Determination of Endothelial Nitric Oxide Synthase Gene Polymorphism and Plasma Asymmetric Dimethyl Arginine Concentrations in Patients with Lung Cancer

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ABSTRACT

Objective: Genetic factors can contribute to both the occurrence and development of lung cancer. This study aimed to investigate endothelial nitric oxide synthase (eNOS) G894T and T-786C polymorphisms and plasma asymmetric dimethylarginine (ADMA) levels of lung cancer patients in comparison with healthy subjects.

Materials and Methods: A total of 200 subjects, 100 patients with lung cancer and 100 healthy volunteers were included in this study. To determine eNOS gene polymorphisms, we collected and analyzed blood samples with polymerase chain reaction (PCR). Plasma ADMA levels were evaluated by high-performance liquid chromatography (HPLC).

Results: The difference in gene polymorphisms between lung cancer patients and healthy controls were insignificant. However, lung cancer patients had statistically significantly higher plasma ADMA levels than healthy controls. The patients and control groups with CC polymorphisms and TT polymorphisms on eNOS T-786C and G894T gene regions had higher plasma ADMA levels. The CC polymorphisms and plasma ADMA levels were higher in patients with small-cell lung cancer compared to those in patients with non-small-cell lung cancer.

Conclusion: Although eNOS gene polymorphisms had no significant difference between lung cancer patients and healthy controls, plasma ADMA levels were higher in lung cancer patients compared to healthy controls. Our study suggests that CC genotypes and elevated plasma ADMA levels might be associated with small-cell lung cancer.

Keywords: ADMA, endothelial nitric oxide synthase, nitric oxide, polymorphism, lung cancer

Introduction

Among cancers, lung cancer has the highest mortality rate and is the most common among cancers globally and also in our country. Several factors can cause lung cancer; however, smoking is the primary cause. In the United States, 75-80% of women and 90% of men who die from lung cancer are smokers [1, 2]. However, less than 20% of smokers have lung cancer. Therefore, various factors other than smoking can contribute in lung cancer formation. Exposure to carcinogenic agents, asbestos, past pulmonary diseases, and family history are some of them [3]. The contribution of various gene polymorphisms and other factors as well as smoking and other toxic exposures to lung cancer ethiopathogenesis have been shown [4, 5].

Endothelial nitric oxide synthase (eNOS) catalyzes nitric oxide production [6]. Nitric oxide synthase (NOS) has four isoforms, including neuronal, endothelial, mitochondrial, and inducible ones. Endothelial nitric oxide synthase is derived from endothelial cells. There are many single nucleotide polymorphisms (SNPs) in eNOS; two of them are the G894T polymorphism in the exon 7 region and the T-786C polymorphism in the promoter region of the gene. Nitric oxide (NO) can lead to tumor cell growth, induction of genotoxic lesions, and angiogenesis [7]. Reactive nitrogen oxide products (RNOS) from NO and NO itself have been shown to be effective in deoxyribonucleic acid (DNA) damage and DNA repair enzymes, such as DNA ligase inhibition. NO also affects the carcinogenic process by disrupting control points, apoptosis, and DNA repair of the cell cycle [8].

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Asymmetric dimethylarginine (ADMA), which is the endogenous inhibitor of nitric oxide synthase in human plasma and urine, was first described by Vallance et al. in 1992 [9]. It has an amino acid structure and is naturally present in the human plasma. ADMA occurs after a post-translational modification of arginine [10]. A healthy adult produces 60 mg (~300 µmol) ADMA per day, released following proteolysis, approximately 20% of which is excreted in urine [11].

In this study, we aimed to measure plasma ADMA levels in patients with lung cancer and investigate their association with NO and polymorphisms and examined the effectiveness of the G894T polymorphism and the T-786C polymorphism in the development of lung cancer.

Materials and Methods

Selection of Patient and Control Group

This study was approved by the ethics committee (22.05.2009 and number 164) of Ataturk University Faculty of Medicine. We included 100 patients with lung cancer (female: 22, male: 78) who had lung cancer diagnosis after pathologic and radiological examinations and were hospitalized at the Department of Chest Diseases, Aziziye Suleyman Demirel Research Hospital, Ataturk University Medical School. All patients and healthy controls were informed about the design of the study, and written informed consent was obtained. The classification of lung cancer of this patient group was done. The smoking status, sex, and age of the patients were obtained from patient files. The control group consisting of 100 people in our work (62.05±9.34) were selected from healthy volunteers without any chronic disease, taking into consideration the mean age of the patient group (63.6±8.1).

