The first report described as an important study: The association of mannose-binding lectin gene 2 polymorphisms in children with Down syndrome

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BACKGROUND: Mannose-binding lectin gene 2 (MBL2) plays a very important role in the first line of host immune response in Down syndrome (DS). The importance of MBL2 gene polymorphisms in children with DS is unclear, and no research has addressed MBL2 gene polymorphisms in patients with DS. This is the first report describing an important association between MBL2 gene polymorphisms and infections in children with DS.
MATERIALS AND METHODS: We compared the frequency of single-nucleotide polymorphisms (SNPs) at two codons of the MBL2 gene in a cross sectional cohort of 166 children with DS and 229 controls. Polymorphisms at codons 54 (GGC→GAC) and 57 (GGA→GAA) in exon 1 of the MBL2 gene were typed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique using the restriction enzymes BshN1 (derivated from *Bacillus sphaericus*) and MboII (derivated from *Moraxella bovis*), respectively.
RESULTS: MBL2 codon 54 GA genotype frequency was found to be lower in patients with DS (22.9%) than those of healthy controls (35.8%), differences were statistically significant (OR = 0.532, 95% CI = 0.339-0.836, *P* = 0.008). On the other hand, codon 57 polymorphism in the MBL2 gene was detected in none of the DS patients, but only one person in the control group showed codon 57 GA genotype (OR = 1.004, 95% CI = 0.996-1.013, *P* = 1.000).
CONCLUSION: Our data provides an evidence for the first time that a homozygote or heterozygote for the variant, MBL2 alleles, is not associated with infections in patients with DS, and do not influence the incidence of infections.

Key words: Down syndrome, Mannose-binding lectin gene 2 gene polymorphisms, single-nucleotide polymorphisms

Introduction

Down syndrome (DS) is the most frequent cause of intellectual disability worldwide, and individuals with DS present increased susceptibility to recurrent infections (RI) and autoimmune diseases, suggesting abnormalities in the immune system.[1] Mannose-binding lectin gene 2 (MBL2) insufficiency is believed to be the most common human immunodeficiency, increasing the susceptibility to numerous infectious diseases, notably by extracellular pathogens.[2,3] MBL2, thus, plays a very important role in the first line of host immune response. Respiratory tract infections remain one of the major causes of death in DS.[4,5] Nisihara *et al.* demonstrated that MBL2 deficiency increases the susceptibility to RI in DS patients, and that in the future, they could be potentially benefited from MBL2 therapy.[6] Clinical studies have shown that single-nucleotide polymorphisms (SNPs) in MBL2 gene are associated with increased susceptibility to infections. The importance of MBL2 polymorphisms in DS patients is unclear. Three frequently occurring SNPs are described in the coding region of the MBL2 gene that are associated with abnormal polymerization of the MBL2 molecule, decreased serum concentrations of high molecular weight MBL2, and strongly impaired

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function. These mutations are in codons 52, 54, and 57, and cause disruption of the collagenous triple helix, which is critical for oligomerization. Each of the three variants reduces the amount of functional high molecular MBL2 in heterozygous individuals 5-10 times, while high molecular weight MBL2 is absent in variant allele homozygotes. These alleles are very common, and up to 35–40% of the Caucasian population are carriers.[7] Heterozygous individuals for these mutations have a substantial decrease in MBL2 serum concentration,[8] whereas MBL2 is undetectable in the serum of homozygous individuals.[8,9] 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%.[9]

Recently, Nisihara *et al.* reported that the MBL2 deficiency increased more than 3-fold the risk of RI and pneumonia in DS patients.[6] According to the clinical history, 30.7% of the DS children suffered from RI, MBL2 deficiency was seen in 34.8% of children, compared to 13.5% of the DS children without RI, and 20% of the children with DS (children with RI and without RI). The findings demonstrated that SNPs of the MBL2 gene are risk factors for the development and increase the susceptibility to RI in DS patients, and in the future, they could be potentially benefited from MBL2 therapy. The MBL2 genotype had no clear effect on the occurrence of RI in children with DS. Focusing on the role of polymorphisms of the MBL2 gene, probably the genotypes in DS children are similar to the healthy children, however, genetic studies need to be done in order to clarify this hypothesis.

In this study, we aimed to investigate whether SNPs within the MBL2 gene are associated with DS and are risk factors for RI. No research has addressed MBL2 gene polymorphisms in patients with DS by now.

Materials and Methods

Patients and controls

A total of 166 DS children (98 males and 68 females, median age 3 years, range 1-7 years), cytogenetically documented and found to have three copies of chromosome 21, were assembled by the Department of Medical Biology and Genetics, Faculty of Medicine, University of Çukurova between June 2004 and March 2009. The 229 healthy children from the same geographic area (123 males and 106 females, median age 5 years, range 4-18 years) were used as a control group. The clinical history of the DS children was obtained from medical records and interviews by the physician.

