Polymorphisms in NRAMP1 and MBL2 genes and their relations with tuberculosis in Turkish children

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ÖZET

Çocukluk çağı tüberkülozunda genetik yatkınlık

Bu çalışmada Çukurova Üniversitesi Çocuk Sağlığı ve Hastalıkları servisinde yatarak veya polikliniğinde ayaktan takip altında olan veya yeni tanı alan, 0-18 yaş arası pediatrik tüberküloz tanısı almış hasta grubunun, kontrol grubuna oranla tüberküloza genetik yatkınlığının belirlenmesi amaçlanmıştır. 1996-2009 yılları arasında Çukurova Üniversitesi Çocuk Sağlığı ve Hastalıkları servisinde yatarak veya polikliniğinde ayaktan takip altında olan veya yeni tanı alan, 0-18 yaş arası pediatrik tüberküloz tanısı almış 50 olgu hasta grubu, altta yatan herhangi bir kronik hastalığı ve akut hastalık tablosu söz konusu olmayan, daha önceden tüberküloz temas öyküsü bulunmayan, sağlıklı 0-18 yaş arası bireylerden seçilen 50 olgu kontrol grubu olarak belirlendi. NRAMP1 ve MBL gen polimorfizmlerinin belirlenmesi için hasta ve kontrol grubundaki bireylerden 4'er cc periferik venöz kan örneği alınarak Çukurova Üniversitesi, Tıp Fakültesi, Tıbbi Biyoloji Anabilim Dalı genetik laboratuvarına analiz için gönderildi. Elde edilen verilerle; Çukurova Üniversitesi, Tıp Fakültesi Biyoistatistik Anabilim Dalında istatistiksel analiz yapıldı. NRAMP1 genin sık görülen polimorfizmlerinden; D543N, 3'-UTR ve INT4 polimorfizmleri açısından hasta grubu ve kontrol grubu olgular arasında istatistiksel bir farklılık saptanmamıştır. MBL geninin sık görülen polimorfizmlerinden KODON 54 ve KODON 57 polimorfizmleri açısından hasta grubu ve kontrol grubu arasında istatistiksel bir farklılık saptanmamıştır. Bu değerler göz önünde bulundurulduğunda her iki grup arasında; NRAMP1 ve MBL gen polimorfizmleri açısından istatistiksel açıdan belirgin farklılık saptanmamıştır. Bu çalışmada hasta grubu ve kontrol grubu arasında NRAMP1 ve MBL gen polimorfizmleri açısından belirgin istatistiksel farklılık saptanmamıştır. Literatürdeki diğer benzer çalışmalardaki pozitif sonuçlar; bu çalışmalardaki olgu sayısı yüksekliği ya da sosyoekonomik, ırksal, çevresel ve coğrafi faktörlerin farklılığını düşündürmektedir. Bu açıdan özellikle olgu sayısının artırılması ve bu etkenlerin daha spesifiye edilebilmesi açısından çalışmanın devamına karar verilmiştir.

Anahtar Kelimeler: *Pediatrik tüberküloz, genetik yatkınlık*

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SUMMARY

Polymorphisms in NRAMP1 and MBL2 genes and their relations with tuberculosis in Turkish children

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In this study, we aimed to determine genetic susceptibility of children group who are under follow up at outpatient and inpatient clinics or newly diagnosed pediatric tuberculosis according to healthy control group. Patient group consists of 50 cases aged between 0-18 years who are under follow up at outpatient and inpatient clinics or newly diagnosed pediatric tuberculosis between 1996-2009 in Cukurova University, Faculty of Medicine, Department of Pediatrics and the control group consists of 50 healthy cases aged between 0-18 years who have neither chronic nor acute diseases and have no history of tuberculosis contact. Analysis of NRAMP1 (D543N, 3'-UTR and INT4 loci) and MBL (codon 54 and 57) gene polymorphisms carried out in Cukurova University, Faculty of Medicine, Department of Medical Biology and Genetics. In this study comprising in total 50 individuals we did not observe any significant association with microsatellite polymorphisms at the INT4, G543A and 3-UTR loci situated in the NRAMP1 gene (p> 0.005). There was no significant difference of MBL gen frequency polimorphisms of codon 54 and 57 polimorphisms between patient and control group statistically (p> 0.05). We reported that the INT4, G543A and 3-UTR loci microsatellite polymorphisms in the NRAMP1 gene were not associated with tuberculosis. No significant associations were also observed for codons 54 and 57 in the MBL2 gene. These results shed light on the role of NRAMP1 in susceptibility to tuberculosis disease and provide a plausible explanation for NRAMP1 and MBL genetic heterogeneity in tuberculosis susceptibility.

