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Determination of the Relationship Between rs4986790 and rs4986791 Variants of TLR4 Gene and Lung Cancer

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Abstract—Chronic inflammation triggers DNA damage and oncogenic mutations and causes tumor formation and tumor progression. One of the important components of the inflammatory response is Toll-like receptor (TLR) family. The objective of our study is to determine the relationship between rs4986790(+896A/G) and rs4986791(+1196C/T) gene polymorphisms and lung cancer risk. PCR-RFLP technique was carried out to identify the genotypes in 100 control individuals and 160 lung cancer patients. DNA extracted from peripheral blood samples were amplified and digested with *NcoI* and *HinfI* then visualized. We did not find any difference between genotype frequencies between controls and patients ($p>0.05$) in rs4986790. But a significant difference between control group and patients with lung cancer as for genotype frequencies ($\chi^2=4.19$, $p=0.041$) in rs4986791 variants was found. Our data indicate that any correlation was not found between rs4986790 polymorphism and lung cancer, while a correlation between rs4986791 and lung cancer has been determined and found to be associated with lung cancer risk.

KEY WORDS: rs4986790; rs4986791; polymorphism; toll-like receptor-4; lung cancer.

INTRODUCTION

Lung cancer is the most frequently seen cancer type in the world. Every year nearly one million people die from lung cancer. Smoking is the major responsible factor in the etiology of pulmonary cancer. Approximately 90 % of the lung cancer patients are smokers. Hereditary factors have been also investigated in the development of lung cancer. In people, whose first-degree relatives had lung cancer, the risk of lung cancer increases 2.4-fold. Some genes with functions involving cell growth are modified when exposed to radiation, chemical substances and viruses, and gain "oncogenic characteristics" which play an important role in carcinogenesis [1–3].

Tobacco smoke induces pulmonary inflammation, which has an important role in progressive pulmonary damage in chronic obstructive pulmonary disease (COPD). Chronic inflammation induces tumorigenesis via formation of reactive oxygen and nitrogen species, which trigger DNA damage and oncogenic mutations. Besides, in experimental animals, the most important effect of inflammation on tumoral induction is its contribution to progression of tumorigenesis [4].

Tobacco smoke is a major risk factor for lung cancer, which is responsible for 87 % of the cases with lung cancer in the USA. Tobacco smoke contains more than 60 carcinogens and also nearly 4000 chemical substances including polycyclic aromatic hydrocarbons and *N*-nitrosamines. Transformation of these compounds into reactive forms (metabolic activation) induces destruction of DNA which induces many genetic changes underlying lung cancer [4].

Chronic inflammation is a long-lasting (for weeks, months, or indeterminate duration) condition where permanent damage of cells and tissues is observed. It is

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characterized by infiltration of mononuclear cells, destruction of tissue by inflammatory cells, and repair of tissue with fibrosis and angiogenesis [5].

At a molecular level, free radicals and aldehydes formed during chronic inflammation can cause harmful gene mutations and posttranslational changes in key proteins involving in cancerogenesis. Other products of inflammation including cytokines, growth factors, and transcriptional factors as NF-κB can control the expression of cancer genes (i.e., tumor suppressor genes and oncogenes) and important inflammatory enzymes as inducible nitric acid synthase and cyclooxygenaase-2. These enzymes also directly affect free oxygen radicals. Precancerous outcomes of chronic inflammation include DNA damage, increased cellular proliferation, impairment of DNA repair process and cellular environment, inhibition of apoptosis, and progression of angiogenesis and invasion. Chronic inflammation is also associated with immune suppression, and it is also a risk factor for cancer [4–7].

Toll-like receptors (TLRs) are important components of inflammatory response which induce activation of TLR, MAPKs, and NF-κB and in turn activation of NF-κB results in synthesis of proinflammatory cytokines as TNF-α, IL-6, and IL-1β and inflammatory enzymes including inducible nitric acid synthase and cyclooxygenaase-2 [8, 9].

TLRs belong to a group of transmembrane glycoprotein family, which ensures formation of natural immune response against many pathogens. In human beings, it is a homologue of interleukin-1 receptor (IL-IR). It has also a very important role in host immunity by activating adaptive immune response. TLRs recognize pathogen- associated molecular patterns (PAMPs) produced by microbial agents [10, 11].

