Association of Mannose-Binding Lectin 2 (MBL2) gene heterogeneity and its serum concentration with osteoporosis in postmenopausal women

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ABSTRACT

The aim of the study was to detect prevalence of MBL2 exon 1 (codons 52, 54 and 57) genetic polymorphism in postmenopausal women in Bosnia and Herzegovina and its possible role as genetic risk factor for susceptibility to occurrence of osteoporosis in this study group. Also, we investigated association between MBL serum concentrations and osteoporosis in postmenopausal women. Genetic codons’ variations were determined by PCR-RFLP and MBL in serum was measured by ELISA method in 118 postmenopausal women (61 with osteoporosis and 57 apparently healthy, non-osteoporotic women serving as a control). Serum MBL levels were not significantly different between osteoporosis and control group (73.2 (67-89.1) and 85.9 (71-100.6) ng/mL respectively, p=0.067). Genotype frequencies were not significantly different (p=0.997) between the studied groups of postmenopausal women. Genotype frequencies A/A, A/A and A/a in osteoporosis group were 0.576; 0.405; 0.018 and in control group 0.562; 0.412; 0.026, respectively. Frequencies of A and a allele were 0.78 and 0.22 in osteoporosis and 0.77 and 0.23 in control group. The results do not suggest association of functional polymorphism of MBL2 gene and MBL serum concentration with osteoporosis in postmenopausal females.

KEY WORDS: mannose-binding lectin, polymorphism, osteoporosis

INTRODUCTION

Mannose-binding lectin (MBL) is in focus of attention because of its role as a recognition molecule in complement system. It is Ca2+-dependent collagenous lectin, synthesized in liver with main role to mediate innate immune defence against microorganisms. MBL recognizes certain sugars on the surface of the bacteria, apoptotic cells, phospholipids and immune complexes and mediates opsonophagocytosis directly and by activation of lectin complement pathway [1]. MBL is also considered as acute-phase reactant and its responsiveness is dependent upon the MBL2 genotype [2].

Serum MBL concentration varies from undetectable to 10,000 ng/mL. It is very well documented that decreased concentration of MBL is associated with susceptibility to infectious diseases. Furthermore, lower concentration may be caused by point mutation on exon 1 and by polymorphism on promoter region of MBL2 gene [3]. It is clear that MBL2 gene harbor complex genetic system associated with infectious conditions but also it may be a diseases modifier in patients with certain diseases. Certain aspects of osteoporosis have genetic influence [4]. Since, the osteoporosis shows an inflammatory character [5] and MBL is considered as modifier of inflammatory responses, we investigated association of MBL2 gene heterogeneity and MBL serum concentration with osteoporosis. It was shown that serum level of MBL is influenced by presence of genetic polymorphism at the protein coding region consist of four exons [6]. MBL2 gene is located in long arm of the chromosome 10q11.2-q21 [7]. MBL1 is pseudo gene. Five functional single-nucleotide polymorphisms can be found in the MBL2 gene. Each of them can affect se-
Materials and Methods

Study population

This cross-sectional study included 75 postmenopausal women (37 with osteoporosis (60.7±7.71 years) and 38 apparently healthy, non-osteoporotic women serving as a control (58.0±7.81 years). All women underwent bone mineral density (BMD) assessment at the hip and lumbar spine which was performed by Dual-energy x-ray absorptiometry (DXA) at Clinics for Radiology, Clinical Center University of Sarajevo. Osteoporosis was defined as total T score equal or below -2.5 measured either on the hip and/or lumbar spine. Women whose total T score was -1 or higher, both on the hip and/or lumbar spine were considered to have preserved bone mass and served as controls. The Ethical Committee of Faculty of Medicine, University of Sarajevo approved the protocol of the study. Sample collection and all laboratory procedures were done in the Laboratory for Molecular medicine, Center for Genetic, Faculty of Medicine, University of Sarajevo. Written informed consent was obtained from each subject included in this study. To obtain genomic DNA for genetic testing, buccal cells were collected with two swabs. Subject’s mouth was vigorously rubbed on the both sides of the cheek at least six times and swabs were placed inside of envelope. Used cotton swabs and the envelope were sterile. Upon receipt, the buccal swabs were placed at room temperature to dry, and keep at -20°C until DNA extraction. Genomic DNA was isolated from buccal swabs following standard salting out procedure (Miller) [10].