Collection of Samples

Blood samples were collected from both lung cancer patients and healthy controls. For each patient or volunteer, 6 mL of venous blood was collected. Collected blood samples were aliquated in EDTA containing tubes. Aliquot samples were stored at -80°C.

Biochemical Analyses

In blood samples collected from the lung cancer patients and healthy controls, eNOS T-786C and eNOS G894T polymorphisms were evaluated with a commercially available CVD strip assay kit (Viennalab Diagnostics; Vienna, Austria). Basically CVD strip assay kit woks in three steps: isolation of DNA, polymerase chain reaction (PCR), and reverse hybridization of amplification products.

Polymorphism Analysis

DNA Isolation, Purity Determination, and Concentration Calculation

For DNA isolation, blood samples were transferred from −80°C to −20°C and +4°C and dissolved gradually. From blood samples, DNA containing supernatant was obtained according to the kit protocol and stored at 2-8°C till PCR studies. Absorbance measurements were performed at 260 and 280 nm wavelengths for determination of DNA concentrations and purity ratings.

In Vitro Amplification (PCR)

Until PCR analyses, DNA samples were kept frozen with PCR reagents and all ongoing steps were performed on ice. A fresh diluent sample of Taq DNA polymerase was prepared (0.2 U/µL) in Taq dilution buffer. After PCR amplification products were analyzed, agarose gel electrophoresis with appropriate protocols was performed. Finally, hybridization procedure was performed with the standard methods. After hybridization, standard wash and staining protocols were also applied. Following the staining process, we evaluated wild type and/or mutation bands that appeared in the strips.

NO Measurement and ADMA Analysis

Because NO is an extremely short half-life molecule, nitrate and nitrite, which are stable end products in NO, are measured. We measured the total nitrite concentration as the nitrates are reduced to nitrites. Nitrite measurement is a spectrophotometric measurement based on the Griess reaction [12]. We performed ADMA analysis with a commercial kit based on high-pressure liquid chromatography (HPLC).

Statistical Analysis

IBM Statistical Package for the Social Sciences version 19.0 (IBM SPSS Corp.; Armonk, NY, USA) statistical program was used for statistical analysis. The distributions of numerical data were determined by the Kolmogorov Smirnov test. Normally disturbed numerical data was assessed using the Sample-T test. One-way analysis of variance test was used to assess the difference between 3 or more groups. Chi-square test was used for the analysis of the categorical data. $P<0.05$ values were accepted as statistically significant.

Results

Demographic Results

There was no statistically significant difference between the age (lung cancer: 64±8; healthy: 62±9) and gender (lung cancer: 78% male and 22% female; healthy: 66% male and 34% female) of the patient group and control group ($p>0.05$). The proportion of smokers (lung cancer: 6% non-smoking, 94% smoking; healthy: 27% non-smoking, 73% smoking) was statistically higher in the patient group ($p<0.05$).

Gene Polymorphisms

Table 1 shows the distribution of TT, TC, CC polymorphisms in the eNOS T-786C promoter gene region between the lung cancer patients and healthy controls. Table 1 also includes data

Table 1. Distribution of eNOS T-786C and G894T polymorphisms in the patient and control group

	Lung cancer patients n=100	Healthy control group n=100	p
eNOS T-786C			
TT	42 (%42)	53 (%53)	0.288
TC	47 (%47)	39 (%39)	
CC	11 (%11)	8 (%8)	
Allele frequency			
T allele	0.655	0.725	
C allele	0.345	0.275	
eNOS G894T			
GG	56 (%56)	60 (%60)	0.848
GT	34 (%34)	31 (%31)	
TT	10 (%10)	9 (%9)	
Allele frequency			
G allele	0.73	0.755	
T allele	0.27	0.245	

eNOS: endothelial nitric oxide synthase

for GG, GT, and TT polymorphisms in the eNOS G894T exon 7 gene region of the lung cancer patients and the healthy control group. There was no statistical difference in the distribution of these gene polymorphisms between the groups (p>0.05).

NO and ADMA levels

Table 2 presents NO and ADMA levels in the lung cancer patients and in the healthy control group. The difference between NO and ADMA levels in the patients and healthy volunteers was statistically significant (p<0.05).

Table 3 shows the statistical comparison of NO and ADMA levels between different lung cancers according to cell type (Figure 1). In patients with small-cell lung cancer (SCLC), the ADMA levels were significantly higher than in those with non-small-cell lung cancer (NSCLC).

