Interferon Gamma (IFN-γ) Promoter and P2X7 Polymorphisms in Turkish Tuberculosis Patients

Türk Tüberküloz Hastalarında İnterferon Gamma (IFN-g) Promoter ve P2X7 Polimorfizimleri

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ABSTRACT

Almost one-third of the world population has been infected with the tuberculosis (TB) bacillus, but TB develops only in 5-10% of the infected people. IFN-γ (interferon gamma) is the most important cytokine that plays a role in resistance to TB. There are many studies demonstrating that a single nucleotide polymorphisms in the "Purinergic Receptor" (P2X7) and promoter site of the IFN-γ genes may cause susceptibility to TB. To our knowledge, there is no study associated with the effects of these polymorphisms in the Turkish population.

In this study, the relationship between -155A/G and -183G/T polymorphisms in the interferon gamma gene and susceptibility to TB disease were investigated in a case-control study. Also, the A1513C polymorphism that usually occurs in the P2X7 gene was examined in the same study.

In conclusion, the -155A/G and -183G/T polymorphisms of the IFN-γ gene were not found in the TB patients and in the control groups. This outcome suggests us that these polymorphisms may barely appear in the Turkish population. However, although the P2X7 A1513C polymorphism that changes glutamine to alanine at codon 496 was detected in both groups, there was no significant relationship between the occurrence of this polymorphism and resistance/or susceptibility to TB (AC; p=0.145 OR=1.521) (CC; p=0.851 OR=1.114) (AA; p=0.171 OR=1.447).

Our findings suggest that TB susceptibility genes may differ in different populations.

Key Words: Tuberculosis, Interferon-Gamma, P2X7, Polymorphism

ÖZ

Dünya nüfusunun 1/3' ü tüberküloz (TB) basili ile infektedir, fakat tüberküloz hastalığı bu bireylerin sadece % 5-10' unda gelişmektedir. IFN-γ, TB hastalığına dirençte en önemli sitokindir. TB hastalığına yatkınlıkta "Purinergic Receptor" (P2X7) ve IFNγ genlerindeki tek nükleotid değişimlerinin etkili olduğunu gösteren birçok yayın vardır. Araştırmalarımıza göre Türk toplumunda bu polimorfizmlerin etkisi ile ilişkili hiç çalışma yoktur.

Bu projede biz TB hastalığına dirençte interferon gamma geni -155A/G ve -183G/T polimorfizmlerinin ilişkisini bir olgu - kontrol araştırması ile incelemeyi planladık. Aynı zamanda P2X7 geninde genellikle görülen A1513C polimorfizminide çalışmamızda araştırdık.

Sonuçta TB hastalığında ve kontrol gruplarında IFNγ -155A/G ve -183G/T polimorfizmleri ile ilgili herhangi bir bulguya rastlanmadı. Bu sonuçlar bize bu polimorfizmin Türk toplumunda nadir olarak rastlanabileceğini gösteriyor. Bununla birlikte P2X7 genindeki Glu496Ala değişimine sebep olan A1513C polimorfizmi her iki grupta da belirlenmesine rağmen TB hastalığına yatkınlık ve dirençte bu hastalıkların görülmesi arasında anlamlı bir ilişki yoktur. (AC; p=0,145 OR=1,521) (CC; p=0,851 OR=1,114) (AA; p=0,171 OR=1,447).

Bu bulgular da gösteriyor ki TB hastalığına yatkınlık genleri popülasyonlara göre farklılık gösterebilir.

Anahtar Sözcükler: Tüberküloz, Interferon-Gamma, P2X7, Polymorfizim

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INTRODUCTION

The genetic single nucleotide polymorphism (SNP) is a single base pair change that causes alteration in the genomic DNA sequence. Genetic polymorphism is fairly common in a population, but its frequency may change depending on ethnical and geographical differences. Genetic polymorphisms may occur in different part of the genes such as promoter, exons, introns, enhancers and silencers. Such SNP's may have functional effects in the genes' activity and lead to changes in important cellular events involving DNA repair, apoptosis, cell cycle control and signal transduction etc. These changes have been shown to play important roles in some diseases and/or susceptibility or resistance to some diseases (1).