Key Words: *Pediatric tuberculosis, genetic susceptibility, polymorphism, NRAMP1 and MBL2 genes.*

Tuberculosis (TB) remains a leading public health problem worldwide, and the global incidence of ıt is rising, with ~ 8.8 million new cases and 2 million deaths each year (1). It is known that genetic and nongenetic factors of both the bacterium and the host have impact on the host response to *Mycobacterium tuberculosis*. Analysis of the genetic basis of susceptibility to major infectious diseases is a potentially complex area. Recent work suggests that in addition to common host susceptibility genes, a second group of susceptibility loci exists the action of which strongly depends on the individual's clinical and exposure history. These findings suggest that a more detailed knowledge of gene-environment interactions in TB is necessary to understand why a small proportion of individuals are susceptible to the disease while the majority of humans are naturally resistant to TB. More genetic studies have focused on adult than on childhood TB but with less success. It is likely that host susceptibility to TB is at least partly under polygenic control. Many lines of evidence support an important role of host genetic variation in TB susceptibility, including animal models of the disease, ethnic clustering of tuberculosis cases, increased concordance rates of tuberculosis among monozygotic vs. dizygotic twins, evidence that certain gene variants are associated or linked with increased risk of TB (2-11).

Polymorphisms in the natural resistance-associated macrophage protein gene 1 (NRAMP1) have been found in a number of genetic studies to be risk factors for the development of TB among adult populations (12). This gene has been shown to be a critical element in the regulation of intracellular membrane vesicle trafficing of macrophages (13). The NRAMP1 region was found to be linked with TB during an outbreak in a Canadian aboriginal community, have been also associated with TB susceptibility in populations from Gambia, Guinea-Conakry, Korea, Brasil and Japan, and these findings have been replicated in some, but not all, case-control studies of human TB (14-19). NRAMP1 does not appear to affect susceptibility to *M. tuberculosis* in mice (20). However, although this and other studies suggest that complex human genetic factors (NRAMP1alleles in particular) may be involved in susceptibility to pulmonary tuberculosis in adults, the associations are weak, and causal relationships between genotypes and phenotypes have not been demonstrated.

Mannose binding lectin is a type of collectine protein with 96 ka of molecular weight and synthesis by liver. Collectine member proteins are encoded on 10. chromosomes short arm (3). MBL acts like an antibody by its ability to bind most of the sugar containing molecules. The mammalians have low density sugars so MBL do not able to bind these structures. After binding to bacteria, MBL coats bacterial surface and let the phagocyte connect easily. As a result the bacteria have been destroyed intracellulary. In this respect MBL is a protein that acts as an opsonin. MBL interacts with the immune system by acting as an opsonin to promote phagocytosis and by activating the complement cascade. Polymorphisms in the first exon of MBL and in its promoter region result in a phenotype of low serum MBL levels, which cause an increased risk of infections (21-23). Bellamy has shown that MBL polymorphisms are protective against TB in a West African community, but this observation was not repeated in a study in India, which suggested the opposite (24,25). Low levels of functional serum MBL are caused by 3 variant alleles (codon 54, 57 and 52, respectively) in exon 1, causing amino acid changes that disrupt the collagenous backbone of the MBL molecule, leading to a dysfunctional protein (23). Each of the three variants reduces the amount of functional high molecular MBL in heterozygous individuals 5-10 times, while high molecular weight MBL is absent in variant allele homozygotes. These alleles are very common, and up to 35-40% of the Caucasian population are carriers (26). Heterozygous individuals for these mutations have a substantial decrease in MBL serum concentrations whereas MBL is undetectable in the serum of homozygous individuals (17,27). The codon 54 mutation occurs in 22-28% of Eurasian populations, whereas the codon 57 mutation is characteristic of sub-Saharan African populations in whom it reaches frequencies of 50-60% (28). To investigate the role of NRAMP1 and MBL gene polymorphisms in TB susceptibility, we focused our genetic analysis on pediatric cases with primary TB disease.