Comparison of ADMA and NO levels with eNOS gene polymorphisms

Figure 2 and Table 4 demonstrate the changes in NO and ADMA levels according to the polymorphisms in the eNOS T-786C promoter gene region. Data for NO and ADMA levels of the patients with the eNOS G894T exon 7 gene region of the eNOS gene polymorphism is shown in Figure 3 and Table 5.

There was no statistically significant difference in the GG, GT and TT genotypes in terms of polymorphisms in the eNOS G894T exon 7 gene region (p=0.107) of the individuals. The difference between the individuals in the genotypes TT, TC, and CC was statistically significant in terms of polymorphisms in the eNOS T-786C promoter gene region (p=0.041) (Table 6).

Discussion

NO was discovered in 1987 and to date many studies focused on the relationship between cancer development and NO. Studies suggest that NO influences tumor formation by direct modification of DNA and the inhibition of systems required for DNA repair. In addition, RNOS has been shown to cause DNA strand breaks. At high concentrations, it has been shown that NO leads to the production of RNOS, such as superoxide and peroxy nitrite, and through these produces various lesions in DNA [13, 14]. Furthermore, studies have shown that the oxidation and deamination of nucleic acids as well as induction of DNA helix breaks require high concentrations of NO or RNOS rarely occurring in the body. Antioxidants found in abundant amounts in the body inhibit the NO or RNOS from being concentrated enough to cause damage to DNA [14]. As a result, the role of NO in cancer is diverse.

This study included 100 patients with lung cancer and 100 healthy subjects. NO levels of the cancer patients were significantly higher than that of healthy subjects. In line with our results, Obara et al. [15] showed significantly increased levels of NO in mycoplasma hyorhinis study on stomach cancer seen in infected people. In another study, Nam et al. [16] evaluated the role of iNOS Helicobacter pylori related

Table 2. ADMA and NO levels in patients with lung cancer and control group

	Lung cancer patients n=100 (X±SD)	Healthy control group n=100 (X±SD)	p
ADMA (µmol/L)	1.72±1.27	0.94±0.45	0.000
NO (µmol/L)	30.95±18.20	24.03±12.61	0.011

ADMA: asymmetric dimethylarginine; NO: nitric oxide

Table 3. Distribution of ADMA and NO levels according to histopathological classification

	Classification		
	SCLC (n=24) (X±SD)	NSCLC (n=76) (X±SD)	
ADMA (µmol/L)	2.51±1.68	1.47±1.01	0.01
NO (µmol/L)	30.65±19.25	31.04±17.99	0.93

ADMA: asymmetric dimethylarginine; NO: nitric oxide

Table 4. ADMA and NO levels in groups according to eNOS T-786C polymorphisms

		Lung cancer patients n=100			Healthy control n=100			Total n=200	
eNOS T-786C	N	NO (µmol/L)	ADMA (µmol/L)	N	NO (µmol/L)	ADMA (µmol/L)	N	NO (µmol/L)	ADMA (µmol/L)
TT	42	31.45±17.22	1.51±1.18	53	24.35±12.98	0.84±0.35	95	27.49±15.33	1.14±0.89
TC	47	32.76±19.73	1.45±1.05	39	23.2±12.41	0.84±0.35	86	28.42±17.39	1.17±0.85
CC	11	21.29±12.44	3.62±0.89*	8	25.96±9.09	2.06±0.37*	19	23.26±11.12	2.96±1.06*

*: The ADMA levels of carriers with CC alleles are statistically significantly higher than the other two types (p<0.05)

Table 5. ADMA and NO levels in groups according to eNOS G894T polymorphisms

		Lung cancer patients n=100			Healthy control n=100			Total n=200	
eNOS G894T	N	NO (µmol/L)	ADMA (µmol/L)	N	NO (µmol/L)	ADMA (µmol/L)	N	NO (µmol/L)	ADMA (µmol/L)
GG	56	31.01±17.4	1.44±0.92	60	23.55±13.6	0.87±0.32	116	27.15±15.93	1.15±0.73
GT	34	31.18±18.82	1.78±1.52	31	24.02±11.06	0.85±0.38	65	27.77±15.9	1.34±1.21
TT	10	29.77±22.02	3.06±1.32*	9	27.23±8.29	1.69±0.76*	19	28.57±16.69	2.41±1.27*