It has been demonstrated that chronic inflammatory diseases contribute to the development of cancer via TLR signaling. In many studies, increased TLR expression has been reported in different types of tumor cells. Various endogenous TLR ligands released from inflammatory cells activate TLR signaling pathways in precancerous cells with resultant expression of cytokines, growth factors, angiogenic factors, and proteases which degrade extracellular matrix. Thus, TLR induces microenvironmental circumstances, which support development and progression of cancer [12–14].

SNP rs4986791 which is situated on TLR4 gene is formed by cytosine/thymine substitution at nucleotide 1196. position, and studies performed have detected the presence of an association between this genetic variant and risks of gall bladder cancer, precancerous gastric lesions, and gastric cancer [15–18]. Another genetic variant (rs4986790), on TLR4 gene, is formed via adenine/guanine substitution at nucleotide 896. position and a correlation between this genetic variant and risks of gastric cancer, prostate cancer, colorectal carcinoma and non-Hodgkin lymphoma [15, 19, 20].

In line with the information presented herein, we thought that functional polymorphisms which can occur on TLR4 gene which has a very important role in the mechanism of inflammation might trigger cancerogenesis through disruption of inflammation, and accordingly, determination of the relationship between rs4986790 and rs4986791 variants of TLR4 gene and lung cancer was aimed.

MATERIALS AND METHODS

Specimens and Patients

In our study, DNA extracts retrieved from 160 lung cancer patients (NSCLC:110, SCLC:50) and 100 healthy individuals who consulted to the Department of Chest Diseases of Eskisehir Osmangazi University, Eskisehir, Turkey during 2005–2007, and stored in Department of Medical Biology were used. Controls were consisted of 94 men and 6 women; patients were consisted of 151 men and 9 women whose ages ranged from 40 to 75 years. The 20 % of control individuals and most patients (96.25 %) had a history of smoking. Frequencies of COPD in control and patient groups were 8 and 25 %, respectively.

The study population was genetically homogeneous and native to Turkey. Informed consent was obtained from each patient in accordance with a study protocol approved by the ethics committee of Eskisehir Osmangazi University.

The following steps of the experiment were used in order to determine rs4986790 and rs4986791 variants of TLR4:

- Quantitative and qualitative analysis of DNA extracts were made using a microscale spectrophotometer (NanoDrop®)
- Amplification of relevant gene domains of DNA extracts using polymerase chain reaction (PCR)
- Exposure of retrieved PCR products to agarose gel electrophoresis and making them visible using UV gel imaging system
- Cleaving amplification products using appropriate restriction enzymes

Table 1. Primers Specific to Gene Domains, Conditions of Amplification and Enzyme Digestion for rs4986790 and rs4986791 Variants of TLR4 Gene

SNP	Base exchange	Primer sequences	Conditions of amplification		Enzyme
rs4986790	896A/G	F, 5′-AGCATACTTAGACTACTACCTCCATG-3′	First denaturation, 94 °C for 30 s		Nco I
		R, 5′-GAGAGATTTGAGTTTCAATGTGGG-3′	Denaturation : 94°C for 30s		37 °C
			Anneling : 58.5–63°C for 1 min	30 cycle	1 h
rs4986791	1196C/T	F, 5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′	Extension : 68°C for 1 min		Hinf I
		R, 5′-GGAAATCCAGATGTTCTAGTTGTTCTAAGCC-3′	Final elongation, 68 °C for 5 min		37 °C
			Final hold, 4 °C for ∞		1 h

Anneling temperature are 58.5 C°, for rs4986790; 63 C° for rs4986791

- Processing of retrieved cleavage products in agarose gel electrophoresis and their visualization using UV gel imaging system
- Determination of genotypes from gel images obtained and realization of statistical analysis

Measurement of Isolated Amount of DNA

Genomic DNA was obtained from whole blood using the EZ-10 Spin Colon Blood Genomic DNA Minipreps kit (Biotechnology Department Bio Basic Inc, Markham Ontario, Canada).

Measurement of isolated amount of DNA was performed using a microscale spectrophotometer (ATC Gene ASP-3700). Before determination of the purity levels of DNA extracts harvested from DNA samples, as blind–placebo control TBE buffer solution was used. Afterwards, 1 μl aliquots were removed from DNA samples and read against the blind-control. DNA samples measured at 260 and 280 nm wavelengths. When measurements made at 260 and 280 nm were compared, DNA samples with a ratio of ≥1.5 were considered to have maximal purity.