MATERIALS and METHODS

Study Population

Patient group consists of 50 cases aged between 0-18 years who are under follow up at outpatient and inpatient clinics or newly diagnosed pediatric tuberculosis between 1996-2009 in Cukurova University, Faculty of Medicine, Department of Pediatrics and the control group consists of 50 healthy cases aged between 0-18 years who have neither chronic nor acute diseases and have no history of tuberculosis contact. The clinical history of the children with TB was obtained from medical records and interviews by the physician.

NRAMP1 and MBL2 Genotyping

Blood samples were collected from 50 children with childhood tuberculosis and 50 healthy controls after their parents had given written informed consent, according to the Ethics Committee of Medical School of Cukurova University. Genomic DNA was isolated from 0.2 mL of whole blood using QIAMP-DNA isolation kit (Qiagen).

For the NRAMP1 gene polymorphisms (D543N, 3'-UTR and INT4), the following pair of primers flanking the polymorphism was used to generate polymerase chain reaction (PCR) products of 240 bp for D543N and 3'-UTR polymorphisms, and 624 bp for INT4: for D543N and 3'-UTR, F--5'-GCATCTCCCCAATTCATGGT-3' and R--5'-AACTGTCCCACTCTATCCTG-3'; for INT4 F--5'-TCTCTGGCTGAAGGCTCTCC-3' and R--5'-TGTGCTATCAGTTGAGCCTC-3'. PCR was performed in a final volume of 25 µL containing 1XPCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 5 pmol primer, 100 ng DNA, and 2U Taq Polymerase (Fermantas) for D543N and 3'-UTR. PCR cycle conditions were 95°C for 5 min, followed by 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s (30 cycles). For INT4, the PCR mixture (25 µL) included 1XPCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 pmol primer, 100 ng DNA sample and 2U Taq polymerase (Takara Taq™ Hot Start DNA polymerase, Takara Bio Inc.), and PCR cycle conditions were 95°C for 10 min, followed by 94°C for 30 s, 64°C for 30 s, 72°C for 30 s (30 cycles). The amplified DNA fragments surrounding the D543N, 3'-UTR and INT4 were incubated with 5 U of the restriction enzymes AvaII, FokI and ApaI, respectively at 37°C for 2 h. PCR restriction fragments were size separated by electrophoresis on 10% polyacrylamide gels.

Polymorphisms at codons 54 (GGC→GAC) and 57 (GGA→GAA) in exon 1 of the MBL2 gene were typed by PCR-RFLP technique using the restriction enzymes BshN1 and MboII, respectively. The following pair of primers flanking the two polymorphisms was used: MBL2 exon1 forward, 5'-AGT CGA CCC AGA TTG TAG GAC AGA G-3' and MBL2 exon1 reverse, 5'-AGG ATC CAG GCA GTT TCC TCT GGA AGG-3'. PCR was performed in a final volume of 25 µL containing 1 µL of genomic DNA (50 ng), 2.5 mM $MgCl_2$, a 5 pmol concentration of forward and reverse primers (each), a 0.2 mM concentration of the deoxynucleotide triphosphates, and 1 U of Taq polymerase. The PCR conditions consisted of an initial denaturation step of 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min. The PCR was followed by a final step at 72°C for 5 min. The PCR product is 349 bp. The amplified DNA fragments

were incubated with 5U of the restriction enzymes BshN1 (for codon 54) and MboII (for codon 57) at 37°C for an overnight, and restriction digests were evaluated using 10% polyacrilamid gels in 1XTBE, and visualised by ethidium bromide staining. To determine the size of the banding patterns, GeneRuler^{TM} 100 bp DNA Ladder Plus marker were loaded together with the digested samples and then compared with it. The PCR product is cleaved into 260 bp and 89 bp by BshN1 for normal allele and is uncleaved when the homozygous variant is present due to the replacement of cytosine with thymine (Codon 54, Gly54Asp). For codon 57, the normal variant is not digested by MboII while the homozygous allele gives fragments of 279 bp and 70 bp (Gly57Glu).

Statistical Analysis

Genotype frequencies of patients as well as healthy control subjects were found to be in Hardy-Weinberg equilibrium, as tested by the chi square test. Genotype and allele frequencies were compared by Fisher's exact test using the Statistical SPSS 17.0 statistics program (SPSS Inc., Chicago, IL).

