TR
INONU UNIVERSITY
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INVESTIGATION OF SOME TLR POLYMORPHISMS IN TUBERCULOSIS PATIENTS IN MALATYA

REFİKA DİLARA VAİZOĞLU

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Thesis prepared by **Refika Dilara VAİZOĞLU**

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The thesis mentioned above is evaluated by our committee and accepted as the master thesis in Molecular Biology and Genetic Department.

Examining Committee Members

Supervisor: **Asst. Prof. Ceren ACAR** .....

Inonu University

Assoc. Prof. Esra BİRBEN .....

Hacettepe University

Asst. Prof . Mustafa Mert SÖZEN .....

Inonu University

Prof. Dr. Halil İbrahim ADIGÜZEL

Director of the Institute

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Refika Dilara Vaizoğlu

Sevgili Babam, Annem ve kardeşime….

ABSTRACT

Master Thesis

INVESTIGATION OF SOME TLR POLYMORPHISMS IN TUBERCULOSIS PATIENTS IN MALATYA

Refika Dilara VAİZOĞLU

Inonu University

Institute of Science

Department of Molecular Biology and Genetics

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Supervisor: Asst. Prof. Dr. Ceren ACAR

Tuberculosis, one of the major health problems causes many deaths every year in the world. According to the report of World Health Organization, 2 million people per year lose their lives due to tuberculosis. The disease can remain in the latent phase for a very long time after infecting the affected individual. Although some of the infected people are showing the symptoms, while in some people the disease never develops, even about 90% of them are recovered by the immune system's response. As in many infectious diseases, the difference between the number of infected people and the number of sick people is caused by differences in balance between host defense and the virulence of the organism. In studies conducted this difference was mostly attributed to the state of the immune system of the site, but not to an adequate explanation. In this case, the genetic basis of the response to infectious agents needs to be investigated in order to understand the relationship between infectious diseases and host. We investigated the effect of polymorphisms in TLR genes from the genes involved in immune response to susceptibility to *Mycobacterium tuberculosis*. In this thesis study, we included 50 tuberculosis patients and 50 healthy individuals living in Malatya. Patient and control groups were compared in terms of TLR 4 (rs4986790, rs4986791) and TLR 6 (rs3821985) genotype and allele distributions and we did not find a statistically significant result between them.

KEYWORDS: Tuberculosis, SNPs, TLR 4, TLR 6, Genotyping

ÖZET

Yüksek Lisans Tezi

MALATYADAKİ TÜBERKÜLOZ HASTALARINDA BAZI TLR GENLERİNİN POLİMORFİZMLERİNİN TARANMASI

Refika Dilara VAİZOĞLU

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Dünyadaki başlıca sağlık sorunlarından biri olan tüberküloz, her yıl çok sayıda ölüme neden olmaktadır. Dünya Sağlık Örgütünün raporuna göre yılda 2 milyon kişi tüberküloz nedeniyle hayatını kaybetmektedir. Hastalık etkeni kişileri enfekte ettikten sonra çok uzun süre latent evrede kalabilmektedir. Enfekte olan kişilerden bazıları hasta olurken bazı kişilerde ise hastalık hiçbir zaman gelişmemekte hatta bunların yaklaşık %90'nı bağışık sistemin verdiği cevapla kendiliğinden iyileşmektedir. Birçok enfektif hastalıkta olduğu gibi enfekte olan kişi sayısı ve hasta olan kişi sayısı arasındaki farklılığa konakçı savunması ve organizmanın virulansı arasındaki denge farklılıkları neden olmaktadır. Yapılan çalışmalarda bu farklılığın sebebi çoğunlukla, konağın bağışıklık sisteminin durumu ile ilişkilendirilmiş ancak yeterli bir cevap olarak görülmemiştir. Bu durumda, enfektif hastalıklarla konak arasındaki ilişkiyi anlayabilmek için enfektif ajanlara verilen cevabın genetik temellerinin araştırılması gerekmiştir. Bu çalışmamızda *Mycobacterium tuberculosis*'e immun cevapta ya da yatkınlıkta söz konusu genlerden TLR genlerindeki polimorfizmlerin etkisini inceledik. Bu çalışmamızda Malatya'da yaşayan 50 tüberküloz hastası ve 50 sağlıklı bireyi dahil ettik. Yaptığımız bu tez çalışmasında TLR 4 (rs4986790, rs4986791) ve TLR 6 (rs3821985) genotipik ve allellik dağılımları açısından hasta ve kontrol grupları karşılaştırıldı ve aralarında istatistiki olarak anlamlı bir sonuç bulamadığımızı gösterdik.

ANAHTAR KELİMLER: Tüberküloz, SNPs,TLR 4,TLR 6,Genotipleme

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ABBREVIATIONS

TB	Tuberculosis
MTB	Mycobacterium tuberculosis
WHO	World Health Organisation
SNPs	Single nucleotide polymorphisms
GWAS	Genome Wide Association Studies
PIMs	Phosphalidyl-myo-inositol mannosides
LM	Lipomannan
Man-LAM	Mannose-capped lipoarabinomannan
PAMPs	Pathogen-associated microbial patterns
PRRs	Pattern recognition receptors
TLRs	Toll-like receptors
DCs	Dendritic cells
ECDs	N-terminal ectodomains
LRR	Leucine-rich repeat
DAMPs	Danger-associated molecular patterns
LPS	Lipopolysaccharide
PGN	Peptidoglycan
IRFs	Interferon regulatory factors
IFNs	Interferons
PIM	Mannosylphosphatidyl- myo-inositol
aa	Amino acid
iDCs	Immature dendritic cells
MAMPs	Microbe-associated molecular patterns
NF – κβ	Nuclear factor kappa - β
JNK	Jun N-terminal kinase
NLR	Nucleotide oligomerization domain-like receptors
PTB	Pulmonary Tuberculosis
BCG	Bacillus Calmette-Guérin

1 INTRODUCTION

Tuberculosis (TB) is the crucial cause of morbidity and mortality worldwide. Tuberculosis is caused by *Mycobacterium tuberculosis* (MTB). According to World Health Organisation (WHO), 32% of world's population is infected with an infectious agent. Approximately 9 million people are infected each year and 2 million deaths are reported because of the disease. Tuberculosis accounts for 2.5 % of all diseases worldwide and 26.0% of evitable deaths. Tuberculosis is a granulomatous infectious disease caused by MTB. Infection with *M. tuberculosis* involves the participation of more than 90 antigens and various virulence factors and results from the interaction between the pathogen and the host's mononuclear phagocytes and T lymphocytes [1].

Although 1/3 of world's population is infected with an agent, the infection usually does not progress to active disease. The pathogen remains in latent form in about 90% of infected individuals, who show no clinical features of the disease [2].

Despite its clinical significance, there are still significant gaps in our understanding of *M. tuberculosis* pathogenesis and the host mechanisms that limit active disease in approximately 10% of those infected. Nevertheless, we continue to gain insight into the dynamic interplay between pathogen and host, with much of the focus centred on the lung microenvironment because this is the initial and primary site of infection [3].

A major goal of biology and medicine is to understand human relationship with infection agents. It is essential to dissect the interaction of bacteria and the genetic basis of human host's phenotypic diversity. Outcomes of Human Genome Project gave clues about the genetics of human host. But still, there are unanswered questions about the naturally occurring genetic variation between the genomes of different individuals and populations that could account for their phenotypic variation. It is easier to compare variation at the level of the entire genome and between populations that are located in different geographic areas with a different ethnic background with the help of the newly developed technologies. By this way, we are able to increase our understanding related to the level of naturally occurring human genome variations and its relationship to disease. So this will lead us to understand the histories and migration patterns of populations and into the genetic

mechanisms underlying the adaptation of *Homo sapiens* to changing environmental conditions, which includes climatic changes, nutritional resources, and pathogen pressures. As a consequence effects of human genetics on disease susceptibility, severity and response to treatment will be resolved greatly [4].

Diversity in resistance to infectious diseases, vital to the host, as well as the influence, spread and promotion of the disease is also important. This diversity is not fully understood. It is possible to compare disease-related genomic regions in patients and healthy controls with the help of specific single nucleotide polymorphisms (SNPs).

1.1 Single Nucleotide Polymorphisms

In genome one of the most common sequence variations is single nucleotide polymorphisms. These are valuable markers for susceptibility to common diseases. SNPs are the results of mutations that produce single base pair changes as can be understood from their names. Each variation can be found with a population to some degree. Depending on the changings they cause, they can be classified as noncoding SNP or coding SNPs and the coding ones can be classified depending on their effect on the protein structure. In order to understand the genetic influences on complex traits Genome Wide Association Studies (GWAS) are employed for the association of SNPs with the diseases [5]. Although the frequencies of SNP variations are low in populations, they play a major role in some cases. Many researchers in the field of genetics are trying to find SNPs that are spesific to the disease. SNPs can be phenotypically classified as SNPs of non-coding regions and as SNPs of coding regions. Non-coding regions contain more SNPs than coding regions. SNPs are heterogeneously distributed in the genome. SNPs are generally used in evolutionary studies and population genetic studies as markers. SNPs in the coding region mostly do not cause important changes in the amino acid sequence due to degeneracy in the genetic code. SNPs that alter the protein structure are used in drug metabolism and therefore in pharmacogenetic studies. SNPs that are found in the coding region there are 2 subtypes; some synonymous SNPs that affect protein activity but do not affect protein sequence and nonsynonymous SNPs that cause changes in protein amino acid sequence [6-8].