Genotyping of MBL2

Detection of the genetic polymorphism in codons 52, 54 and 57 of the MBL2 gene was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and the restriction enzymes HhaI, BanI and MboII, respectively. For determination of polymorphisms following pair of primers were used: MBLexF 5’-CAT CAA CGG CTT CCC AGG CAA AGA TGC G-3’ and MBLexR 5’-CAG GCA GTT TCC TCT GGA AGG TAA AG-3’ as reported previously [11]. The PCRs were performed in final volume of 25 μl using 50 ng of genomic DNA, 0.25 μM of forward and reverse primer each (Eurofins MWG Operon, Germany) 1.5 mM of MgCl2, 2 U of Taq polymerase in buffer containing 100 mM Tris-HCl (pH 8.3) and 500 mM KCl (Qiagen, Germany) and 40 μM of dNTP (Sigma-Aldrich Chemie GmbH, Germany). PCR conditions were as follow: an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 62°C for 20 s, and extension at 72°C for 30 s. The PCR was followed by a final step at 72°C for 7 minutes. Obtained PCR product with this procedure was 119 pb. Amplified products were cleaved with restriction enzyme (TAKARA BIO INK, Japan) HhaI into 91 and 28 bp for the A allele and were uncleaved when the D variant is present. Fragments with wild-type, for codon 54, were cleaved into two fragment of 84 and 35 bp, but with B allele stayed undigested by BanI. For codon 57, fragments with A variant stayed uncut by MboII, while fragment with mutant allele showed two bands of 63 and 56 bp. The genotypes were determine by electrophoresis on 3.5% agarose gel stained with ethidium bromide.

Measurement of MBL

Venous blood samples were taken and centrifuged at 3000 g for 10 minutes. Collected serum samples were stored at -80°C. Quantitative determination of serum MBL levels was performed by a solid-phase enzyme-linked immunoassay (ELISA). Samples were diluted and processed according to the manufacturer’s instructions (HyCult Biotechnology). Briefly, after activation of samples and standards, they are incubated in microtiter wells coated with mannann. After capturing of human MBL, biotinylated tracer antibody, streptavidin conjugate and tetrathiomolybenzidine (TMB) in separate phases of protocol were added. The reaction was stopped by oxalic acid addition. Sample MBL levels were calculated from the standard curve based on samples of known MBL concentration. Absorbances were read at A450 by using ELISA reader (STAT FAX 2100, USA). Values of MBL were expressed in ng/ml.
Statistical analysis
Genotype and allele frequencies were obtained by direct counting. Genotype frequencies differences were analyzed by Pearson $\chi^2$ test. In descriptive statistic median with quartiles (first and third) were used. Non-parametric Mann-Whitney U-test was used to compare continuous data between carriers of the different MBL2 genotypes. Statistical significance was defined as $p<0.05$. Statistical calculation was performed with SPSS for Windows (version 19.0. SPSS Chicago, IL).

RESULTS
Seventy five participants included in the study were divided into two groups; women with osteoporosis and healthy control. Initially, evidence of the presence of the MBL allele variants B, C and D was sought in DNA samples from postmenopausal women (Figure 1.). Table 1 presents the genotype frequencies of the exon 4 variants of MBL2 in postmenopausal women with osteoporosis and control group. There were no statistically significant differences regarding genotype frequency of the MBL2 gene polymorphism ($p=0.997$) in studied groups. The genotype frequencies of A/A, A/0 and 0/0 were 0.576, 0.405; 0.018 in osteoporosis and 0.562; 0.412; 0.026 in control group of postmenopausal females. The data of allele frequencies of point mutations on MBL2 gene in postmenopausal women are presented in Table 2. The frequency of A variant allele was 0.773, but 0 allele variant frequency was 0.227. Allele variant frequencies for three different structural variants were 0.29, 0.22 and 0.17 for B, D and C respectively. Concerning the osteoporosis and control groups, frequencies of the A allele were 0.78 and 0.77 and of 0 allele were 0.22 and 0.23, respectively. MBL serum concentrations in postmenopausal women were shown in Table 3. Comparison of mannose-binding lectin serum concentration between postmenopausal women with osteoporosis and control shown no significant difference (492 (37-565.1) ng/mL and 522.6 (477-559.4) ng/mL respectively, $p=0.206$). Analysis of the MBL serum concentration and corresponding MBL2 gene polymorphism in postmenopausal women confirmed that genotype variants have effects on the concentration of MBL. Mannose-binding lectin concentration in serum of postmenopausal women was lower in variant alleles (484.9 (357-555.5) ng/mL) compared to wild-type allele carriers (541.6 (522.2-589.8) ng/mL) ($p=0.001$). After distribution of participants into two groups (women with osteoporosis and group with preserved bone mass - control), it was shown that the wild-type MBL2 genotype AA was associated with high MBL level (554.4 (531-582.5) ng/mL) while, A0/00 genotype was associated with lower concentration of MBL in serum (475.3 (25.8 – 522.8) ng/mL) in a group of women with osteoporosis ($p=0.001$). This genotype influence was not confirmed in control group ($p=0.110$).

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>Total (n=75)</th>
<th>Osteoporosis group (n=37)</th>
<th>Control group (n=38)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>0.569</td>
<td>0.576</td>
<td>0.562</td>
<td>0.997</td>
</tr>
<tr>
<td>A0</td>
<td>0.409</td>
<td>0.405</td>
<td>0.412</td>
<td>0.026</td>
</tr>
<tr>
<td>00</td>
<td>0.022</td>
<td>0.018</td>
<td>0.026</td>
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</tr>
</tbody>
</table>

A- individuals with homozygous wild-type genotypes; A0 – individuals with heterozygous genotypes; 00 – individuals with homozygous genotypes; $p$ – probability.