Many studies in this issue showed that certain genetic factors might affect the progression to TB disease (2, 3). Furthermore, different strains of tuberculosis bacillus establish different relationships with the host and their transmission features differ from each other (4, 5).

Interferon consists of a small group of cytokines that includes interferon alpha, beta and gamma. They are produced by both T-cell types (CD4 + and CD8 +) and natural killer cells (6, 7). IFN-γ and IL-12 are important cytokines that help to prevent individuals from developing TB. Therefore, defects of these cytokine and/or receptors may cause increasing susceptibility to TB (8). When IFN-γ genes were ablated in the mice models, TB susceptibility was found to be increased (9). IFN-γ is capable of binding to the receptors and is encoded by separate chromosomal loci. Initially, it was thought that IFN-γ is produced only by CD4 + helper T cells and CD8 + cytotoxic T lymphocytes. However, recent work has revealed that B cells, NK cells, T cells and other antigen-presenting cells can secrete IFN-γ. IFN-γ production by some antigen-presenting cells (APCs: monocytes / macrophages, dendritic cells (DCs) may be required to activate themselves and other closely localized other cells. While IFN-γ is a major component of the adaptive immune response, its secretion by NK cells and some APCs may indicate its possible roles in early host defense against infections (10).

P2X7 receptors are a family of ATP-sensitive ionotropic receptors of p2x and include seven homomeric receptor sub-types (P2X1–P2X7) (11). The P2X7 receptor is unique among the p2x family and have high concentrations of ATP (12). P2X7 receptors are expressed in hematopoietic stem cells (including mast cells, lymphocytes, erythrocytes, fibroblasts and epidermal Langerhans cells, peripheral macrophages) (13). Purinergic receptors are expressed in high amounts on macrophages. When P2X7 is induced, the binary cation channels are opened and calcium enters inside the cell. The entry of calcium causes caspase stimulation that results in apoptosis (14).

A few polymorphisms have been detected in the promoter regions of the P2X7 gene. These regions are ATP-binding domain, a trafficking domain and a repeat domain (A1513C, E496A,...). Occurrence of these polymorphisms results in functional impairments such as the loss of ATP-induced apoptosis, reduction of the ATP-stimulated ethidium uptake (15, 16). Moreover, the relationship between SNP in the P2X7 gene and susceptibility to TB was revealed in two case-control studies. Although the effect mechanism of the polymorphism remained elusive, researchers observed an increased risk of pulmonary TB (17). In the other study, A1513C SNP was found to be related to extra pulmonary TB, but there was no relation with pulmonary (18). A1513C polymorphism was found to be associated with decreased MTB and BCG (Bacillus Calmette Guerin) killing activity in the ATP-stimulated macrophages (18, 19). Taken together, only A1513C has been determined as a factor that triggers the attenuation macrophage's function that provides the ability of killing the MTB.

In light of all the studies described above, we aimed to investigate the polymorphisms of -155A/G and -183G/T in the IFN-γ gene and A1513C in the P2X7 gene to determine their effect on the changes in susceptibility and resistance to TB in the Turkish society.

MATERIALS and METHODS

The research protocol was approved by the Human Investigations Ethics Committee of Akdeniz University (15.08.2006.309). The study was performed at the Health Sciences Research Centre in the Faculty of Medicine.

Patient

In this study, 188 patients with TB and ethnically matched 81 healthy individuals were used to analyze the polymorphisms of -155A/G and -183G/T in the IFN-γ gene and A1513C in the P2X7 gene. Patients with pulmonary TB had been followed by certain centers including rhe State Tuberculosis Control Centre in Antalya, Akdeniz University, Faculty of Medicine and Marmara University, Faculty of Medicine in Istanbul. TB patients were defined with the presence of at least one of the following: (1) Clinical, radiological and laboratory findings consistent with TB disease and positive sputum or cerebrospinal fluid (CSF) smears for acid-fast bacilli (at least two separate occasions for pulmonary TB, CSF biochemical and cellular findings compatible with TB), (2) culture positivity of sputum, bronchial lavage, pleural fluid and/or cerebrospinal fluid. The 81 healthy individuals comprising the control group were gathered from the living-related transplant donors at Akdeniz University Transplantation Centre. These individuals were particularly chosen because they were examined for the presence of any kind of disease including TB and none of them were reported to have TB during the 2-year period during which this study was performed.