SNP databases are so popular among researchers who work on GWAS. Nowadays the commonly used database is dbSNP which contains more than 6 million human SNPs. In addition to this, Human Gene Mutation Database, Human OMIM (Online Mendel Heritage), Swiss-Prot and Human Genome Variation Database are the other frequently used databases [8].

The widespread availability of SNPs in genomes can be used as a dominant marker in genetic screens and will continue to be expanded in favour of efficient screenings. Since new drug discovery projects involve a large number of SNPs and individuals, SNPs have played a role in the development of new technologies for screening and genotyping [7].

1.2 Mycobacteria

Studies to understand the pathogenesis of the tuberculosis were started by Theophile Laennec in the 19th century and further in 1865 Jean-Antoine Villemin demonstrated the transmissibility of *M. tuberculosis* infection and 1882 Robert Koch identified the tubercle bacillus as the etiologic agent [9]. The only genus of the Mycobacteriaceae family of Actinomycetales is Mycobacterium. Among the general characteristics of mycobacteria are; Aeropers, catalase-producing, stationary, spore-free and acid-resistant, slow reproductive, cell wall contains plenty of lipids. High Guanine (G) + Cytosine (C) contents of mycobacteria species are similar to microorganisms such as *Gordonia, Tsukamurella, Nocardia* and *Rhodococcus* which contain mycolic acid [10].

All members of the genus Mycobacterium are significantly smaller than other bacteria. The widths vary between 0,2-0,6 μm, and the length varies between 1-10 μm. In appearance mildly curved or regular rod-shaped bacteria, sometimes seen in filamentous form or coccobacili [11]. The optimum temperature range for growth varies widely among species (<30-45°C). Colonies can be seen within 2-60 days at appropriate temperatures [12,13].

Mycobacteria have abundant amounts of lipids in their cell wall structures because of that they are difficult to handle. However, once they are stained they do not easily leave the stain and resist decolorization with acid alcohol. For this reason, they are classified as acid-resistant bacteria (ARB, Acido Resistant Bacteria).

Mycobacteria are usually considered to be Gram positive, although they are not easily stained by the Gram method [14].

1.2.1 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is usually spreaded by sneezing, coughing and speech of patients with pulmonary tuberculosis and droplet nuclei with live bacilli in them are inhabited by sensitive people and also the only natural source is people [11]. These particles which are 1-5 μm in diameter, hang in the normal air flow. Bacillus reaching alveoli with respirable particles are trapped by alveolar macrophages. Frequently the host cellular immune response prevents the multiplication and spread of *M. tuberculosis*. In addition, some bacil may live for years after the first infection but remain "dormant" bacili [12].

1.2.2 Mycobacterial Cell Wall

Mycobacterium tuberculosis - an intra-cellular pathogen- grows slowly. It has the ability to stay alive inside the host's macrophages. Since hydrophobic mycolic acids are found in its cell wall (50% of the dry weight), it is an acid-fast bacterium. Mycobacteria's slow growth is caused by its thick layer of mycolic acids which impairs nutrient intake. On the other hand, this layer gives the cells a resistance against lysosomal enzymes. Mainly the external regions of the cell wall carry the mycolic acids and the internal layers mainly consist of phosphatidyl-myo-inositol mannosidase (PIMs), arabinogalactan and peptidoglycans. Beneath the external layer mannose-containing molecules are found. These biomolecules can be "mannoglycoproteins, related lipomannan (LM), and mannose-capped lipoarabinomannan (Man-LAM)". Bacterium's outer capsule is formed by both arabinomannan and mannan which are present on the cell surface.

On cell surface, one of the most abundant mannans is Man-LAM and it is an important virulence factor. All pathogenic bacteria share the feature of mannose-capped motifs which are not found in less pathogenic, fast-growing mycobacterial strains [15]. Even though host-pathogen interactions of MTB and humans are studied in detail it is not completely solved. In innate host defence activation, the first stage starts with pathogen's pattern recognition. The PAMPs (Pathogen-related molecular

patterns) of *M. tuberculosis* are detected by specific pattern recognition receptors (PRRs). After recognition, a couple of events like proinflammatory cytokines and chemokines production, phagocytosis and destruction of bacteria and antigen presentation are triggered.

1.3 Toll-like Receptors (TLRs)

Toll-Like Receptors are belong to pattern recognition receptors family. In mammals, twelve members are found in that family. They are either expressed on cell membrane surface or endocytic vesicle membranes of immune cells like dendritic cells (DCs) and macrophages. In 1985 Christiane Nüsslein-Volhard discovered them to be crucial for defence against microbial infection as the homologs of the Drosophila Toll protein and they are evolutionarily conserved structures [16]. They initiate immune responses to a wide variety of pathogens. They have characteristic planar horseshoe shape with N-terminal ectodomains (ECDs) leucine-rich repeat (LRR). Although ligand interactions of Toll-like receptors are so distinctive, they all form a characteristic m-shaped dimeric complex with N-terminals on outside and C-terminals in the middle [17].

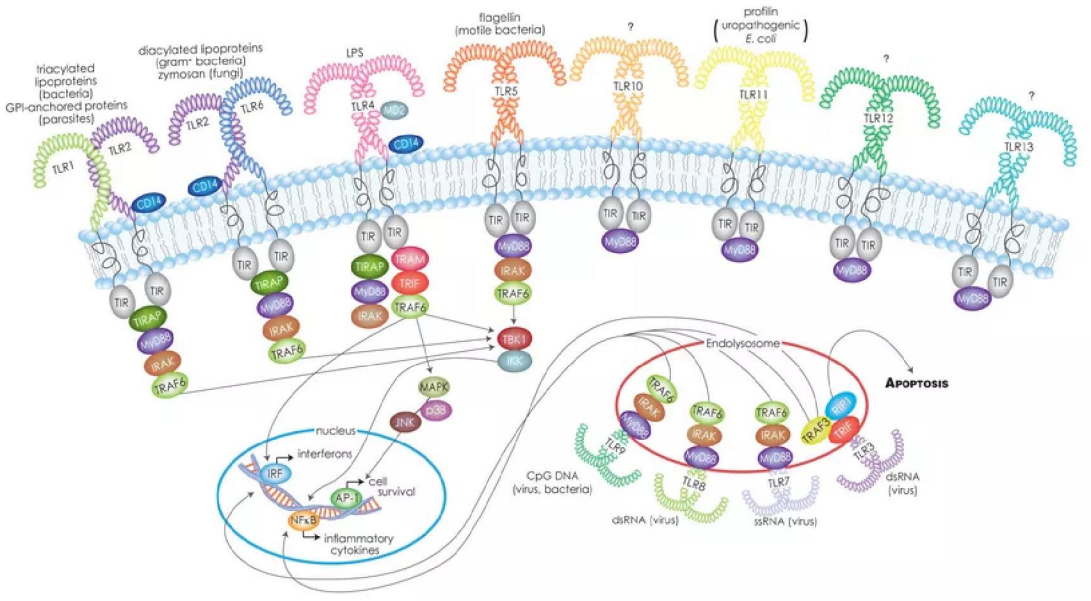

Figure 1. 1: Toll-Like Receptors Localisation and Signalling
This figure was taken by; "TLR localisation and signalling," [18].

Endogenous molecules from dying cells or microbial pathogens express Pathogen-related molecular patterns which are highly conserved structural motifs. Danger-associated molecular patterns (DAMPs) are recognised by TLRs. PAMPs includes cell wall components. These components can be listed as lipopeptides, lipopolysaccharide (LPS) and peptidoglycan (PGN). On the other side viral double-stranded RNA, bacterial DNA and flagellin can be classified as PAMPs. PAMPs sensed by the TLR family range from lipids to lipopeptides, to proteins and nucleic acid. Danger-associated molecular patterns include intra-cellular proteins like heat shock proteins as well as protein fragments from the extra-cellular matrix [19].