Mannose-binding lectin gene 2 genotyping

Blood samples were collected from 166 children with DS and 229 healthy controls after their parents had given written informed consent, according to the Ethics Committee of Medical School of Çukurova University. Genomic DNA was isolated from 0.2 ml of whole blood using QIAMP-DNA isolation kit (Qiagen). Polymorphisms at codons 54 (GGC→GAC) and 57 (GGA→GAA) in exon 1 of the MBL2 gene were typed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (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 minutes followed by 35 cycles of 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 2 minutes. The PCR was followed by a final step at 72°C for 5 minutes. The PCR product is 349 bp. The amplified DNA fragments were incubated with 5 U 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 1XTris/Boric Acid/EDTA (TBE), and visualized by ethidium bromide staining. To determine the size of the banding patterns, GeneRulerTM 100 bp DNA Ladder Plus marker was 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 variant allele is present due to the replacement of cytosine with thymine (codon 54, Gly54Asp). For codon 57, the normal allele is not digested by MboII, while the variant 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 χ^2 test. Genotype and allele frequencies were compared by Fisher's exact test using the SPSS 17.0 statistics program (SPSS Inc., Chicago, IL).

Results

To examine whether MBL2 gene polymorphisms are of higher frequency in children with DS, we assessed the MBL2 genotype in children with DS and in children without DS by PCR-RFLP analysis. The studied genotype and allele frequencies were in Hardy–Weinberg equilibrium in both DS and healthy control groups [Table 1]. As shown in Table 1, MBL2 codon 54 GA genotype frequency was found to be lower in patients with DS (22.9%) than those of healthy controls (35.8%), differences were statistically significant (OR = 0.532, 95% CI = 0.339-0.836, $P = 0.008$). On the other hand, codon 57 polymorphism in the MBL2 gene was detected in none of the DS patients, but only one person in the control group showed codon 57 GA genotype (OR = 1.004, 95% CI = 0.996-1.013, P = 1.000). No significant differences were found between the genotype or allele frequencies of males or females in any of the groups analyzed.

Discussion

The immune dysfunction in DS consists of abnormalities in both the innate and adaptive systems. However, the majority of reports describing abnormalities in DS have been related to the adaptive response, with very few studies on the defects of innate immunity. Infections are still a major cause of death among DS patients of all ages.[4] It was evidenced that the MBL2 deficiency increased more than 3-fold risk of RI and pneumonia in DS patients.[6] However, MBL2 genotype had no clear effect on the occurrence of infections in children with DS. In this respect, this is the first report describing an important association between MBL2 gene polymorphisms and DS children.

The last 10 years of research have shown that the MBL2 gene constitutes a complex genetic system, which displays polymorphisms both in the structural and the regulatory elements. The MBL2 variant alleles are so frequent in the healthy population, it is conceivable that multiple genetic factors may influence susceptibilities and outcomes in which MBL2 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.[10] 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. For example, the codon 54 variant is significantly common in Asia and in Japan.[11-13] In our study, the heterozygous genotype (54 GA) for structural gene mutations was also found in 35.8% of 229 healthy donors. In an Australian study involving 236 healthy blood donors, 30% were found to be heterozygous for structural gene mutations, and an additional 8% were homozygous or

Table 1: Comparison of genotype and allele frequencies of mannose binding lectin gene 2 codon 54 GA and codon 57 GA polymorphisms between patients with Down syndrome and healthy controls

Genotype/Allele	DS (n = 166)	Controls (n = 229)	χ^2	OR (95% CI)	*P*
Codon 54					
GG	123(74.1)	144 (62.9)	5.525	1.688 (1.089-2.618)	Ref.
GA	38 (22.9)	82 (35.8)	7.591	0.532 (0.339-0.836)	0.008
AA	5 (3.0)	3 (1.3)	1.405	2.340 (0.551-9.930)	0.289
G	284 (85.5)	370 (80.8)	3.055	1.407 (0.958-2.066)	Ref.
A	48 (14.5)	88 (19.2)	3.055	0.711 (0.484-1.043)	0.086
Codon 57					
GG	166 (100)	228 (99.56)	0.727	1.004 (0.996-1.013)	Ref.
GA	0 (0)	1 (0.43)	0.727	1.004 (0.996-1.013)	ns
AA	0 (0)	0 (0.00)	-	-	ns
G	332 (100)	457 (99.78)	0.726	1.002 (0998-1.006)	Ref.
A	0 (0)	1 (0.22)	0.726	1.002 (0998-1.006)	ns

χ^2 = Chi-square, OR = Odds ratio, CI = Confidence interval, NS = Not significant, Ref = Reference

had double mutations of the structural genes.[14] The codon 54 variant has an observed frequency of 42–46% in South American Chiriguanos and Mapuches;[15] in Danish, Midwestern American, and Greenland Eskimo population groups, the frequency is 11–13%.[16-18] The codon 57 variant is more common in sub-Saharan African populations than in white populations; it is 23–29% in Gambians and Kenyans.[17,19,20] The high frequency of MBL2 variant alleles in different populations indicates that MBL2 polymorphisms represent a balanced genetic system favoring variant alleles arising from genetic selection.[21] This is consistent with the observation that the distributions of the three MBL2 mutant coding alleles are in Hardy-Weinberg equilibrium worldwide, indicating that there is currently no preferential selective pressure on any genotypic combination.

