple cancers such as lung, prostate, gastric, colorectal and *etc*. In this meta-analysis, it has been shown that the *CASP9* mutant A variant had a protective effect against various cancers and it was suggested that this effect may originate from increased apoptotic activity. *CASP9* Ex5+32 G>A polymorphism could increase apoptosome affinity and decrease cancer risk (22). Although there were no statistically significant differences regarding

CASP9 Ex5+32 G>A genotypes, our results showed that serum *CASP9* levels were significantly higher in the control group than in the patient group.

Another meta-analysis has evaluated functional *CASP9* gene polymorphisms in various cancer types and confirmed the protective role of *CASP9* Ex5+32 G>A mutant A allele in cancer susceptibility (23). The association between *CASP9* genes and hematological malignancies have also been investigated in two case-control studies. Similiar to our results, these studies showed that the homozygote mutant AA and heterozygote GA genotypes associated with decreased risk of multiple myeloma (24) and non-Hodgkin lymphoma (25).

Consequently, the present study showed that reduced serum *CASP9* levels were associated with NSCLC risk and carrying *CASP9* Ex5+32 G>A mutant A allele was a risk reducing factor, whereas wildtype homozygote GG genotype was having a negative impact on NSCLC. These results suggested that *CASP9* Ex5+32 G>A could play an important role in NSCLC susceptibility. To the best of our knowledge, this was the first study that investigate the association between *CASP9* Ex5+32 G>A polymorphism and NSCLC in a Turkish patient population. Due to the limitations of small sample size, further investigations are needed to display the relationship between *CASP9* gene polymorphisms and NSCLC predisposition.

Conflicts of Interest

The Authors declare that no conflicts of interest exist.

Authors' Contributions

Sina Ercan: Recruitment of study participants and clinical investigation, data collection, contributed to the design and implementation of the research. Sibel Arınç, Feride Yaman: recruitment of study participants and clinical investigation, data collection. Seda Gülec Yılmaz: performed the experiments. Çiğdem Altunok: performed statistical analysis. Turgay İsbir: contributed to the design and implementation of the research, writing manuscript, approval of the final version. All Authors read and approved the final manuscript.

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