RESULTS

Blood samples were taken from patient and control group cases and were sent to Cukurova University, Faculty of Medicine, Department of Medical Biology for analysis of NRAMP1 and MBL genetic polimorphisms. DNA isolation had been made via QIAMP-DNA isolation tecnic (QIAGEN) from these blood samples. The data received from gene analysis and TBC patients questioner forms analisied statistically.

GA genotype of NRAMP1-D543N polymorphism was detected at 2 of patient group cases (4%) and in 5 of control group cases (10%). GG genotype of NRAMP1-D543N polymorphism was detected at 48 of patient group cases (96%) and 45 of control group cases (90%).

By the data received no statistical differences determined between both groups according to NRAMP1-D543N polymorphism (p> 0.05).

TGTG/del genotype of 3'-UTR region of NRAMP1 gene was detected at 3 of patient group cases (6%) and 5 of control group cases (10%). TGTG/TGTG genotype of 3'-UTR region of NRAMP1 gene was detected at 46 of patient group cases (92%) and 45 of control group cases (90%). No del/del genotype was determined in both groups. The analysis couldnt be performed in one of the cases. By the data recieved no statistical differences determined between both groups according to NRAMP1-D543N polymorphism (p> 0.05).

CC, GC and GG genotype of NRAMP1-INT4 polymorphisms were detected at 1 (2%), 15 (%30), 34 (%68) of patient cases respectively; GC and GG genotypes were detected at 17 (34%) and 33 (66%) of control group cases. No CC genotype was determined in control group cases. By the data received no statistical differences determined between both groups according to NRAMP1-INT4 polymorphism (p> 0.05).

Individuals in patient group those are carriers for MBL gene codon 54 GG genotype (Normal: N) were 35 (70%), GA genotype (Heterozygote: He) were 13 (26%) and AA genotype (Homozygote: Ho) were 2 (4%) respectively. By the data received no statistical differences determined for MBL codon 54 G-> A polymorphism between both two groups (p> 0.05).

Individuals in patient group those are carriers for MBL gene codon 57 GG genotype (Normal: N) were 50 (100%). GA and AA genotype were absent in both groups. By the data received no statistical differences determined for MBL codon 57 polymorphism between both two groups (p> 0.05).

DISCUSSION

Yet, little is known about the mechanisms that influence the rate of progression from infection to disease pediatric and adult tuberculosis differ markedly in epidemiological features, clinical appearance, and pathogenesis. Host genetic factors explain, at least in part why some people resist infection more successfully than others. Rare gene disruptions cause fatal vulnerability to certain pathogens, but more subtle differences are common and arise from minor variations in many genes. Although more studies have been conducted on NRAMP1 than any other gene with respect to susceptibility to TB, its role has not been definitely established. Polymorphisms in NRAMP1 gene have in several different population studies from different parts of the world been shown to be associated with clinical TB. In the present study, we did not observe any significant association with microsatellite polymorphisms at the INT4, G543A and 3-UTR loci situated in the NRAMP1 gene (p> 0.005), and these loci were not associated with pediatric TB in a Turkish population. This finding dosen't confirm a previous investigation in West Africans, the four NRAMP1 variants, namely the 3'UTR deletion, D543N, INT4 and 5'(GT), were found to be significantly associated with TB (29). In studies of Asian subjects, the results have been inconsistent (18,30-33), and in Koreans, only the D543N and 3'UTR variants were associated with susceptibility to TB (18). However, in Cambodians, the D543N and 3'UTR variants were associated with resistance to TB (30). In Taiwanese,