Genotyping

Blood samples (5–15 ml) from control and TB disease groups were collected from each person and genomic DNA was extracted using the genomic DNA purification Kit (Gentra kit) according to the manufacturer instruction and kept at –20°C.

Genotyping of -155 A/G and -183 G/T polymorphisms in the IFNg gene A1513C polymorphism in the P2X7 gene were carried out by using specific primers. PCR amplifications for both genes were performed as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 60 s at 51°C and 60 s at 72°C and final elongation at 72°C for 2 min in a volume of 50 µl reaction mixture involving 100 ng of genomic DNA, 1X reaction buffer (Roche), 0.5 mM of each primer, 1,5 mM $MgCl_2$ (Roche), 200 mM of each dNTPs (Fermentase) and 1 unit of Taq DNA polymerase (Roche). Phenol-Chloroform extraction was applied amplified DNA.

Restriction site analysis

To determine the restriction sites of the promoter region of IFN-γ gene, 2.5 µl of the PCR products was digested with the restriction enzyme *AluI (Roche) (IFN-γ -155A/G) and AvaII (Roche) (IFN-γ -183G/T) at the total 10* µl reaction under 37°C in a water bath (respectively 3 and 2 hours). For the A1513C polymorphism, the *Hae II (Roche) restriction enzyme was used. A volume of 5* µl of PCR product was digested with the restriction enzyme *HaeII in a total of 25* µl reaction under 37°C in a water bath (2,5 hours). While the Hae II and AluI enzymes cut the polymorphic individual, the AvaII enzyme cut the wild type individual. After restriction enzyme digestions, PCR products were visualized under UV transillumination to reveal whether these polymorphisms exist.

Statistical analyses

The statistical difference between TB patients and healthy individuals were analyzed with the chi-square test. Statistical significance was accepted as $p< 0.05$.

RESULTS

1. RFLP-PCR result of Interferon g -155 A/G polymorphism

To assay 188 TB patients and 81 healthy controls for the -155 A/G polymorphism, the amplified PCR products were cut by the *Alu* I restriction enzyme. When restricted PCR products were run on the 3% gel, there were three bands in the length of 147 bp, 184 bp, 331 bp in the heterozygote AG genotype, single-band (331 bp) in wild-type genotype (AA) and two bands (147 bp and 184 bp) in homozygous polymorphic genotype (GG) (Figures 1, 2). It is important to note that frequencies of all three genotypes were tested for the Hardy-Weinberg equilibrium and they showed a normal distribution (Table II) (Figure 3).

IFN-γ promoter heterozygote (AG) and homozygous mutants (GG) of the -155 A/G polymorphism were not detected in the patient and control groups (Table I). This finding suggests that the IFN-γ promoter -155 A/G polymorphism may not play a role in the pathogenesis of TB. Moreover, this polymorphism is barely present in the Turkish population.

2. RFLP-PCR Result of IFN- γ -183 G / T Polymorphism

To determine the -183 G/T Polymorphism in the IFN-g gene in 165 TB patients and 87 healthy controls, PCR products were cut by the Ava II restriction enzyme. There were three bands in the length of 156 bp, 175 bp, 331 bp in heterozygote (GT) genotype, single-band (331bp) in homozygous polymorphic genotype (TT) and two bands