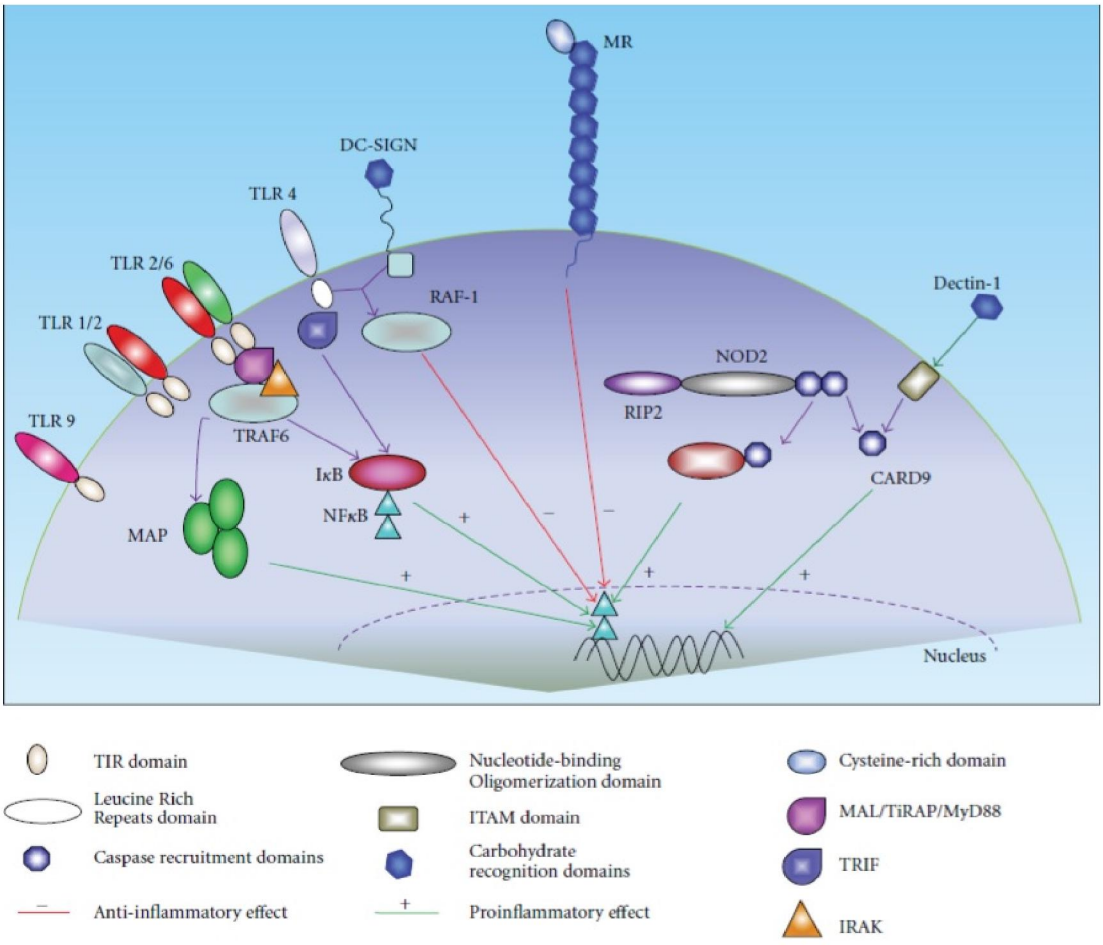

Figure 1. 2: Downstream signalling pathways of PRRs in recognition of MTB [15].

Inducing Toll-Like Receptors by PAMPs or DAMPs trigger signalling cascades leading to the activation of AP-1 and NF-κB, interferon regulatory factors (IRFs) like transcription factors. Adaptive immune response directing production of pro-inflammatory cytokine, effector cytokine and Interferon (IFN) are the variety of cellular responses that are consequences of TLR signalling. TLRs with IL-1 receptors form a receptor superfamily, known as "interleukin-1 receptor/toll-like receptor superfamily". Five molecules (My-D88, TRIF, Mal, TRAM & SARM) with the TIR domain which are adapters for TIR domain of TLR receptor family interact with this family's all members [20].

The TLRs family includes TLR 1 to TLR 10 in human. In mice, this family includes TLR 1- 9, the homolog of TLR 10 which is a pseudogene, TLR 11, TLR 12, and TLR 13. TLRs are expressed mainly in spleen and peripheral blood leukocytes, which play a major role in immunity. In addition, the lungs and gastrointestinal tract in the external environment are also secreted.

Researchers have simply classified TLRs into two groups according to their cellular location. TLRs in different locations play an important role in the identification of different markers. The first group 1, 2, 4, 5, 6 are all synthesised on the cell surface and recognise lipid structures. In recognition of protein flagellin, TLR 5 has a vital role. The second group, Toll-Like Receptors 3, 7, 8, 9 recognise nucleic acids derived from the genome of bacteria and viruses and they are found in intra-cellular locations. Access to these molecules thus requires some form of degradation within the late endosomes or lysosomes of the cell. It has become ever increasingly apparent that the localisation and trafficking of these receptors in the cell is an important mechanism to allow their ligand detection. Remarkably, trafficking of certain TLRs during signalling can also prevent overactivation of the TLR signalling pathways [20,21]. Within the TLR family 2, 4, 9, and possibly 8 are involved in recognition of *M.tuberculosis*. At the same time, TLR 2 forms heterodimers either with TLR 1 or TLR 6 and these heterodimers have been implicated in recognition of mycobacterial cell wall glycolipids like LAM, LM, 38-kDa, and 19-kD mycobacterial glycoprotein and phosphatidylinositol mannose (PIM), triacylated (TLR 2/TLR 1), or diacylated (TLR 2/TLR 6) lipoproteins [22,23].

Table 1.1: General Features of TLRs

Receptor	Ligands	Ligands Location	Adapters	Localization	Cells
TLR-1	Multiple Triacyl lipopeptides	Bacterial lipoprotein	MyD-88/ MAL	Cell Surface	Macrophages/Monocytes subsets of dendritic cells B lymphocytes
TLR-2	Multiple lipoproteins Multiple glycolipids Multiple lipopeptides Lipoteichoic acid Beta glukan HSP70	Bacterial peptidoglycans Gram-positive bacteria Host cells Fungi	MyD-88 / MAL	Cell Surface	Myeloid dendritic cells Macrophages/Monocytes Neutrophils Mast cells
TLR-3	dsRNA	Viruses	TRIF	Cellular Compartment	B lymphocytes Dendritic cells
TLR-4	Lipopolysaccharide Heat shock proteins Hyaluronic acid Heparan sulfate Fibrinogen Nickel	Bacteria Gram-negative bacteria Host cells	MyD-88/ MAL / TRAM / TRIF	Cell Surface	Myeloid dendritic cells Macrophages /Monocytes Intestinal epithelium B lymphocytes Mast cells Neutrophils
TLR-5	Profilin Bacterial flagellin	*T. gondii* Bacteria	MyD-88	Cell surface	Macrophages /Monocytes Intestinal epithelium
TLR-6	Multiple diacyl lipopeptides	Mycoplasma	MyD-88/ MAL	Cell surface	Monocytes /Macrophages B lymphocytes Mast cells
TLR-7	Imidazoquinoline Bropirimine Loxoribine ssRNA	Small synthetic compounds RNA viruses	MyD-88	Cellular Compartment	Macrophages /Monocytes Plasmacytoid dendritic cells B lymphoctes
TLR-8	ssRNA	Small synthetic compounds	MyD-88	Cellular Compartment	Macrophages /Monocytes subsets of dendritic cells Mast cells
TLR-9	Unmethylated CpG Oligodeoxynucleotide DNA	Bacteria DNA viruses	MyD-88	Cellular Compartment	Monocytes /macrophages Plasmacytoid dendritic cells B lymphoctes
TLR-11	Profilin	*T. gondii*	MyD-88	Cellular Compartment	Urinary bladder epithelium Monocytes/macrophages Liver cells Kidney
TLR-12	Profilin	*T. gondii*	MyD-88		Conventional dendritic cells Plasmacytoid dendritic cells Macrophages Neurons
TLR-13	Bacterial ribosomal RNA	Bacteria, virus	MyD- 88 / TAK - 1	Cellular Compartment	Macrophages/Monocytes Conventional dendritic cells

1.3.1 TLR-4

Toll-like receptor 4 is a protein encoded by the TLR 4 gene in humans and located on chromosome 9q-32-33. The approximate length is 13 kb. It has 3 exons which encode a protein of 222 amino acids [23,24].

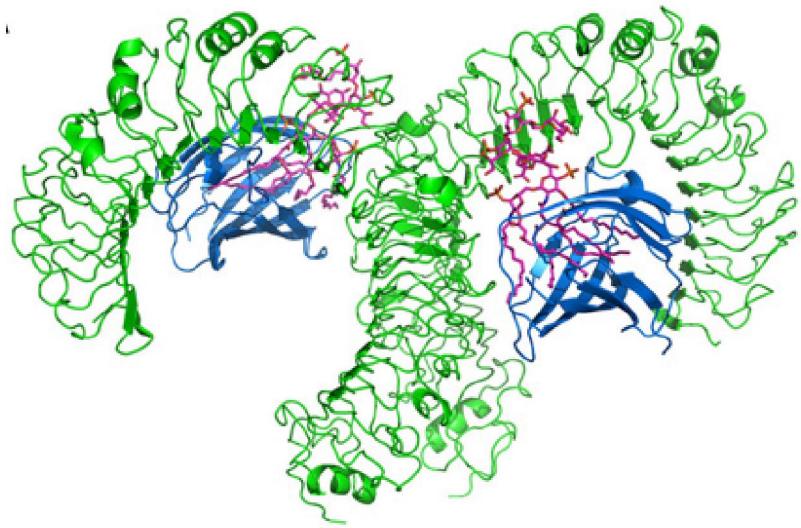

Figure 1.3 : Toll-Like 4 structure [17]

TLR 4 play a crucial role in eliciting an innate immune response to MTB infection. It is related to recognition of bacterial lipopolysaccharides (LPS). It senses lipopolysaccharide from gram-negative bacteria and allows the activation of the innate immune system [25]. TLR 4 has also been specified as CD284. It forms a complex with another LRR protein known as MD-2. Between LPS and TLR 4 there is no direct interaction, but in TLR 4–MD-2 complex MD-2 acts like a bridge for LPS-binding [26]. It was shown that other kinds of lipids also have the ability to activate it. In Africa *Plasmodium falciparum* is a common parasitic infection, may produce glycosylphosphatidylinositol, which in turn can activate TLR 4 [27]. In populations that lived in Africa, two SNPs (Thr399Ile -rs4986791- and Asp299Gly -rs4986790-) in TLR4 are co-expressed with high penetrance. TLR 4 -mediated IL-10 is an immunomodulator. The increase in the production of this immunomodulator and a decrease in proinflammatory cytokines are associated with the mentioned polymorphisms [28]. The TLR 4 Asp299Gly is a point mutation and strongly correlated with an increased infection rate with *P. falciparum.* It appears that the mutation prevents TLR 4 from acting as vigorously against, at least some plasmodial infections. The Malaria infection rate and associated morbidity are higher in the TLR 4 Asp299Gly group, but mortality appears to be decreased. This may indicate that at

least part of the pathogenesis of Malaria takes advantage of cytokine production. By reducing the cytokine production with the TLR 4 mutation, the infection rate may increase, but the number of deaths due to the infection seem to decrease [27].