Amplification of DNA Samples Using PCR

PCR is a technique used to amplify a DNA segment extending between two domains with known sequences. Two oligonucleotides are used as primers of a reaction series catalyzed by a DNA polymerase. Typically, these oligonucleotides have different sequences and they are complementary to sequences extending in sequences opposite to DNA pattern but adjacent to DNA segment which will be amplified. For the PCR amplification procedure, DNA, PCR mix, forward-reverse primer, and water are used. This procedure was performed in a Thermal Cycler device. PCR reactions, primer sequences, and annealing temperatures are summarized in the following:

PCR reaction (25 μl)	
Master mix	12.5 μl
Primer F (10 μM)	0.5 μl
Primer R (10 μM)	0.5 μl
ddH2O	10.5 μl
DNA	1 μl

For the PCR mixture, OneTaq® 2× Master Mix with Standard Buffer containing Taq polymerase, dNTP mixture, and suitable buffer solutions was used (New England Biolabs, Ipswich, MA, USA). Primers specific to gene domains (Alpha DNA, USA) are indicated in Table 1. For rs4986790 and rs4986791, variants of TLR4 gene conditions of amplification procedure are as showed in Table 1. Two percent agarose gel was prepared to analyze post-PCR products, and the samples were subjected to agarose gel electrophoresis to make them visible.

PCR products whose amplification is confirmed by proliferating them in gel under appropriate PCR conditions have been cleaved using cleavage enzymes and under cleavage circumstances indicated in Table 1. Cleavage

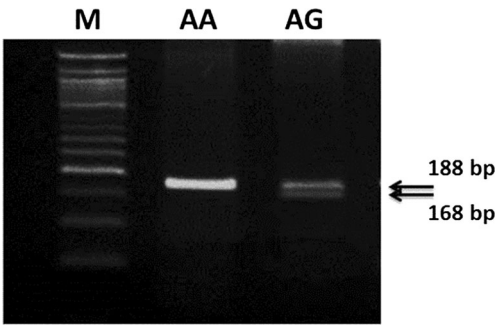

Fig. 1. TLR4 896 A/G gene domain as seen in agarose gel; AA, 188 bp; AG, 188+ 168+ 20 bp (a product of 20 bp is not visible in 2 % agarose gel) (M: marker 50 bp).

Table 2. Distribution of Genotypes of the Patient and the Control Groups and their Statistical Evaluation

Groups	*n*	TLR4 896 A/G Genotypes			
		AA		AG	
		n	%	*n*	%
Control	100	99	99.0	1	1.0
Lung cancer	160	159	99.4	1	0.6
Total	260	258	99.2	2	0.8
Statistics		$p>0.05$			

products were subjected to electrophoresis in 2 % agarose gel and evaluated using GeneGenius Gel Light Imaging System (Syngene, Cambridge, UK).

Statistical Analysis

The comparisons of genotype between the groups and odds ratios were evaluated using chi-square analysis (SPSS Inc., Chicago, IL, USA). Statistical analyses were performed using IBM SPSS Statistics 21 software, and the *p* values that were less than 0.05 were considered significant.

RESULTS

TLR4 896A/G Gene Domain (rs4986790)

For TLR4 896 A/G domain, 188 bp is an amplification product and in case of A-G Base Exchange, this product is cleaved by *Ncol* enzyme to a product of 168+20 bp (Fig. 1).

Any difference between genotype frequencies was not found between control group and patients with lung cancer ($p>0.05$). Distribution of genotypes of the patient and the control groups and their statistical evaluation are presented in Table 2.

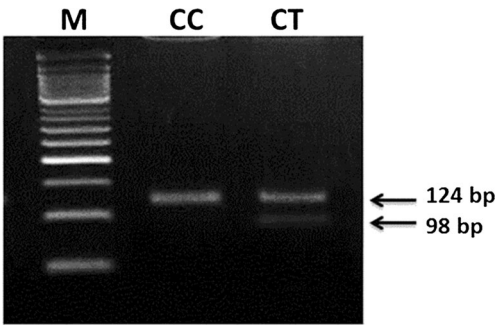

Fig. 2. TLR4 1196C/T gen domain as seen in agarose gel; CC, 124 bp; CT, 124+ 98+ 26 bp (a product of 26 bp is not visible in 2 % agarose gel) (M: marker 50 bp).