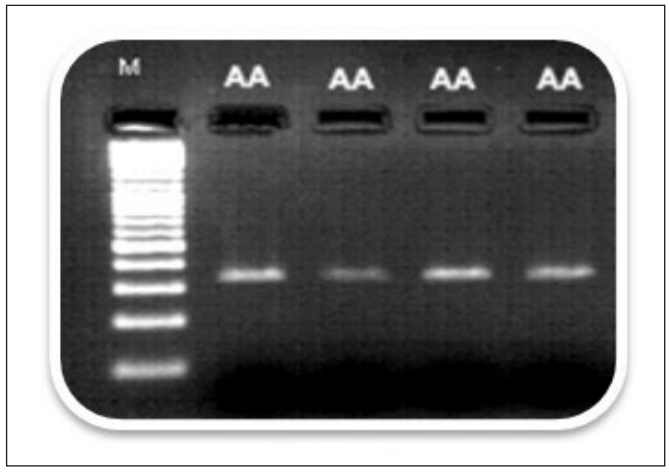

Figure 1. Representing picture of the IFN g -155 A / G polymorphism in patients with tuberculosis in as a result of the cutting reaction.

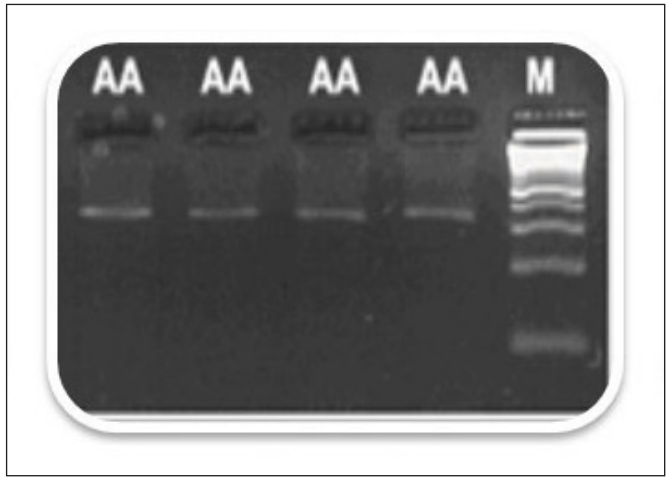

Figure 2. Interferon gamma -155 A / G polymorphism in healthy controls in as a result of the cutting reaction.

Table I.: The distribution of the patients and controls which genotyping have been done according to Interferon gamma -155 A / G.

		Control	Tuberculosis
Position	**Genotype/ allele**	**n(%)**	**n(%)**
—155A-G	AA	81(100)	188(100)
	AG	0(0)	0(0)
	GG	0(0)	0(0)

Table II: Hardy-Weinberg equilibrium in terms of Interferon gamma - 155 A / G polymorphism in our study.

A<>G	**AA<>AG**	**AA+GG<>GG**	**AA<>AG+GG**
p=1.00	p=1.00	p=1.00	p=1.00
G<>A	**GG<>AG**	**GG+GA<>AA**	**AA+AG<>GG**
p=1.00	p=1.00	p=1.00	p=1.00

Table III: The distribution of the patients and controls which genotyping have been done according to Table 4. Hardy-Weinberg equilibrium in terms of Interferon gamma - 183 G / T polymorphism in our study

		Control	Tuberculosis
Position	**Genotype/ allele**	**n(%)**	**n(%)**
—183A-T	GG	87(100)	165(100)
	GT	0(0)	0(0)
	TT	0(0)	0(0)

(156 bp, 175 bp) in homozygous wild type genotype (GG) (Figures 4, 5). As a result of analyzing the restricted PCR products, only the GG wild type genotype was observed in both the control and patient groups (Figure 6). It is important to note that frequencies of the three genotypes were tested for the Hardy-Weinberg equilibrium and they showed a normal distribution.

These findings suggest that the -183 G/T polymorphism may not play a role in the pathogenesis of TB disease. Furthermore, G to T change seems to be rarely seen in Turkish people.

All of these mentioned above are schematized in Figure 6 and summarized in Table III. The chi-square test was used to compare the tuberculosis and healthy controls for genotype (Table IV).