1.3.2 TLR 6

Toll-like receptor 6, a protein encoded by the TLR6 gene in humans and it consists of 2391 base pairs (25). It encodes a 796 amino acid (aa) type I transmembrane protein with a 630 aa extracellular LRR region (including a 31 aa signal peptide), a 21 aa transmembrane domain and a 145 aa intracellular TIR signalling domain [29].

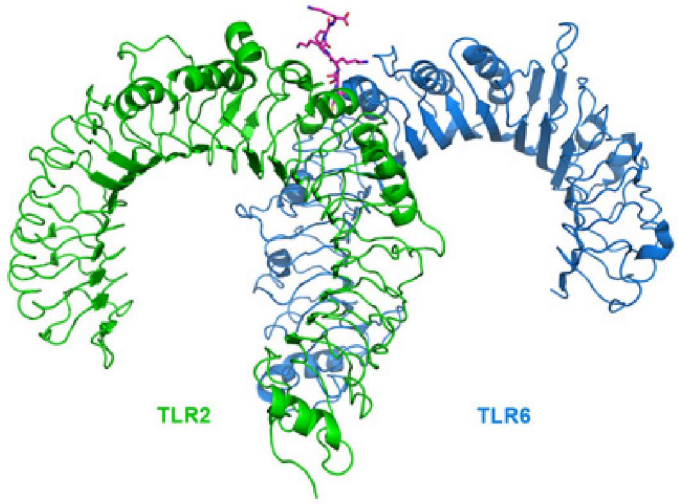

Figure 1.4: Toll-Like 2 and Toll-Like 6 (heterodimer) structure [17]

Monocytes, monocyte-derived immature dendritic cells (iDCs) and neutrophils express TLR 6 on their cell surface. It consists of an N-terminal signal peptide, nineteen tandemly repeated extracellular LRR motifs and a Toll/IL-1R homology domain in the cytoplasmic region. They recognise microbe-associated molecular patterns (MAMPs) that are expressed on infectious agents and mediate the production of cytokines necessary for the development of effective immunity. Its function has been studied mainly in mouse cells. Nuclear factor kappa-β (NF- κβ) and Jun N-terminal kinase (JNK) pathways are both activated by constitutive expression of TLR 6. Studies in human cells showed that TLR 6 and TLR 2 incorporated at the plasma membrane of monocytes. In some populations, Ser249Pro polymorphism in the extracellular domain of the encoded protein may be associated with an increased risk of asthma [30].

On the other hand, in recognition of the different pathogens specific role of human TLR 6 is less well understood than that of TLR 1 and TLR 2 [31]. A recent study evidenced that rare SNPs in TLR 6 were associated with altered NF-κβ signalling and an increased risk of tuberculosis in certain ethnic groups [32].

2 LITERATURE REVIEW

The innate immune system plays a significant role in host defence against MTB. In the first step, innate immune system cells recognise MTB. Many PPRs classes which include Toll-Like Receptors, Nod-Like Receptors and C-type lectin receptors play roles in recognising *M.tuberculosis*. At the beginning of immune response to TB, TLR 4 and TLR 6 with their adaptor molecules have the most dominant functions. In recognising *M. tuberculosis* the significance of TLR 4 was shown by the studies with murine macrophages and transfected CHO cells. TLR 6 forms heterodimers with TLR 2. Cell wall glycolipids found on mycobacteria are recognized by these heterodimers. In the light of this information, many scientists have investigated the effect of the TLR family on tuberculosis [15]. Many researchers performed genotyping on different populations, looked at the expression of the TLR family and the results were obtained accordingly. Again, many studies have applied HapMap data in different groups and have done a meta-analysis on this topic.

Takeuchi and colleagues [30] reported a new member of Toll-Like Receptor family which is TLR 6 by performing molecular cloning. Both murine and human TLR 6 have an extra-cellular an LRR domain and a cytoplasmic Toll/IL-1 receptor (IL-1R) like region and they have the structure of type-I transmembrane receptors. TLR 6 expression is detected in different tissues like thymus, lung, spleen and ovary predominantly. Active TLR 6 activates JNK and NF–κβ like other TLR family members.

Selvaraj and colleagues [33] investigated the effect of TLR 1, TLR 2, TLR 4, TLR 6, TIRAP and TLR 9 polymorphisms on PTB resistance and susceptibility in 212 healthy controls and 206 PTB patients in South India. The allele and genotype frequencies of TLR 1 polymorphism were not significantly different between PTB patients and controls. They found that there were no homozygoute genotype for the minor allel in both control and the case groups. However, alleles and genotype frequencies of TLR 4 polymorphisms were not significantly different between control and PTB patients. Related to TLR 9 polymorphisms, the frequencies of alleles and genotypes were similar in healthy subjects and patients. T allele frequency of TIRAP polymorphism was significantly high among patients compared to healthy subjects.

In India Najmi and colleagues [25] have studied 135 patients and 250 healthy controls and reported an association between TLR 4 Asp299Gly and Thr399Ile polymorphisms and susceptibility to PTB notably in the severe form of the disease.

Randhawa and colleagues [34] have reported that TLR variations are associated with altered in vivo immune responses to BCG (Bacillus Calmette-Guérin) vaccination. They investigated South African infants who were vaccinated with BCG and they found that TLR polymorphisms are associated with IL-2 or IFN-c responses induction 10 weeks after in-vivo infant BCG vaccination. They also figured out that C745T and G1083C of TLR 6 were related with an IL-6 decrease in response to lipopeptide stimulation showed by cytokine assays. Besides when transfected HEK cells were stimulated with diacylated lipopeptide or MTB cell lysate C745T variant mediated NF–κβ signalling was low. When all the data provided from these studies is examined it suggests that in innate pathway gene defects regulate pathogen related adapter responses by altering the production of BCG-induced cytokines by T cells.

Baker and colleagues [35] have studied genetic variations in Toll-like receptor genes in Ugandan and South African Populations. They carried out full-exon sequencing in 4 genes (TLR 2, TLR 4, TLR 6, and TIRAP) in the TLR pathway in samples obtained from South Africa and Uganda. They observed notable differences in haplotype frequency between the Ugandan population and the HapMap populations in TLR 2 and TLR 6. One of the important findings of the study was a group of new polymorphisms in TIRAP and TLR 6.

Many studies, in general, demonstrated the synergic effect of 2 TLR 4 gene variants (Asp299Gly & Thr399Ile) encoded within the fourth exon. Endotoxin hyporesponsive phenotype is associated with these polymorphisms. Jahantigh and colleagues [24] investigated the potential association between PTB, a TB infection of the lungs, and three SNPs in TLR 4 and TLR 9 genes in Iranian population. They have studied 124 TB patients and 149 healthy control. There was no significant association of TLR 4 variants and TLR 9 T486C polymorphisms with PTB risk.

Feruglio and colleagues [36] have investigated soluble markers of the TLR 4 pathway in various stages of TB disease and during antiTB therapy. They have studied 19 patients with active TB, 10 with PTB and 9 with extrapulmonary TB.

Since Mycobacterial cell wall components are immunogenic they are potential biomarkers.

Zhang and colleagues [37] have performed a meta-analysis of the TLR 1, TLR 2 and TLR 6 polymorphisms on PTB susceptibility. In this study, sixteen studies from fourteen papers were included in the meta-analysis. The results showed significant associations between TB risk and TLR 6 and TLR 2.

Zhao and colleagues [38] performed a meta-analysis on 16 case-control studies. In this cases, TLR 4 rs4986790 and rs4986791 and TLR 9 rs187084, rs574386, and rs352139 SNPs were studied. As a result, they haven't found an association between the polymorphisms in TLR 4 and TLR 9 and PTB risk overall, but TLR 4 rs4986791 and TLR 9 rs352139 could be associated with increased PTB risk in Africans and Asians, respectively.

Schurz and colleagues [39] conducted a meta-analysis for investigating the relationship between TLR variants and TB susceptibility. In their study 18907 individuals from 32 papers were included and 14 TLR polymorphisms data was extracted. The information related with TLR 6 was shown significant association for rs5743810. In all ethnic groups, this polymorphism was related with protection. The T allele of TLR 4 rs4986791 was also found to increase the risk of TB in the Asian subgroup. However, in the mentioned meta-analysis, other TLR variants were not found to be associated with tuberculosis.

In another meta-analysis involving 51 relevant case-control studies Liu and colleagues [40] investigated the effects of rs4986790 and rs4986791 polymorphisms of TLR 4 on sepsis susceptibility. The results of their research showed that polymorphisms they studied, no significant association with the susceptibility of sepsis was found, but both polymorphisms were related to increased risk of developing sepsis in other ethnicity subgroups under corresponding genetic models after stratified analysis by ethnicity.

Shey and colleagues [29] determined whether SNPs of TLR 6 are associated with altered immune responses to lipopeptides and whole mycobacteria. They sequenced coding region of the gene to understand the association between polymorphisms and lipopeptide-induced IL-6 production in whole blood of 100 healthy South African. They found C745T and G1083C polymorphisms were

associated with altered IL-6 secretion. As a conclusion polymorphisms of TLR 6 may take part in altered lipopeptide-induced cytokine responses and recognition of *M.tuberculosis*.