*: The ADMA levels of the TT allele carriers are statistically significantly higher than the other two types (p<0.05). ADMA: asymmetric dimethylarginine; eNOS: endothelial nitric oxide synthase; NO: nitric oxide

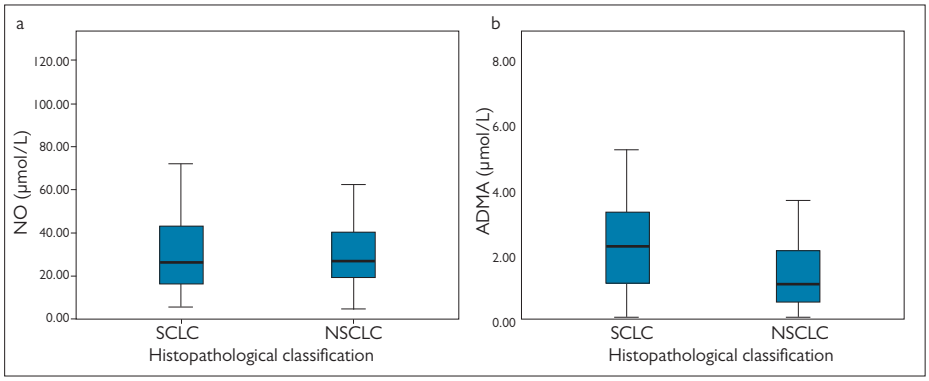

Figure 1. a, b. Distribution of NO (a) and ADMA (b) levels according to histopathological classification

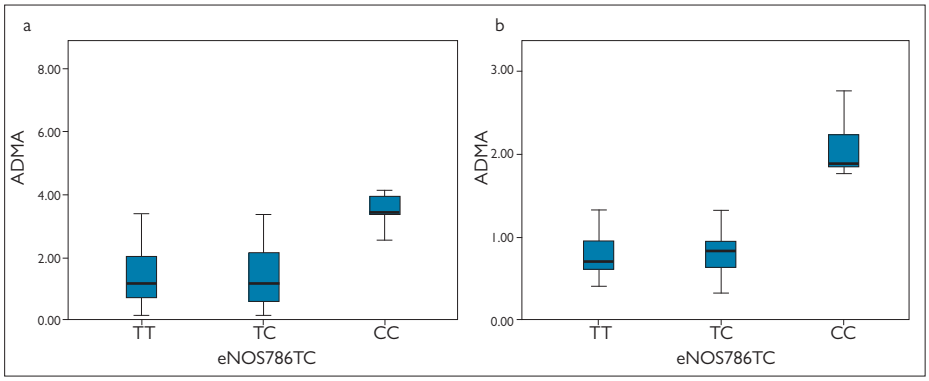

Figure 2. a, b. ADMA levels according to eNOS T-786C polymorphism in the patient (a) and healthy (b) groups

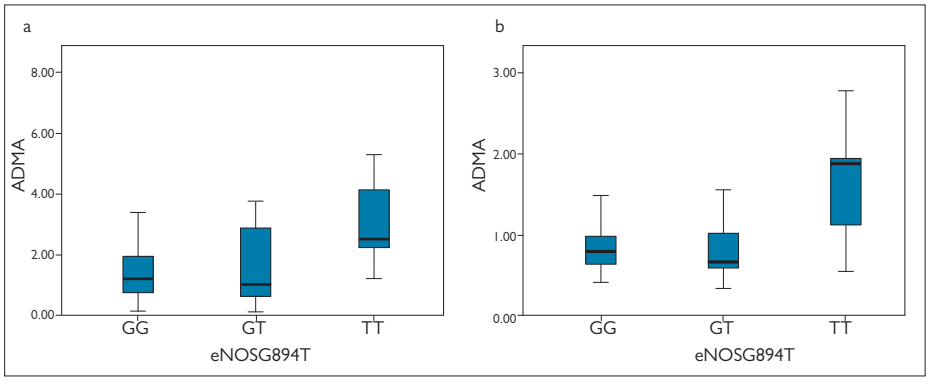

Figure 3. a, b. According to eNOS G894T polymorphisms, ADMA levels in patient (a) and healthy (b) groups

carcinogenesis and suggested that iNOS plays a role in the formation of Helicobacter-related cancers.