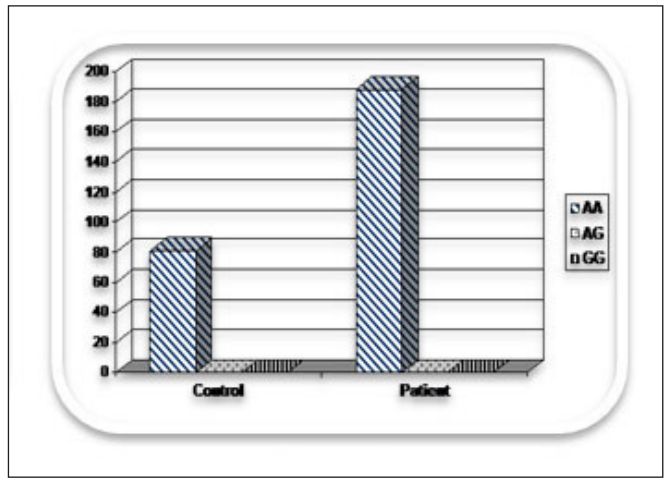

Figure 3. The distribution of the cutting reaction for Interferon gamma - 155 A / G polymorphism.

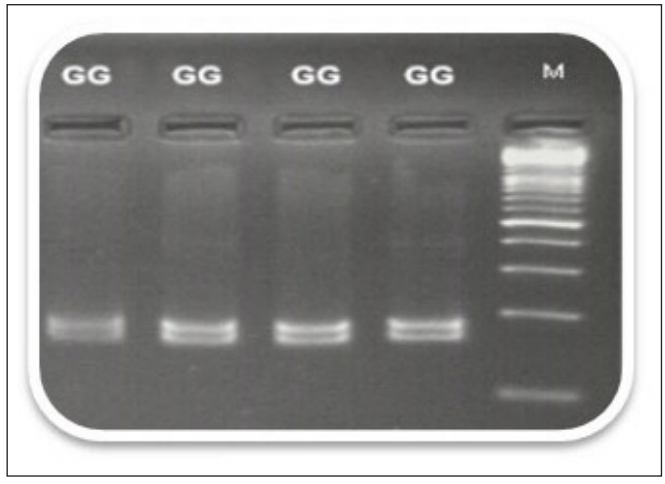

Figure 4. Interferon gamma -183 G / T polymorphism in patients with tuberculosis as a result of cutting reaction.

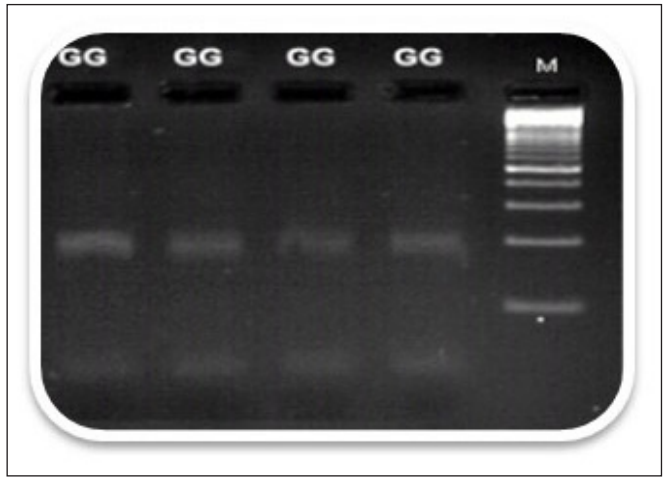

Figure 5. Interferon gamma -183 G / T polymorphism in healthy controls as a result of the cutting reaction.

3. RFLP-PCR Results of P2X7 A1513C Polymorphism

To determine the A1513C polymorphism in the P2X7 gene in 139 TB patients and 87 healthy controls, PCR products were cut by the HaeII restriction enzyme. There were three bands in the length of 62 bp, 165 bp, 227 bp in heterozygote (AC) genotype, single-band (227 bp) in homozygous polymorphic genotype (CC) and two bands (62 bp, 165 bp) in homozygous wild type genotype (AA) (Figures 7, 8). As a result of analyzing the restricted PCR products, only the AA wild type genotype was observed in both control and patient groups (Figure 6). It is important to note that frequencies of the three genotypes were tested for the Hardy-Weinberg equilibrium and showed a normal distribution (Figure 9).