Table 2.1: Literature Summary

Study	Sample	Result	Reference	Year
TLR1, TLR2, TLR4, TLR6, TIRAP and TLR9	212 Controls 206 Patients	TLR4:No association TLR9,TIRAP: Association	Selvaraj et al.	2010
TLR4 Asp299Gly and Thr399Ile	250 Controls 135 Patients	TLR4: Association	Najmi et al.	2010
TLR genes in HapMap with different population		TLR6 and TLR2 Association in African population	Baker et al.	2012
TLR4 and TLR9 genes	149 Controls 124 Patients	TLR4 and TLR9: No association	Jahantigh et al.	2013
TLR4	19 patients	TLR4: No association	Feruglio et al.	2013
TLR 1, TLR 2, and TLR 6 in Meta-analysis	16 studies from 14 articles	TLR 1, TLR 2: association TLR 6: association	Zhang et al.	2013
TLR 4 and TLR 9 in Meta-analysis	16 case-control	TLR4: Association TLR9: No association	Zhao et al.	2015
TLR genes in Meta-analysis	32 articles	TLR2,TLR9,TLR1and TLR6: No association	Schurz et al.	2015
TLR6	100 individuals	TLR6: Association	Shey et al.	2015
TLR4 in Meta-analysis	51 case-control	TLR4: No association	Liu et al.	2016

3 MATERIALS AND METHODS

50 TB patients who are under control of Malatya Provincial Health Directorate and 50 healthy individuals are the subjects of this thesis study. The inclusion criteria of this study were being older than eighteen years of age with no known inherited diseases. Patients younger than eighteen years of age were not accepted to the study. All the patients and control subject were informed about the study by a clinician and informed consent forms were signed by all the volunteers.

3.1 DNA Isolation:

Blood samples were isolated by a commercial DNA isolation kit according to the manufacturer's protocol (Qiagen DNA mini kit). This protocol was including four basic steps; blood lysate, binding DNA, washing DNA and eluting DNA.

The steps of the protocol were as the following:

1. 20µl proteinase K was pipetted into the bottom of a sterile 1.5 ml microcentrifuge tube and 200 µl whole blood sample was added to the microcentrifuge tube.
2. 200 µl Buffer AL was added to the sample and it was mixed well thoroughly to yield a homogeneous solution by pulse vortexing for 15 seconds.
3. The sample was incubated at 56°C for 10 minutes.
4. After incubation, the tubes were centrifuged briefly to remove drops from the inside of the lid of microcentrifuge tube.
5. 200 µl ethanol (96–100%) was added to the sample and mixed again by pulse vortexing for 15 seconds.
6. After mixing, the tubes were centrifuge briefly to remove drops from the inside of the lid.
7. The mixture from blood lysate was carefully applied to the QIAamp Mini Spin column (in a 2 ml collection tube) without wetting the rim.
8. The cap was closed and it was centrifuged at 6000 x g (8000 rpm) for 1 min.
9. The spin column was placed in a clean 2 ml collection tube and the tube containing filtrate was discarded.
10. Spin column was opened carefully and 500 µl Buffer AW1 was added after that the cap was closed and the tube was centrifuged at 6000 x g (8000 rpm) for 1 min.

11. Spin column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded.
12. Spin column was opened carefully and 500 µl Buffer AW2 was added without wetting the rim.
13. The cap was closed and centrifuged at full speed (20,000 x g;14,000 rpm) for 3 minutes.
14. Spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded.
15. Spin column was opened carefully and 150 µl Buffer AE or distilled water was added and it was incubated at room temperature (15–25°C) for 1 min, and then centrifuged at 6000 x g (8000 rpm) for 1 minute.
16. In a second elution step 50 µl Buffer AE or distilled water was added and incubated at room temperature (15–25°C) for 1 min, and then it was centrifuged at 6000 x g (8000 rpm) for 1 minute.
17. The obtained DNA was stored at +4 ^{0}C.

3.2 Measuring DNA concentration by Qubit Assay Kit

DNA concentrations were measured by using "Qubit Fluorometer". In order to measure the concentration dsDNA BR Qubit® (Broad-Range) Assay Kit was used. For optimal performance, the kit components and the DNA samples were equilibrated at room temperature (22–28°C).

The steps of the protocol were as the following:

1. The required number of 0.5-mL tubes were set up for standards and samples. The Qubit® dsDNA BR Assay requires 2 standards.
2. The tubes were labelled.
3. Working solution was prepared by diluting the Qubit® dsDNA BR Reagent 1:200 in Qubit® dsDNA BR Buffer.
4. 190 µL of Qubit® working solution was added to each of the tubes used for standards.
5. 10 µL of each Qubit® standard was added to the appropriate tube, then was mixed without creating bubbles by vortexing 2–3 seconds.
6. All tubes were incubated at room temperature for 2 minutes.
7. The tube containing the Standards were measured.

8. When the calibration was completed, the instrument displays the Sample screen.
9. For the DNA samples; 198µl Qubit® working solution was added to individual assay and was added 2 µl DNA sample.
10. All tubes were vortexed without creating bubbles and the samples were incubated 2 minutes at room temperature.
11. The sample tubes were measured.

3.3 Genotyping

DNA genotyping was done by using StepOnePlus™ Real-Time PCR System. In order to do genotyping TaqMan SNP Genotyping Assay kits were used. General PCR amplification process requires were as the following:

• Prepare the reaction mix

• Add the reaction mix to the prepared DNA reaction plate

• Perform PCR

To prepare a plate:

1. Each DNA sample was diluted with DNase - free water to get a final DNA mass of 10 ng per well.
2. All wells belonging to the same assay must contain the same amount of sample or control and were prepared a 96 - well reaction plate, DNA sample and DNase-free water per reaction were 11.25µl.
3. The samples were pipetted into each well of the 96-well optical reaction plate.

During the first step of a Genotyping Assay experiment, AmpliTaq Gold® DNA polymerase amplifies target DNA using sequence - specific primers. TaqMan MGB probes from the SNP Genotyping Assay provide a fluorescence signal for the amplification of each allele.

For each reaction following components were used:

- TaqMan Universal PCR Master Mix (2x), ...12.50µl
- 40x working stock of SNP Genotyping Assay.....1.25 µl

1. PCR Master Mix (2X) was swirled gently.
2. The 40X SNP Genotyping Assay was vortexed and centrifuged briefly.
3. The 40X SNP Genotyping Assay was diluted to 20X working stock with 1X TE buffer.
4. The required total volumes of PCR Master Mix (2X) and 20X SNP Genotyping Assay were pipetted into a sterile microcentrifuge tube.
5. The tube was capped and inverted several times to mix.
6. The tube was centrifuged briefly to spin down the contents and was to eliminate any air bubbles from the reaction mix.

To add the reaction mix to the prepared DNA reaction plate:

1. Into each well of DNA reaction plate reaction mix was pipetted.
2. Inspect all the wells for uniformity of volume, and note which wells do not appear to contain the proper volume.
3. The plate was sealed with the appropriate cover.
4. The plate was vortexed to mix the wells.
5. The plate was centrifuged briefly to spin down the contents and was eliminated any air bubbles.
6. The plate was run on a StepOnePlus™ Real-Time PCR System was using the following thermal cycling conditions:

- 95^0C 10 minutes
- 92^0C 15 seconds
  60^0C 1 minutes
  } 40 cycles

3.4 Statistical Analysis

For the data were summarized by count and percent. Hardy-Weinberg equilibrium was tested by a chi-square distribution with 1 df. Differences between groups due to allelic and genotypic distributions were analysed by Pearson's exact or Fisher's exact tests. In all comparisons, the significance level was considered to be $\leq$ 0,05.

4 RESULTS

50 TB patients and 50 healthy individuals were the subjects of this study. Two SNPs of TLR 4 - rs4986790, rs4986791- and one SNP of TLR 6 -rs3821985- were investigated and no significant association was found between the disease susceptibility and the genotype frequencies of the SNPs that were mentioned. The results of the genotyping for each SNPs investigated were summarized in tables.

4.1 Genotyping of rs4986790

50 patients and 50 control were screened for rs4986790. According to the statistical results between patients and controls for rs4986790; 46 (92,0%) AA, 4 (8,0%) AG genotype were found in patients but GG genotype was not found. 44 (88,0%) genotype AA, 5 (10,0%) genotype AG and 1 (2%) GG genotype were found in controls. According to the Hardy-Weinberg equilibrium, the ratio in the patients is 0,101 and the ratio in the control group is 0,768 (table:4.1). The allele frequencies for rs4986790 in controls were 93 (93%) for A and 7 (7%) for G alleles; in patients, as follows: 96 (96%) for A, and 4 (4%) for G alleles (Table:4.1). When the comparison between male and female was made we reached the following results; 32 (88,9%) genotype AA, 4 (11,1%) AG genotype were found in female but GG genotype was not found. 58 (90.6%) genotype AA, 5 (7,8%) AG genotype, and 1 (1.6%) GG genotype found in male. The allele frequencies in female were 68 (94,4%) for A and 4 (5,6%) for G alleles; in male as follows; 121 (94,5%) for A, and 7 (5,5%) for G alleles (Table:4.2). In a female group, the frequencies of the genotype and alleles were compared between patients and healthy controls. 12 (92.3%) genotype AA, 1 (7.7%) AG genotype was found in female controls but GG genotype was not found. 20 (87.0%) genotype AA, 3 (13.0%) genotype AG and GG genotype were not found in female patients. The allele frequencies in female controls were 25 (96,2%) for A and 1 (3,8%) for G alleles; in female patients as follows; 43 (93,5%) for A, and 3 (6,5%) for G alleles (Table:4.2). And also for the male patients and control; 26 (96.3%) genotype AA, 1 (3.7 %) AG genotype were found in male patients but GG genotype was not found. 32 (86.5%) AA, 4 (10.8%) genotype AG and 1 (2.7%) GG genotype were found in male controls. The allele frequencies in male controls were 68 (91,9%) for A and 6 (8.1%) for G alleles; in male patients, as follows: 53 (98,1%) for A, and 1 (1.9%) for G alleles (Table:4.2). The result of the genotyping assay was shown in allelic discrimination plot in figure 4.1.