there was no association between the NRAMP1 variants and TB (31). Some studies have shown associations only with the severe forms of TB, but not with susceptibility to TB. In Japanese subjects, the D543N variant, but not the INT4 variant, was associated with the presence of cavity lesions, whereas in Chinese subjects, the D543N and INT4 variants were associated with more severe forms of TB (32,33). In the Thai population, there was no association of the INT4, D543N or 3'UTR variants with susceptibility to TB, or with the severe form of TB. The allele frequencies of NRAMP1 polymorphisms in Asians were different from those of Caucasians and Africans (29,34,35). Drawing conclusions as to which polymorphisms in NRAMP1 play a role in susceptibility to TB is complicated by the lack of consistency in the associations demonstrated in studies conducted in different ethnic groups. The discrepancy between the findings from our study and those from previous studies could be due to many factors. Some of these, it may be ethnicity-related differences in gene polymorphisms, and were differences in clinical severity of the patients between studies. A other possible reason for the discrepancy between studies is that NRAMP1 may not be the disease-associated gene. In addition, we cannot exclude that unknown cofactors (e.g. socio-economical factors, nutritional status, other co-infections or different genetic interactions). Nevertheless the complex interactions between gene and other host factors as well as environmental factors emphasise the difficulties to compare one study from another. Further studies are required on the function of these genes.

Low serum concentrations of MBL may be associated with recurrent infections in young children, and the high frequency of MBL2 variant alleles in different populations indicates that MBL polymorphisms represent a balanced genetic system favoring variant alleles arising from genetic selection (36,37). However, the MBL variant alleles are so frequent in the healthy population, it is conceivable that multiple genetic factors may influence susceptibilities and outcomes in which MBL deficiency plays a role. To explore the underlying forces accounting for the high worldwide prevalence of MBL2 deficiency alleles, Verdu et al. characterized genetic diversity in and around the MBL2 genomic region in 1166 chromosomes from 24 worldwide populations (38). The joint frequency of the exon 1 variant alleles can be above 40% in the human population, dependent on the ethnicity, and in geographic areas where mycobacterial infections are endemic. In a our resent study, the variant MBL allele (codon 54G/A) has a combined frequency of 35.8% among a 229 healthy Turkish population [ın press]. In a Australian study involving 236 healthy blood donors, 30% were found to be heterozygous for structural gene mutations, and an additional 8% were homozygous or had double mutations of the structural genes (39). The codon 54 variant has an observed frequency of 42%-46% in South American Chiriguanos and Mapuches (40); in Danish, Midwestern American, and Greenland Eskimo population groups, the frequency is 11%-13% (41,42).

Epidemiological studies in African-American and Asian populations have disclosed a lower frequency of the B allele (codon 54) among healthy controls than in patients, suggesting a risk of the B allele in TB infection (17,43). There is some evidence that such variants may be protective against meningeal TB in Cape Coloureds but no association with protection against pulmonary TB in The Gambia was found (24,44). In contrast, other groups have presented evidence supporting an association between MBL genetic variants in the structural region and protection from TB infection (44-46). Therefore, the question of whether the mutant alleles are advantageous or disadvantageous in TB infection deserves further investigation in other populations. At this time, it is still speculative as to what influences have contributed to the preservation of heterozygosity in exon 1, resulting in the structural alleles. We found no significant difference of the patterns of the codon 54 and 57 variant frequencies between the cases and controls, and found no convincing evidence of association with TB. At this time, our results clearly demonstrate that the patterns of the codon 54 and 57 variants are compatible with neutral evolution, as opposed to negative, positive or balanced natural selection. Already, preliminary studies have suggested that heterozygotes for B, C or D could be protected against severe TB infection (46-48). It has been suggested that heterozygote advantage may maintain MBL variant alleles at high frequency by conferring resistance to mycobacterial diseases (36). It should be mentioned, however, that the theory of selective forces shaping the frequencies of MBL2 polymorphisms have recently been debated supporting the notion that the main reason for the high frequencies of MBL2 polymorphisms are due to random genetic drift and bottle neck effects (38). But significantly underrepresented among TB patients compared with controls in a large association study from Gambia and thus might associated with protection against TB (49). However, this association may be surpassed by co-infections. Hypotheses explaining the selective advantage of MBL2 polymorphisms arose from population group studies describing a higher frequency of MBL2 structural gene mutations in geograp-

hic areas where mycobacterial infections are endemic. An alternative, and equally likely hypothesis to explain the high worldwide frequency of MBL2 alleles resulting in the production of little or no MBL2 therefore result exclusively from human migration and genetic drift.

In this study, the INT4, G543A and 3-UTR loci microsatellite polymorphisms in the NRAMP1 gene were not associated with TB. No significant associations were also observed for the MBL2 genetic system. There is not a suggestion for a protective effect of the 54 and 57 codons of the MBL gene against TB in Turkish children. Further studies are warranted to clarify the possible mechanisms involved.

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