The frequencies of the AA, AC and CC genotypes in the patient group were found to be 66, 31 and 5%, respectively. In the control group, the frequencies were 72% for AA, 22% for AC and 5% for CC genotypes. It is important to note that frequencies of the three genotypes were tested for Hardy-Weinberg equilibrium and showed a normal distribution (Tables V, VI).

As is known, the A1513C polymorphism causes to Glu496Ala change in the P2X7 gene and has been observed in both tuberculosis patients and healthy individuals in the Turkish society. No significant differences were found between patient and control groups, and this polymorphism may not increase the risk of susceptibility or resistance to tuberculosis.

Table IV: Hardy-Weinberg equilibrium in terms of Interferon gamma - 183 G / T polymorphism in our study.

G<>T	GG<>GT	GG+TT<>TT	GG<>GT+TT
p=1.00	p=1.00	p=1.00	p=1.00
T<>G	**TT<>GT**	**TT+TG<>GG**	**GG+GT<>TT**
p=1.00	p=1.00	p=1.00	p=1.00

DISCUSSION

Tuberculosis is an important disease and causes mortality and morbidity in one-third of the world population (20-22).

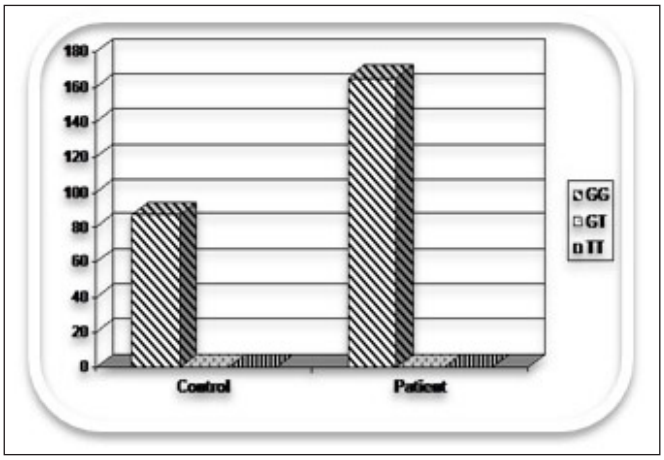

Figure 6. The distribution of the cutting reaction for Interferon gamma - 183 G / T polymorphism.

Table V: The distribution of the patients and controls which genotyping have been done according to P2X7 A1513C.

		Control	Tuberculosis	The P value
Position	**Genotype/allele**	**N(%)**	**n(%)**	**Tuberculosis**
—1513 A-C	AA	85 (72)	66 (64)	0.27
	AC	27 (22)	32 (31)	
	CC	6 (5)	6 (5)	

Table VI: Hardy-Weinberg equilibrium in terms of P2X7 A1513C polymorphism in our study.

A<>C	AA<>AC	AA+AC<>CC	AA<>AC+CC
p=0.249 Odds ratio=1,303 (0,83–2,04)	p=0.145 Odds ratio=1,521 (0,864–2,678)	p=0.851 Odds ratio=1,114 (0,36–3,45)	p=0.171 Odds ratio=1,447 (0,851–2,46)
C<>A	**CC<>AC**	**CC+CA<>AA**	**AA+AC<>CC**
p=0.249 Odds ratio=0,768 (0,49–1,204)	p=0.608 Odds ratio=1,365 (0,414–4,496)	p=0.851 Odds ratio=0,897 (0,29–2,779)	p=0.986 Odds ratio=1,01 (0,33–3,093)