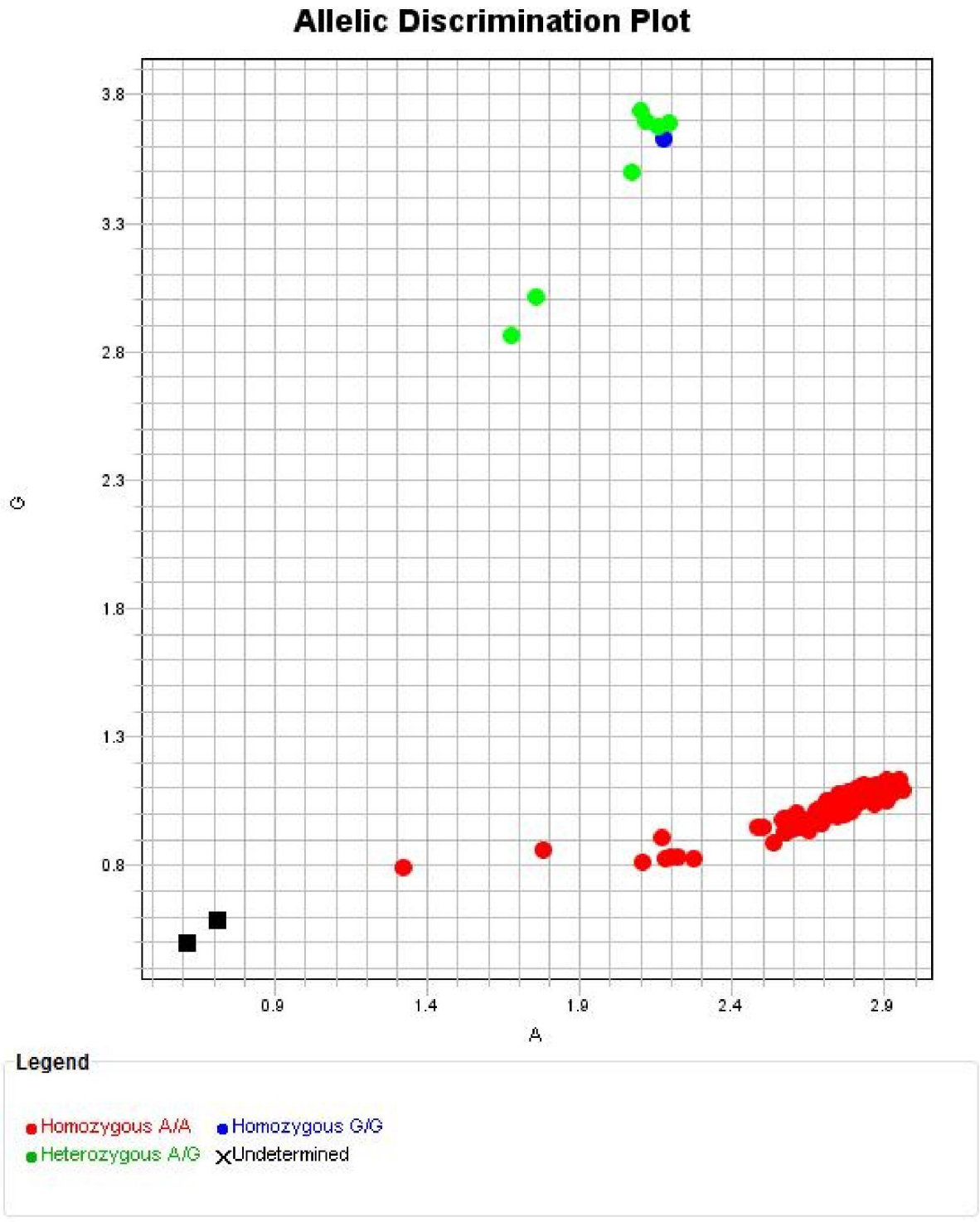

Figure 4.1: TLR 4; rs4986790 Allelic Discrimination Plot for patients and controls

Table:4.1 rs4986790 Genotype and allele frequency table for patients and controls

Group	AA	AG	GG	p	Hardy-Weinberg	A	G	p
	n (%)	n (%)	n (%)		p	n (%)	n (%)	
Control	44 (88,0)	5 (10,0)	1 (2,0)	0,741	0,101	93(93,0)	7(7,0)	0,535
Patients	46 (92,0)	4 (8,0)	0 (0,0)		0,768	96(96,0)	4(4,0)	

Table:4.2 Distribution of rs4986790 genotype and allele frequencies according to gender

Gender	AA	AG	GG	p	A	G	
	n (%)	n (%)	n (%)		n (%)	n (%)	p
Female	32(88,9)	4(11,1)	0(0,0)	0,821	68(94,4)	4(5,6)	1,000
Male	58(90,6)	5(7,8)	1(1,6)		121(94,5)	7(5,5)	
Female	AA	AG	GG	p	A	G	p
	n (%)	n (%)	n (%)		n (%)	n (%)	
Control	12(92,3)	1(7,7)	0(0,0)	1,000	25 (96,2)	1 (3,8)	1,000
Patients	20(87,0)	3(13,0)	0(0,0)		43 (93,5)	3 (6,5)	
Male	AA	AG	GG	p	A	G	
	n (%)	n (%)	n (%)		n (%)	n (%)	p
Control	32(86,5)	4(10,8)	1(2,7)	0,491	68 (91,9)	6 (8,1)	0,237
Patients	26(96,3)	1(3,7)	0(0,0)		53 (98,1)	1 (1,9)	

4.2 Genotyping of rs4986791

According to the statistical results between patients and controls for rs4986791; 50 patients and 50 control were screened for rs4986791; 46 (92,0%) CC, 4 (8,0%) CT genotype were found in patients but TT genotype was not found. 45 (90,0%) genotype CC, 5 (10,0%) genotype CT but TT genotype was not found in controls. According to the Hardy-Weinberg equilibrium, the ratio in the patients is 0,710 and the ratio in the control group is 0,768 (table:4.3).

The allele frequencies for rs4986791 in controls were 95 (95%) for C and 5 (5%) for T alleles; in patients, it was as follows; 96 (96%) for C, and 4 (4%) for T alleles (Table:4.3). When the comparison between male and female was made we reached the following results; 30 (90,9%) genotype CC, 3 (9,1%) CT genotype were found but TT genotype was not found in female. 61 (91.0%) genotype CC, 6 (9,0%) CT genotype were and TT genotype was not found in the male. The allele frequencies in female were 63 (95,5%) for C and 3 (4,5%) for T alleles; in male it was as follows: 128 (95,5%) for C, and 6 (4,5%) for T alleles (Table:4.4).

In the female group, the frequencies of the genotype and alleles were compared between patients and healthy controls. 11 (91.7%) genotype CC, 1 (8.3%) CT genotype were found in female controls but TT genotype was not found. 19 (90.5%) genotype CC, 2 (9.1%) genotype CT were found and TT genotype was not found in female patients. The allele frequencies in female controls were 23 (95,8%) for C and 1 (4,2%) for T alleles; in female patients as follows: 40 (95,2%) for C, and 2 (4,8%) for T alleles (Table:4.4).

And also for the male patients and control; 27 (93.1%) genotype CC, 2 (6.9%) CT genotype were found in male patients but TT genotype was not found. 34 (89.5%) genotype CC, 4 (10.5%) genotype CT were found and TT genotype was not found in male controls. The allele frequencies in male controls were 72 (94,7%) for C and 4 (5.3%) for T alleles; in male patients, as follows: 56 (96,6%) for C, and 2 (3.4%) for T alleles (Table:4.4). The result of the genotyping assay was shown in allelic discrimination plot in figure 4.2.