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. It is likely that changes in the circulating levels and function of variant proteins could have a selective advantage in response to environmental pressures, such as infection. Nearly 60% of the sub-Saharan populations contain the C allele, which suggests that MBL2 deficiency could provide protection against intracellular pathogens, such as tuberculosis. Already, preliminary studies have suggested that heterozygotes for B, C, or D could be protected against severe tuberculosis infection.[22-24] Our results also clearly demonstrate that the patterns of the codon 54 variant is compatible with neutral evolution, as opposed to negative, positive or balanced natural selection. 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. It should be noted that 90% of MBL2 deficient individuals do not suffer from RI. This can probably be explained by the redundancy of the complement system. Heterozygous individuals for these mutations have a substantial decrease in MBL2 serum concentration,[8] whereas MBL2 is undetectable in the serum of homozygous individuals.[8,9] Hypotheses explaining the selective advantage of MBL2 polymorphisms arose from population group studies describing a higher frequency

of MBL2 structural gene mutations in geographic areas where mycobacterial infections are endemic.

MBL2 levels were significantly lower in DS patients than in the non-DS children.[6] The dysregulation of immune cells could modify the expression of cytokines such as interleukin (IL)-6,[25-27] which may affect the production of MBL2 in these individuals. The importance of MBL2 polymorphisms in DS children as an outcome is unclear. However, genetic studies need to be done in order to clarify this ambiguity. We investigated the variant alleles at codons 54 and 57 of exon 1 of MBL2 gene. A significantly decreased frequency of patients with the low-expressing heterozygosity (54 GA) for MBL2 variant allele was observed in patients compared to control subjects ($P = 0.008$). This result suggests that MBL2 polymorphisms and haplotypes are not associated with susceptibility to infections in DS children. Recently, Nisihara *et al.* reported that the MBL2 circulating levels were evaluated in 150 children with DS from Brazil, in order to verify whether MBL2 deficiency was associated with the presence of RI in these patients.[6] The MBL2 deficiency increased more than 3-fold of the RI and pneumonia in DS patients. According to the clinical history 30.7% of the DS children suffered from RI, MBL2 deficiency was seen in 34.8% of children, compared to 13.5% of the DS children without RI, and 20% of the children with DS (children with RI and without RI).[6] We reported that 22.9% of all the children with DS had low heterozygote (54 GA), defective MBL2 alleles, compared to 35.8% of the controls. A significantly decreased frequency of patients with the low-expressing heterozygosity (54 GA). These findings are in agreement with the finding of Nisihara *et al.* (in 20% of all children with DS).[6]

It is doubtful whether MBL2 polymorphisms may influence or predict infections in DS children. Moreover, lower levels of thyroid hormones have been shown to be implicated in decreased production of MBL2.[27] Since hypothyroidism is a common condition in DS children, occurring in about 40% of them, such association is reasonable. The clinical manifestation of MBL2 deficiency seems to be of greater significance, while the immune system is still immature, such as in the two first years of childhood.[28] It is most probable that the defective MBL2 alleles are one of the several other predisposing risk

factors. It is likely to act in synergy with other factors, both genetic and environmental, in the development of the disease. Therefore, it could be argued that the effect of the MBL2 variant alleles is due to linkage disequilibrium with polymorphisms in one of these or in another gene. If MBL2 plays an essential role in protective immunity, then we would expect MBL2 deficiency alleles to be selectively removed at the population level, which is clearly not the case. If the presence of MBL2 is detrimental, then we would expect MBL2 deficiency haplotypes to be favored and subject to positive selection, which might account for the high population frequencies of deficiency alleles. Also, the presence in most populations of both deficiency and wildtype MBL2 haplotypes may result from a more general pattern of balancing selection, favoring heterozygous individuals presenting intermediate MBL2 levels. Thus, although this observation is not a classical heterosis situation (i.e. heterozygous advantage as seen with hemoglobin S (HbS) allele in malaria and sickle cell anemia), it indicates that individuals heterozygous for a structural variant allele who also carry a low-expression promoter in front of the normal allele on the other chromosome are partly protected against infections.

In conclusion, our results suggest that the immune dysfunction in DS is independent of the codon 54 and 57 variants, at least in the adult population. At the same time, a low-expression of heterozygosity (54 GA) is not a major risk factor for infections in Turkish DS children, and may be associated with protection against infections, whereas high-expression genotypes conferring MBL2 deficiency does not need further elucidation in relation to possible biological functions and relative advantages or disadvantages for the host. If MBL2 plays an essential role in protective immunity, then we would expect MBL2 deficiency alleles to be selectively removed at the population level, which is clearly not the case.

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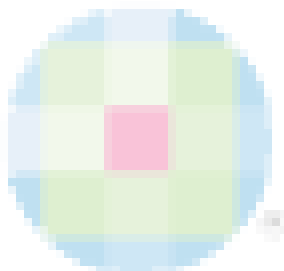
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