Most individuals (90-95%) infected by MTb show no clinical symptom. It means that MTb can live in a long-term latent form. When the immune system weakens, TB disease may occur in those MTb infected individuals. IFN- γ release from lymphocytes activate monocytes and macrophages. These cells normally cannot kill the MTb effectively, if not activated by IFN-γ. So IFN-γ is the most important cytokine in defense against TB. Moreover, P2X7 receptors are largely expressed by macrophages (23, 24). When the P2X7 receptor is activated, it causes an opening the cation channels so that Ca2+ enters inside. Entry of the Ca2+ ion activates the cascade to induce the apoptotic pathway (25, 26). The P2X7 gene is highly polymorphic in humans and it has several SNPs defined (27). Studies have shown that variants of interferon gamma and P2X7 gene seem to have an important role in acquiring susceptibility or resistance to TB disease. This may arise from increasing definite cytokine secretion based on the polymorphic changes in these genes (28). Therefore, in this study, we have examined the affect of -155A/G and -183G/T polymorphisms in the IFN-γ gene and A1513C polymorphism in the P2X7 gene in TB patients and healthy individuals. IFN polymorphisms (-155A/G and -183G/T) were not detected in the control and patient groups. However, the A1513C polymorphism of the P2X7 gene was determined in both groups in slightly different frequencies, which was not found to be statistically different. These findings suggest that the IFN-γ polymorphisms may be rarely present in the Turkish people who have TB disease or in healthy individuals. In contrast, the A1513C polymorphism seems to not have a major role in progression of TB.

Two polymorphisms (-155 A/G and -183G/Tin, the IFN-γ gene promoter) were studied in the TB patients and healthy controls in Sudan. Allelic frequencies were found to be 0.927 for G and 0.073 for T at a position of -183 and 0.977 for A and 0.023 for G at a position of -155 (29) 27 boulevard Jean Moulin, 13385 Marseille Cedex, France. Two new polymorphisms in the human interferon gamma (IFN-gamma. Allelic frequencies of these polymorphisms in the Sudan society seems to be almost similar to the Turkish population results we have found in the present study. These two polymorphisms did not affect the transcriptional activity of the IFN-γ gene.

The +874 T/A polymorphism in the IFN-γ gene was also investigated in patients with TB disease and control individuals who had no history of TB disease and the same ethnic and geographic background with the patients. The researchers observed that this polymorphism may be associated with IFN-γ response and appearance of the TB disease in Turkey (30).

Similarly, IFN-γ promoter (-155 and -183) polymorphisms were investigated regarding susceptibility to Hepatitis B

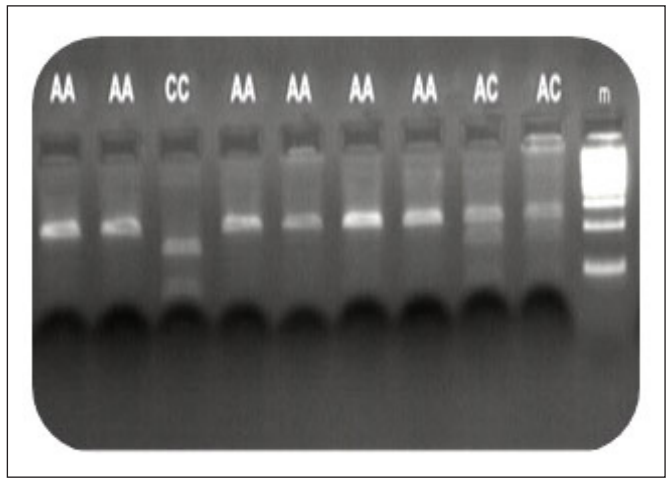

Figure 7. P2X7 A1513C polymorphism in patient with Tuberculosis as a result of cutting reaction.