<table>
<thead>
<tr>
<th>Alleles frequencies</th>
<th>Postmenopausal women (n=75)</th>
<th>Osteoporosis group (n=37)</th>
<th>Control group (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.773</td>
<td>0.78</td>
<td>0.768</td>
</tr>
<tr>
<td>0</td>
<td>0.227</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.29</td>
<td>0.17</td>
<td>0.22</td>
</tr>
<tr>
<td>C</td>
<td>0.17</td>
<td></td>
<td></td>
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<tr>
<td>D</td>
<td>0.22</td>
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</table>

A – wild-type MBL2 allele; B-the codon 54 allele; C-the codon 52 allele; D - the codon 57 allele; 0 – any combination of the structural variant alleles;
DISCUSSION

Data on MBL2 polymorphism and MBL serum concentration in a relation with osteoporosis are scarce. There is no published data about influence of MBL in susceptibility to appearance and development of osteoporosis in postmenopausal women. In presented study of postmenopausal women, examined frequencies of the genotype with structural variant alleles B, C or D; A0/00 – heterozygous and homozygous exon 1 variants; A – wild-type MBL2 allele; 0 – any combination of the structural variant alleles B, C or D; A0/00 – heterozygous and homozygous exon 1 variants; p – probability. Data are presented as median level and with first and third quartile. NS - non-significant difference osteoporosis vs. control.

TABLE 3. Serum MBL concentrations in postmenopausal women with osteoporosis and control group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Postmenopausal women (n=75)</th>
<th>Osteoporosis group (n=37)</th>
<th>Control group (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBL concentration in serum (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0/00</td>
<td>507.8 (435.6-558.8)</td>
<td>492 (37-565.1)</td>
<td>522 (477–559.4)</td>
</tr>
<tr>
<td>A/A</td>
<td>541.6 (522.2-389.8)</td>
<td>554.4 (531-582.5)</td>
<td>526.4 (518.5–582.2)</td>
</tr>
<tr>
<td>A/A0</td>
<td>484.9 (357-555.5)</td>
<td>475.3 (25.8-522.8)</td>
<td>502.5 (456–558.8)</td>
</tr>
</tbody>
</table>

p = 0.001

As it is already reported, MBL polymorphism in exon 1 variation in this study were obviously similar to control group of women. Since study included a small number of women, further studies with larger sample size should be performed to clarify possible association between osteoporosis and MBL2 gene polymorphism. As it is already reported, MBL polymorphism in exon 1 causes lower MBL serum concentration [6, 21]. These study results confirmed the influence of different MBL genotypes on MBL concentration in postmenopausal women. Inconsistent result was observed when we tested this association between two study groups. Influence of polymorphism on MBL concentration in serum was observed only in osteoporosis group. Significantly higher MBL concentration was detected in women of osteoporosis group carrying wild-type genotype compared to allele variant carriers. The influence of genotype on serum MBL levels in a healthy group of women was not confirmed, since no difference in MBL concentration was found among different genotype carriers. There is strong correlation of MBL2 polymorphism and MBL concentration and function, confirmed by many studies [20, 21]. Despite these obvious associations, substantial interpersonal variations of the MBL concentration have been observed. Many individuals with wild-type MBL genes may have low or undetectable MBL levels and function [22]. Also, variations of MBL concentration between the same genotype have been observed [19]. Difference in circulating serum concentration of MBL cannot be explained just by influence of MBL2 gene polymorphism in exon 1 but there is also significant influence of the polymorphism in promoter region [12]. Investigation of promoter region genetic variation haven’t been included in this study, so large research has to be done to elucidate mechanism and association of MBL2 gene polymorphism and circulating serum MBL level. In conclusion, we could not find an association of MBL2 polymorphism with susceptibility to occurrence of osteoporosis in postmenopausal women. Also, lack of the

There is no evidence regarding gender difference in the genetic variation in MBL2 gene currently available in the literature. There is strong correlation of MBL5 gene polymorphism with susceptibility to appearance of osteoporosis and control subjects (AA, A0 and 00 for genotype and 0.77 and 0.22 for allele frequencies respectively). Also, genotype frequencies for all postmenopausal women in this study (Table 2) were similar to the frequencies found in a control group in the study by Nielsen et al. [17] (0.56 for AA, 0.40 for A0 and 0.04 for 00 genotype groups).
evidence regarding the association between MBL serum concentration and osteoporosis was observed. Genetic influence of structural MBL2 gene on MBL concentration was confirmed in group of postmenopausal women with osteoporosis. Study with larger number of participants and further assessment of polymorphism in the promoter region of the MBL2 gene is necessary to distinguish whether there is association between MBL2 gene polymorphisms, serum MBL concentration and osteoporosis.

CONCLUSION

The results do not suggest association of functional polymorphism of MBL2 gene and MBL serum concentration with osteoporosis in postmenopausal females.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

REFERENCES