In the light of previous data, we hypothesized that eNOS gene expression and presence of ADMA in the plasma might contribute to the formation and progression of lung cancer. All subjects in our study were evaluated in terms of GG, GT, TT polymorphisms in the eNOS G894T exon 7 gene region and TT, TC, CC polymorphisms in the eNOS T-786C promoter gene locus. In the healthy control group, TT distribution in the eNOS T-786C promoter gene region was 53%; it was 42% in the patients with lung cancer. TC distribution was 39% in the control group and 47% in the lung cancer group, while CC distribution was 8% in the control group and 11% in patients with lung cancer. For the eNOS T-786C promoter gene region polymorphisms, there was no statistically significant difference between healthy controls and lung cancer. In the eNOS G894T exon 7 gene locus, the GG distribution was 60% and 56%; GT distribution was 31% and 34%; and TT distribution was 9% and 10% in the control group and lung cancer group, respectively. The difference in polymorphisms between eNOS G894T exon 7 gene locus of lung cancer patients was not statistically significant.

There are very few studies related to eNOS gene polymorphisms in lung cancer. Cheon et al. [17] have studied eNOS and angiotensin converting enzyme gene polymorphisms in lung cancer and found a statistically significant difference between cancer patients and control subjects in terms of eNOS gene polymorphism. However, when the statistics of this study is examined, the phrase "when the control group is compared to the asthmatic patients" is used. In addition, there is no mention of the region of the gene locus and where it is polymorphic. Therefore, this study is not a remarkable study in our opinion, although there is a difference in terms of eNOS gene polymorphism between patients with lung cancer and the control group. In a study evaluating the association of eNOS and ACE gene polymorphisms and plasma nitric oxide with risk of NSCLC in South India, Peddireddy et al. report that in the South Indian population, polymorphisms in the eNOS and ACE genes may contribute to the increased risk of NSCLC. Fujita et al. [18] have looked at the polymorphism in the eNOS intron 4 VNTR gene region in advanced NSCLC patients who have received dual chemotherapy on the basis of platinum and have stated that the polymorphism in this gene region may be related to the progression and survival of the disease. Lee et al. [19] have studied eNOS gene polymorphism in prostate cancer. Their studies of NOS-2 and NOS-3 enzymes in terms of polymorphisms in different gene regions found that the single nucleotide polymorphisms in the various regions of the eNOS gene in prostate cancers

Table 6. Distribution of eNOS T-786C and eNOS G894T polymorphisms according to histopathological classification in the patient group

	Lung cancer patients	
	SCLC	NSCLC
eNOS T-786C		
TT	9 (37.5%)	33 (43.4%)
TC	9 (37.5%)	38 (50%)
CC	6 (25%)	5 (6.6%)
Allele frequency		
T allele	0.562	0.684
C allele	0.437	0.315
eNOS G894T		
GG	13 (54.2%)	43 (56.6%)
GT	6 (25%)	28 (36.8%)
TT	5(20.8%)	5(6.6%)
Allele frequency		
G allele	0.666	0.75
T allele	0.333	0.25

eNOS: endothelial nitric oxide synthase; SCLC: small-cell lung cancer; NSCLC: non-small-cell lung cancer

were statistically significant compared to the control group and they were associated with tumor aggressiveness. In a study conducted by Vidyullatha Peddireddy et al.[20] in the South Indian population, the homozygous "AA" genotypic frequency for NSCLC patients was significantly associated with an overall risk of 3.6 fold. Tecder Unal et al. [21] have studied eNOS gene polymorphism in stomach cancer and found no significant relationship between eNOS G894T polymorphism and stomach cancer. They found significant difference between gastric cancer patients and control group in terms of T-786C polymorphism. In their study, they reported that CC polymorphism was significantly associated with gastric cancer. Yao et al.'s [22] meta-analysis of eNOS T-786C polymorphism in breast cancer in humans suggested that reduced serum nitrite/nitrate ratio and reduced endothelial NO production may be associated with a change from T to C in the eNOS gene promoter region. In this respect, eNOS T-786C indicated that polymorphism may be associated with reduced risk of breast cancer. Ikenouchi-Sugita et al. [23] looked at the eNOS T-786C polymorphism in depressed patients and found that the levels of NO in the TC polymorphism were statistically significant. When they looked at the polymorphisms in the eNOS G894T region, they found no difference in the NO levels of GT polymorphisms. Wang et al. [24] have reported that eNOS T-786C polymorphism is in an inconsistent relationship with eNOS-associated phenotypic changes. They reported that transcription activity in individuals carrying the T allele is lower than in those carrying the C allele. They also reported that cigarette smoking significantly increased the transcriptional activity in individuals carrying the T allele, but reduced it in individuals carrying the C allele.