Table 3. Distribution of Genotypes of the Patient and the Control Groups and their Statistical Evaluation

Groups	*n*	TLR4 1196 C/T Genotypes			
		CC		CT	
		n	%	*n*	%
Control	100	91	91.0	9	9.0
Lung cancer	160	156	97.5	4	2.5
Total	260	247	95.0	13	5.0
Statistics		$\chi 2=4.19$; $p=0.041$			

TLR4 1196C/T Gen Domain (rs4986791)

Amplification product of TLR4 1196C/T domain is 124 bp, when C-T Base Exchange happens, this product is cleaved with *Hinf I* enzyme into a product of 98+26 bp (Fig. 2).

A significant difference was found between control group and patients with lung cancer as for genotype frequencies ($\chi^2=4.19$, $p=0.041$). Distribution of genotypes of the patient and the control groups and their statistical evaluation are presented in Table 3.

Risk analysis performed revealed a 3.857 higher risk of lung cancer in patients with a CT genotype (Table 4) ($p<0.05$).

DISCUSSION

Lung cancer is a fatal and the most frequently encountered cancer type worldwide. Frequently, it occurs because of smoking and exposure to asbestos or chemical carcinogens, while genetic predisposition is an effective factor on its pathogenesis [1, 4, 21].

Chronic inflammation is a long-lasting (for weeks, months, or indeterminate duration) condition where permanent damage of cells and tissues is observed. Free radicals and aldehydes formed during chronic inflammation can cause harmful gene mutations and posttranslational changes in key proteins involving in cancerogenesis. Chronic

Table 4. Risk Analysis of TLR4 1196 C/T Domain CC and CT Genotypes

Genotypes	Odds ratio	%95 CI	*p* Value
CC	1	–	–
CT	3.857	1.155–12.881	$p<0.05$

CI confidence interval

inflammation can cause cancerogenesis via inducing DNA damage, cellular proliferation, impairment of DNA repair pathways, cellular environment, inhibition of apoptosis, and promoting angiogenesis and invasion. Chronic inflammation is also associated with immune suppression, and it is a risk factor for cancer [4–7].

Though Toll-like receptors have very important roles in the mechanism of inflammation, functional polymorphisms that might occur in genes encoding these proteins can presumably induce carcinogenesis via disrupting mechanism of inflammation. To this end, in our study, the frequency of rs4986790 and rs4986791 variations on TLR4 gene was determined and the relationship between these polymorphisms and lung cancer was investigated.

TLR4 Gene rs4986790 and rs4986791 Polymorphisms. In studies performed, it has been indicated that single-nucleotide polymorphisms (SNPs) may increase the risk of chronic inflammation and cancer via disruption of TLR4 signalization [15].

SNP rs4986791 located on TLR4 gene is formed by cytosine/thymine substitution taking place on 1196. position, and a correlation between this genetic variant and risks of gall bladder cancer, precancerous gastric lesions and gastric cancer has been detected [15–18]. Another genetic variant on TLR4 gene (rs4986790) is formed by adenine/guanine substitution at 896. Position and the correlation between this genetic variant and risks of gastric cancer, prostate cancer, colorectal carcinoma, and non-Hodgkin lymphoma have been determined [15, 19, 20].

In our literature survey, any publication investigating polymorphisms of rs4986790 and rs4986791 of TLR4 gene in lung cancer has not been encountered so far. However, in our study, any correlation was not found for rs4986790 polymorphism between adenine/guanine substitution at 896. and lung cancer, while a correlation between cytosine/thymine substitution at 1196. position and lung cancer has been determined.

CONCLUSION

In a conclusion this study indicated that rs4986790 polymorphism in TLR4 gene was not associated with lung cancer. Moreover, rs4986791 was determined to be associated with lung cancer risk. The 3.857-fold risk was evaluated in the presences of CT genotype compared to CC for lung cancer. In the case of our study, rs4986791 variation of TLR4 gene may play an important role in the development of lung cancer.

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