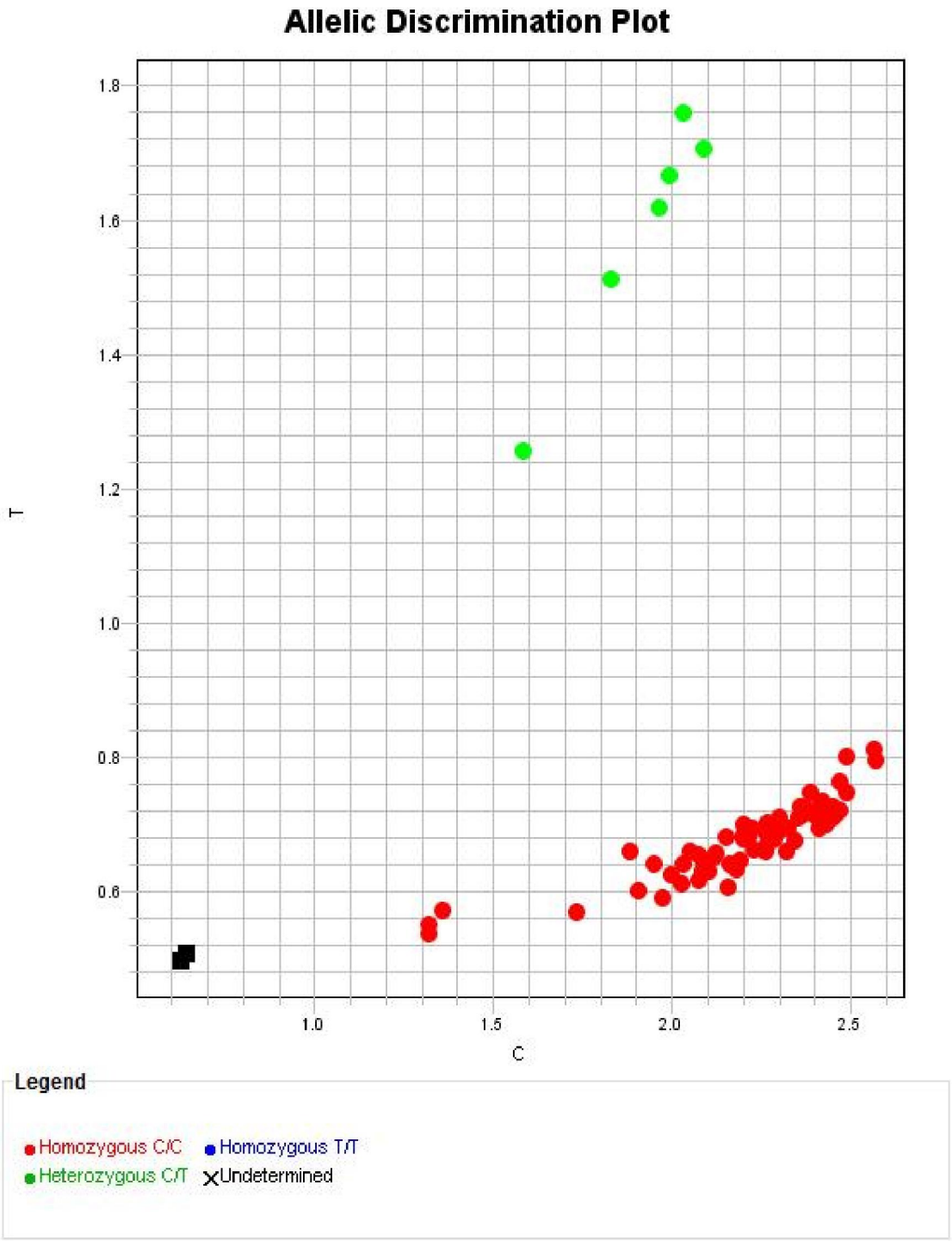

Figure 4.2: TLR 4; rs4986791 Allelic Discrimination Plot for patients and controls

Table:4.3 rs4986791 Genotype and allele frequency table for patients and controls

Group	CC	CT	TT	p	Hardy-Weinberg	C	T	p
	n (%)	n (%)	n (%)		p	n (%)	n (%)	
Control	45 (90,0)	5 (10,0)	0 (0,0)	1,000	0,768	95(95,0)	5(5,0)	1,000
Patient	46(92,0)	4 (8,0)	0 (0,0)		0,710	96(96,0)	4(4,0)	

Table:4.4 Distribution of rs4986791 genotype and allele frequencies according to gender

Gender	CC	CT	TT	p	C	T	p
	n (%)	n (%)	n (%)		n (%)	n (%)	
Female	30(90,9)	3(9,1)	0 (0,0)	1,000	63(95,5)	3(4,5)	1,000
Male	61(91,0)	6(9,0)	0 (0,0)		128(95,5)	6(4,5)	
Female	CC	CT	TT	p	C	T	p
	n (%)	n (%)	n (%)		n (%)	n (%)	
Control	11(91,7)	1(8,3)	0 (0,0)	1,000	23 (95,8)	1 (4,2)	1,000
Patients	19(90,5)	2(9,1)	0 (0,0)		40 (95,2)	2 (4,8)	
Male	CC	CT	TT	p	C	T	p
	n (%)	n (%)	n (%)		n (%)	n (%)	
Control	34(89,5)	4(10,5)	0 (0,0)	0,691	72 (94,7)	4 (5,3)	0,698
Patients	27(93,1)	2(6,9)	0 (0,0)		56 (96,6)	2 (3,4)	

4.3 Genotyping of rs3821985

Amplification has not been performed because of the low concentration of DNA in some individuals. For this reason, 48 individuals from 50 tuberculosis patients and 50 controls were genotyped for TLR6 rs3821985. As we can see the allelic discrimination plot Figure: 4.3.

According to the statistical results between patients and controls for rs3821985 in the table:4.5; 5 (10.0%) CC, 18 (36.0%) CG genotype were found in patients and 27 (54.0%) GG genotype was found. 9 (18,8%) CC, 18 (37.5%) genotype CG and 21 (43,8%) GG genotype were found in controls (Table:4.5). According to the Hardy-Weinberg equilibrium, the ratio in the patients is 0,449 and the ratio in the control group is 0,166 (table:4.5).

The allele frequencies for rs3821985 in controls were 36 (37,5%) for C and 60 (62,5%) for G alleles; in patients as follows: 28 (28,0%) for C, and 72 (72,0%) for G alleles (Table:4.5). When the comparison between male and female was made we reached the following results: 5 (12,8%) genotype CC, 12 (30,8%) CG genotype were found and 22 (56,4%) GG genotype was found in the female. 9 (15,3%) genotype CC, 24 (40,7%) CG genotype were found and 26 (44,1%) GG genotype was found in male group. The allele frequencies in females were 22 (28.2%) for C and 56 (71,8%) for G alleles; in males, as follows: 42 (35,6%) for A, and 76 (64,4%) for G alleles (Table:4.6).

In the female group, the frequencies of the genotype and alleles were compared between patients and healthy controls. 5 (29.4%) genotype CC, 3 (17.6%) CG genotype were found in female controls and also 9 (52,9%) GG genotype was found. 13 (59.1%) genotype GG, 9 (40.9%) genotype CG and CC genotype was not found in female patients. The allele frequencies in female controls were 13 (38.2%) for C and 21 (61,8%) for G alleles; in female patients as follows: 9 (20,5%) for A, and 35 (79,5%) for G alleles (Table:4.6). And also for the male patients and control; 5 (17,9%) genotype CC, 9 (32,1%) CG genotype were found in male patients and 14 (50,0) GG genotype was found. 4 (12.9%) genotype CC, 15 (48.4%) genotype CG and 12 (38.7%) GG genotype was found male controls. The allele frequencies in male controls were 23 (37.1%) for C and 39 (62.9%) for G alleles; in male patients, as follows: 19 (33,9%) for C, and 37 (66.1%) for G alleles (Table:4.6).

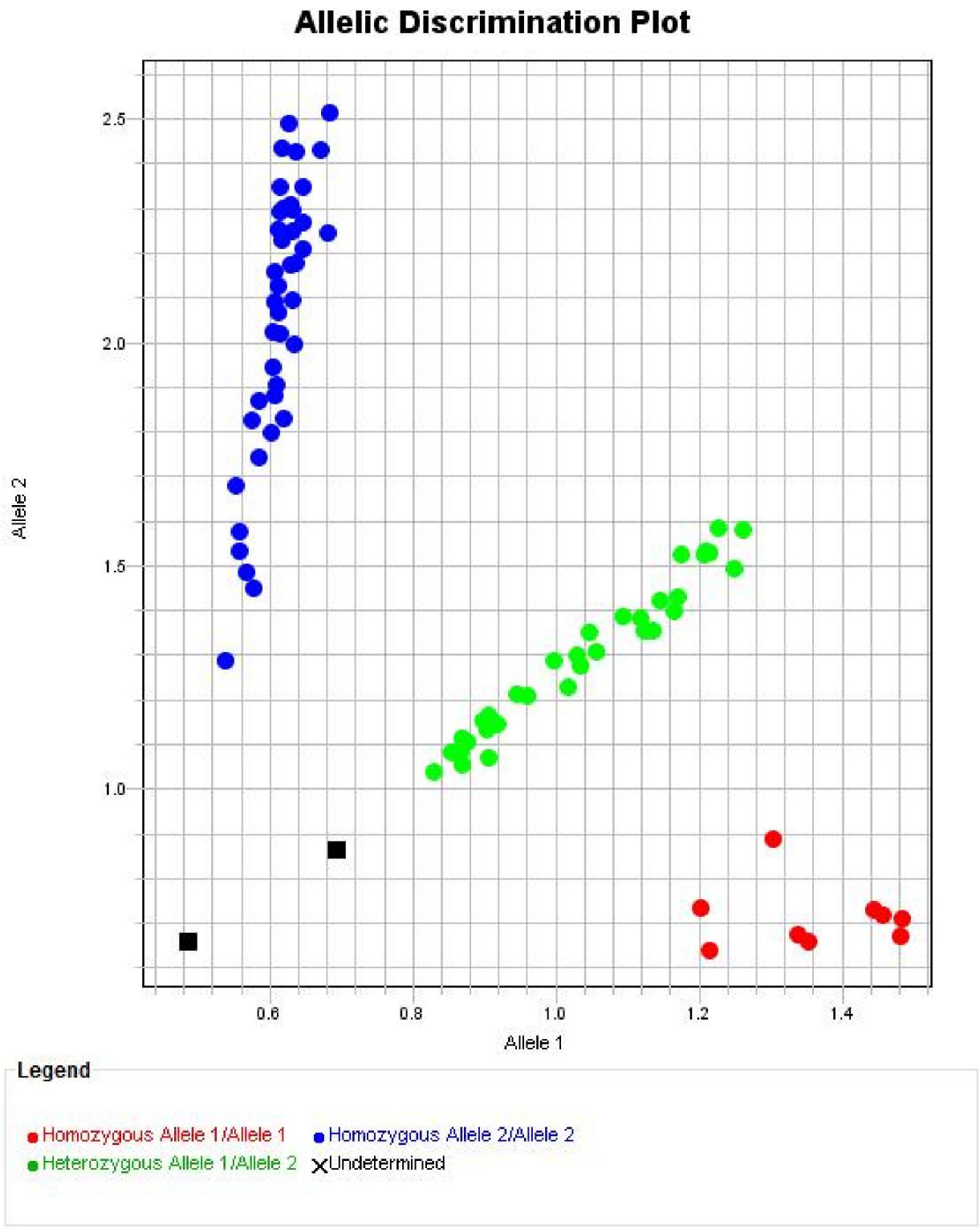

Figure 4.3: TLR6; rs3821985 Allelic Discrimination Plot for patients and controls