ADMA was first described as an endogenous inhibitor of NO synthase in human urine in 1992 [4]. ADMA is naturally present in human plasma and has an amino acid chemical structure. As a result of posttranslational modification, arginine turns into ADMA [10]. In a previous study, with a total of 118 subjects (33 of these cancer cases were lung cancer), ADMA levels were found to be higher in serums of stomach, breast, hematopoietic, and lung cancer cases than those of healthy controls [25]. In the mentioned study, Yoshimatsu et al. suggested that ADMA elevation is related to the overexpression of protein arginine methyl transferase (PRMT1) in the patients' serum samples. However, they also paid attention to the necessity of further detailed studies that would explain the increase in expression and regulation of ADMA levels in different cancers. Furthermore, Szuba et al. [26] investigated the different types of hematological malignancies for their plasma ADMA levels and found significantly higher levels of ADMA in these cancer patients when compared to healthy volunteers. A significant inverse correlation between ADMA and NO levels has also been shown. In that study, Phebe L suggested the correlation between NO and ADMA [27]. Previously elevated dimethyl arginine dimethylaminohydrolase-1 (DDAH1) was shown to result in enhanced NO production [28] during cancers. In addition, patients with different hematological malignancies exhibited a substantial increase in plasma ADMA levels [26]. In hematological patients, increased levels of ADMA could be a result of increased degradation of intracellular proteins [29]. Zheng et al.[13] reveals that the plasma ADMA level is elevated in colon cancer patients, which can attenuate serum starvation-induced apoptosis in LoVo cells. In summary, ADMA levels were found to be significantly increased in patients with different cancer types. In line with previous studies, we found statistically increased plasma ADMA levels in lung cancer patients when compared to healthy control ones. We also investigated the relationship between eNOS G894T and T-786C polymorphisms and plasma ADMA and NO levels of lung cancer patients. Basically there was no significant difference between the polymorphisms and NO levels of lung cancer and healthy groups. However, ADMA levels were significantly higher in patients with CC and TT polymorphism, in terms of gene regions above. This finding suggests that in the subjects with the CC genotype eNOS gene expression is affected in a way resulting in increased NO levels and in these subjects, eNOS may cause a rebound increase in plasma ADMA levels, which is the endogenous inhibitor of eNOS. In terms of eNOS T-786C and G894T polymorphisms, the individuals with CC and TT genotypes, respectively, suggest that plasma ADMA levels are highly dependent on plasma NO levels. However, other factors can also affect plasma ADMA levels.

When we compared our results according to the cell type of lung cancer, the difference between the NO levels of NSCLC and SCLC patients was insignificant while ADMA levels were statistically higher in SCLC patients than NSCLC patients. For G894T polymorphisms, the difference between SCLC and NSCLC patients was also insignificant. However, the difference of T-786C polymorphism between NSCLC and SCLC patients was statistically significant. In SCLC patients, CC genotype polymorphism was higher than NSCLC patients. We can suggest that the CC genotype is more likely to occur in SCLC patients. Therefore, the patients carrying this genotype may be more likely to develop SCLC.

The plasma ADMA and NO levels of lung cancer patients were statistically higher than healthy subjects. Patients with the CC and TT genotypes have higher plasma ADMA levels than the other polymorphic groups in terms of eNOS T-786C and G894T polymorphisms, respectively. In addition, there was no statistically significant difference between the NO levels of NSCLC and SCLC patients. However, ADMA levels in patients with SCLC were significantly higher than in in patients with NSCLC. On evaluation, we could not determine any statistical difference in G894T polymorphism in both groups. However, there was a statistically significant difference for T-786C polymorphism. The CC genotype was higher in SCLC cases than NSCLC.

The most important limitation of our study was that the number of patients was determined as 100 for economic reasons. When lung cancer typing was performed histologically, the number of patients in the cancer types was statistically low. However, when all the lung cancer patients were evaluated without typing, significant results were obtained.

In conclusion, it is clear that more detailed genetic studies are necessary for determining the interaction of genetic and environmental factors in lung cancer. In patients with lung cancer, eNOS T-786C and G894T polymorphisms and plasma ADMA levels should be considered along with other parameters during diagnosis and treatment protocols. Thus, our study makes a significant contribution to both the etiology and the development of lung cancer.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Ataturk University Faculty of Medicine (22.05.2009/164).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

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