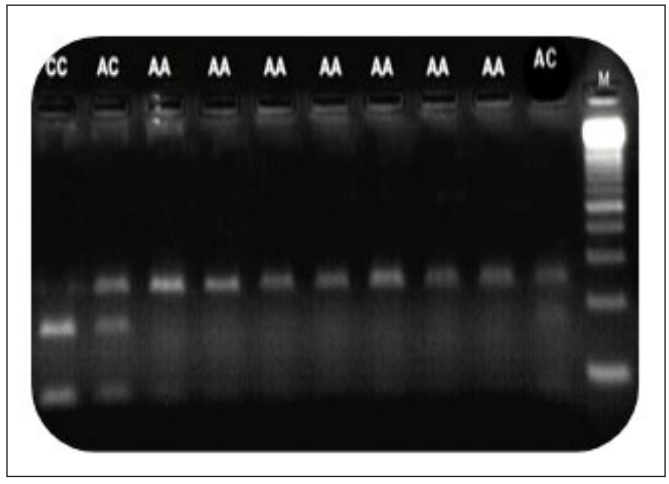

Figure 8. P2X7 A1513C polymorphism in healthy controls as a result of cutting reaction.

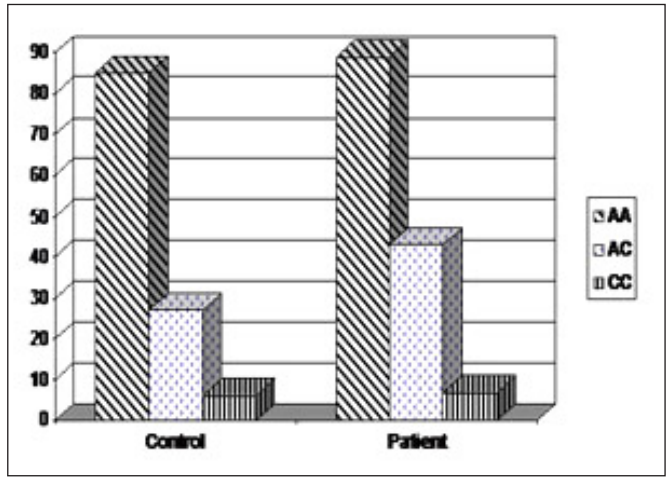

Figure 9. The distribution of the cutting reaction for P2X7 A1513C polymorphism.

in the Chinese population. They found the 183 promoter polymorphism in cases and controls but found no 155 promoter polymorphism (31).

The A1513C polymorphism of the P2X7 gene, known to be the most common polymorphism, changes alanine into glutamic acid at the position 496 of the C terminal site of the P2X7 protein (24). This polymorphism may affect certain functions that the P2X7 play roles including the cation migration of many cells, secretions of IL-1β, IL-18 and matrix metalloproteinase (MMP9) from macrophages, and expression of CD23 and CD62L in the lymphocytes (19, 24, 32-36). Different studies have shown that the homozygous C/C genotype may lead to almost complete loss of the P2X7 function that prevents mycobacterial death in the infected individuals (37-39). The Glu496Ala SNP of the P2X7 gene was evaluated and this polymorphism was found to be markedly increased in the patients with pulmonary TB. This first demonstrated the relationship between tuberculosis and the polymorphism that caused increasing susceptibility to tuberculosis. Taken together, this receptor probably plays an important role in the pathogenesis of TB disease (40). The P2X7 Glu497Ala mutation affects the function of this channel and its function is almost completely lost in the individuals who have homozygous CC genotypes (19, 41). Similar results were also found in the Russian Slavic population where the A1513C polymorphism of the P2X7 gene that may cause loss of the host control for M. tuberculosis infection is found. Therefore, they suggested that the risk factor for the TB disease in the individuals that had CC genotype was found to be increased (42). Intriguingly, there was no relation between this polymorphism and TB disease in the Gambian population (17)

In conclusion, the -155A/G and -183G/T polymorphisms of the IFN-g gene were not found in the TB patient and control groups. This finding suggests that these polymorphisms may barely appear in Turkish population. Although A1513C polymorphism that changes glutamine to alanine at codon 496 was detected in both groups, there was no significant relation between occurrence of these polymoprhisms and resistance to TB. A1513C polymorphisms may not provide resistance to TB. New studies in a high number of TB patients/individuals from different parts of Turkey should be conducted to reveal the allelic frequencies of these polymorphisms in the Turkish population. Additionally, the effect of these polymorphisms on the functional features of IFN-γ and P2X7 proteins should be investigated to completely understand their roles on other diseases.

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