Table:4.5 rs3821985 Genotype and allele frequency for patients and controls table

Group	CC	CG	GG	p	Hardy-Weinberg	C	G	p
	n (%)	n (%)	n (%)		p	n (%)	n (%)	
Control	9 (18,8)	18 (37,5)	21 (43,8)	0,396	0,166	36(37,5)	60(62,5)	0,156
Patient	5 (10,0)	18 (36,0)	27 (54,0)		0,449	28(28,0)	72(72,0)	

Table:4.6 Distribution rs3821985 genotype and allele frequencies according to gender

Gender	CC	CG	GG	p	C	G	
	n (%)	n (%)	n (%)		n (%)	n (%)	p
Female	5(12,8)	12(30,8)	22(56,4)	0,483	22(28,2)	56(71,8)	0,355
Male	9(15,3)	24(40,7)	26(44,1)		42(35,6)	76(64,4)	
Female	CC	CG	GG	p	C	G	p
	n (%)	n (%)	n (%)		n (%)	n (%)	
Control	5(29,4)	3(17,6)	9(52,9)	0,017	13 (38,2)	21 (61,8)	0,140
Patient	0(0,0)	9(40,9)	13(59,1)		9 (20,5)	35 (79,5)	
Male	CC	CG	GG	p	C	G	
	n (%)	n (%)	n (%)		n (%)	n (%)	p
Control	4(12,9)	15(48,4)	12(38,7)	0,499	23 (37,1)	39 (62,9)	0,868
Patient	5(17,9)	9(32,1)	14(50,0)		19 (33,9)	37 (66,1)	

5 DISCUSSION AND CONCLUSION

Tuberculosis is an infectious disease that is caused by intra-cellular pathogen *Mycobacterium tuberculosis*. About 30% of the world's populations is carrying the pathogen and at least one tenth of it develops the active form of the disease. In the Global Tuberculosis 2014 Report, the incidence rate of Turkey in 2013 was 0.02%, the prevalence rate was 0.023% and the TB mortality rate was 0.00042%. According to Turkey Tuberculosis War 2015 report; case numbers decreased from 212 to 117 cases in seven years (2006-2013) in Malatya [41].

The molecular mechanisms how *M.tuberculosis* cause infection are unclear that is why it is difficult to fight the disease with effective therapies or design strategies for prevention. It is still unknown why some carriers are developing the active form of the disease and the others not. Previous studies showed that at least some infected people exhibit a powerful immune response to the infectious agent and this determines the end result of the disease [15]. At the focus of the start point of innate immune response "pathogen-associated molecular patterns" (PAMPs) are found. These are recognised by "pathogen recognition receptor" (PRRs) that are found on cell surfaces or intra-cellular compartments or they are patrolling in both tissue fluids and bloodstream. By recognition of PAMPs by these PRRs host immune response is started and regulated [42]. Recognation of *"M.tuberculosis"* is accomplished by PRRs like "Toll-Like Receptors" (TLR), "Nucleotide oligomerization domain-like receptors" (NLR) and "C-type Lectin receptor" in the host.

Since tuberculosis has a high frequency in Malatya, we aimed to investigate the role of TLR polymorphisms in TB. In we try to figure out if some TLR polymorphisms are associated with the susceptibility to tuberculosis in patients who are living in Malatya.

TLR polymorphisms have shown a great impact on susceptibility to TB. Members of the populations who has the certain genotype for TLRs may have affinity differences to *M. tuberculosis* ligands, therefore this may lead variations in signal transduction [42].

Toll-like receptors are transmembrane proteins that induce a natural immune response to many pathogens. Twelve TLRs have been described to date. The

mycobacterial ligands recognised by TLRs are lipoarabinomannan, lipomannan, phosphatidylinositol mannose and the 19-kDa lipoprotein. After recognition of these ligands by receptors, the TLR signal pathway is activated by binding the TIR domain to MyD88 adapter protein. IRAK-1, Toll/IL-1 receptor domain-containing adapter protein and TIR-domain-containing adapter-inducing IFN-β adapter protein than the participate in activation of the mitogen-activated protein kinase and nuclear factor-B in the nucleus. Increasing levels of inflammatory cytokines, especially TNF-α, then initiate the natural immune response to bacteria [43-45].

We have examined rs4986790, rs4986791 (TLR 4) and rs3821985 (TLR 6) SNPs of toll-like 4 and toll-like 6 receptors which we think are related to tuberculosis. As a result of our study, although we have found differences in the frequencies it wasn't significant statistically. The main limitation was the number of patients we had.

The number of patients was so low. Compared to the studies with other populations, it is seen that patient numbers are suitable to examine statistically in studies with linkages. In addition, there are many different molecules in the host-pathogen associations as well as TLRs in the immune system.

Many studies showed the critical role of TLR 4 in recognition of MTB. Jahantigh et al. investigated of the mutant alleles of TLR 4 Asp299Gly (rs4986790) and Thr399Ile (rs4986791) polymorphisms which were the same ones we investigated. They couldn't find an association with pulmonary tuberculosis susceptibility as well. In their study, they had 124 subjects and 149 controls [24].

As a result of the meta-analysis that Schurz and colleagues performed on TLR 1, 2, 4, 6 and 9 variants, TLR 1 rs4833095 have been shown to be associated with resistance to TB. It results in an asparagine to serine change. This affects the folding of TLRs and its binding efficiency to its ligand and also it destroys the ability of heterodimer formation with TLR 2. TLR 2 forms heterodimers with TLR 1 and TLR6. By this way, they recognise various ligands so if the polymorphisms cause defective TLR 2 activation it can affect multiple PRRs and cause a compound negative effect on immune response.

In Asian population study susceptibility to TB was associated with TLR 4 rs4986791 [39]. Contrary to our study Najmi et al. have studied TLR 4

polymorphisms in the Asian Indian population with 135 PTB patients and 250 healthy individuals and in the patients, it was observed that TLR 4 Asp299Gly mutation has a significantly high frequency [25].

Wu and colleagues used TLRs as marker on the population of China. They suggested that TLR 2, 4, 8 and 9 play a role in the activation of TLR pathways in the activation of TB immunity and natural immunity [46]. DeFranco and colleagues suggested that Thr399Ile mutation could alter the extracellular domain of the TLR 4 structure, which in turn may modulate the interaction of ligands with TLR 4. This may lead impaired immune response [47].

On the other hand the reason of having no association between tuberculosis and SNPs we and others investigated it should be taken into account that there are many molecules taking part in the signalling pathway of TLRs. And there are many candidate genes to search for the associations. The most studied gene is the "solute carrier family 11 members 1 gene" (SLC11A1) at Chr2q35, which is more popularly known as the "natural resistance-associated Macrophage Protein 1" gene (NRAMP1) [48]. Also in infection diseases the "histocompatibility leukocyte antigen" (HLA) genes at Chr6p21.3 play important roles [49]. IFN-γ receptor genes mutations are specific and lethal for mycobacterial infection. In the other studies IL-12, IL-10 and TNF-α polymorphisms were also shown to be associated with tuberculosis susceptibility [50]. Besides vitamin D mediate innate immunity against *M. tuberculosis* infections. In literature, many studies report the associations of vitamin D gene variations and tuberculosis [51,52].

In PubMed database, there are limited numbers of published reports related to the TLR 6 and TLR 4 and tuberculosis associations. There are 25 papers related to TLR 6 and TB association and 37 papers related with TLR 4 and TB association when search with the keywords TLR 6 polymorphisms and tuberculosis and TLR 4 polymorphisms and tuberculosis respectively. None of these reports are from Turkey. So this study can be accepted as an initial study for our population. Since tuberculosis is an important public health issue in our country the number of these studies with increased numbers of patients and controls should contribute to the literature in the area.

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7 CURRICULUM VITAE

Name Surname	: Refika Dilara VAİZOĞLU
Date and Place of Birth	: 12.08.1990 Malatya/ TURKEY
Adress	: Inonu University, Institute of Science, Department of Molecular Biology and Genetics, 44280 Malatya
E-mail	: dilaravaizoglu@hotmail.com
Undergraduate	: Inonu University, Department of Biology, (2009-2013)

Graduate:

Inonu University, Institute of Science, Molecular Biology Section (2013-)

Work Experiences:

Biology Teacher, Yeni Açılım-Bil private teaching institution(2014-2015)

Biology Teacher, Benim Kütüphanem private teaching institution (2015-2016)

Biologist, AVİSA Veterinary Clinic (2015-2017)

Publications:

- Acar, C., Kurt, A. Giray.,Vaizoğlu, R.Dilara., Gözükara, G.Harika., Arıca, Z., Durmaz, B., Kayabaş, Ü., Otlu, B., (2015) Apo E Genotyping İn Tuberculosis Patients From Malatya. Turgut Özal Tıp Merkezi Dergisi, 22 (3).180-183 ss
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