Polymorphisms in the promoter region and at codon 54 of the MBL2 gene are not associated with IgA nephropathy

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Abstract

Background. IgA nephropathy (IgAN) occurs sporadically in unrelated individuals. Several different polymorphic genes have been investigated in recent years in order to demonstrate their possible association with IgAN. Three recent, different studies with conflicting conclusions have discussed the role of the mannose binding lectin (MBL), a serum lectin involved in natural immunity, in the IgAN pathogenesis by examination of MBL deposits in biopsies. In the present study we investigated several polymorphisms of the MBL gene located in the promoter region and in the first exon.

Methods. MBL polymorphism detection was performed in 22 Italian patients with familial IgA nephropathy and in 138 Italian patients with the sporadic form of the disease. The polymorphisms in the MBL2 promoter region and in the exon 1 were investigated, respectively, by direct sequencing and by amplification refractory mutation system-polymerase chain reaction on genomic DNA collected from peripheral blood. Seventy-four unrelated healthy subjects matched for ethnic origin were used as controls.

Results. Allelic and genotypic frequencies of the polymorphisms at position −550, −328, −221 and at codon 54 did not show any differences between patients and controls. Similar frequency distributions of these polymorphisms were also found in the subgroups of IgAN patients subdivided according to the clinical manifestations and the progression of the disease.

Conclusions. This study indicates that the analysed polymorphisms of the MBL gene do not appear to be primarily involved in the susceptibility and severity of IgAN.

Keywords: DNA; genetics; IgA nephropathy; MBL2; polymorphisms; susceptibility

Introduction

IgA nephropathy (IgAN) occurs sporadically in unrelated individuals. Infectious agents [1,2] and alimentary antigens [3] have been identified as environmental factors involved in the pathogenesis of the disease. Prevalence of the disease [4–6] has been observed to be affected by demographic and racial differences, and several cases of family clustering have been identified [7–10]. For these reasons, several studies attempted to investigate a genetic predisposition to IgAN. Associations between the disease and HLA alleles have been described, particularly in class II and III loci such as DR, DQ and Bf [11–13]. Other polymorphic genes, such as C3, also seem to be involved in the susceptibility to IgA nephropathy [13] and abnormalities in IgA production might be demonstrated not only in patients with IgAN, but also in healthy family members [14]. All these data suggest that several genetic factors are involved in the susceptibility to the disease.

The advent of recombinant DNA technology has greatly facilitated the search for genetic markers and several different polymorphic genes have been investigated in recent years in order to demonstrate their possible association with IgAN, as HLA-DQ [15] or the switch regions of immunoglobulin heavy chain constant genes [16,17]. The various studies have shown conflicting results and it may be postulated that genetic
factors vary among different populations. Moreover, when multifactorial heredity is involved, it may be difficult to identify the role of single genes by studying sporadic patients.

Three different studies with conflicting conclusions have recently debated the role of the mannose binding lectin (MBL), previously named mannose binding protein (MBP), in IgAN pathogenesis [18–20]. This serum lectin involved in natural immunity is codified by the MBL2 gene, which is organized in four exons. Low levels of this protein have been correlated with recurrent infections [21]. MBL deficiency and low MBL serum levels are strongly associated with the presence of MBL alleles that encode three different structural variants of MBL polypeptide (codons 52, 54 and 57). Among these variants in the first exon, Gly54Asp has the most important role in the Caucasoid population causing a reduction of the serum MBL level by 5–10 times in heterozygous individuals [22]. Other variants detected upstream of the gene also have a dramatic effect on the serum MBL level. In particular, two allelic variants in the MBL2 promoter region were described by Madsen et al. [23] with the guanine at position −550 (allele H) and at position −221 (allele Y). These alleles have been correlated with higher MBL levels, while the cytosine at position −550 and at position −221 (alleles L and X, respectively) have been correlated with lower levels of this protein. Moreover, a deletion of 6 bp has recently been described in the promoter region of the MBL2 gene [24].

In order to evaluate the influence of the MBL promoter and of Gly54Asp mutation on IgAN susceptibility and progression, MBL polymorphisms detection was performed in 22 Italian patients with familial IgAN and in 138 Italian patients with the sporadic form of the disease. The polymorphisms in the MBL2 promoter region and at codon 54 of exon 1 were investigated, respectively, by direct sequencing and by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) on genomic DNA collected from peripheral blood. Seventy-four unrelated healthy subjects matched for ethnic origin were used as control population. All patients were Italian, a population in which IgAN is the most common glomerulopathy [25].

Diagnosis of IgAN in the other 94 patients was made when they were older than 18 years. Of those 94 patients 55 of them had a stable renal function (group IGAN2); the remaining 39 had disease progressing to renal failure (13 were on chronic haemodialysis and 26 received a kidney graft; group IGAN3).

Twenty-two patients (15 males and seven females) with familial IgAN were identified by the recurrence of the disease in two or more members of the same family. The majority of the families to whom they belonged have been previously described [26,27].

Diagnostic criteria

IgAN was diagnosed when, in the absence of liver or systemic disease, there was a variable degree of mesangial proliferation by light microscopy and immunofluorescence study showed a prevalent IgA deposition in mesangial areas.

The familial group included 14 patients with biopsy-proven IgAN and eight patients (not subjected to renal biopsy and who had a family member with a biopsy-proven IgAN) with isolated or recurrent macroscopic haematuria and/or pathologic proteinuria that was confirmed in three subsequent tests. These patients had no signs of systemic disease; anatomical abnormality or nephrolithiasis were excluded by intravenous pyelography.

The ‘stable’ group (IGAN2) included those patients who maintained a CrCl > 80 ml/min for more than 7 years after biopsy. The ‘progressive’ group (IGAN3) was composed of patients with CrCl < 60 ml/min at the last observation.

Controls

Seventy-four healthy unrelated subjects matched for ethnic origin served as controls.

Methods

DNA was isolated from EDTA-collected peripheral whole blood using standard laboratory techniques [28].

For the promoter polymorphism analysis, the PCR reactions were performed under standard conditions in 50 μl final volume using 10 pmol of each primer (5’CCAGGGCC-AACGTAGTAAGAA3’, forward primer; 5’TGGCGTT-GCTGCTGGAA3’, ‘reverse primer), and 1 U of AmpliTaq GOLD DNA polymerase (PE Biosystems, Foster City, CA) in a thermal cycler 2400 (PE Biosystems). After 35 cycles at 95°C for 30 s, 56°C for 30 s, and at 72°C for 30 s, PCR products were detected in a 3% agarose gel. DNA sequencing of both DNA strands was performed according to the modified Sanger dyeideox method with BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). The allelic variants in the MBL2 promoter region were at position −550 (alleles H and L, defined by the presence of guanine and cytosine, respectively), at position −221 (alleles Y and X, defined by the presence of guanine and cytosine, respectively) and at position −328, where a deletion of 6 bp (∼328delAAAGAG) was also described.

The analysis of polymorphism of the first exon was restricted to codon 54, because it is the most relevant in Caucasoid population. To detect the polymorphism Gly54Asp an ARMS-PCR was performed in 50 μl final volume using 10 pmol of each primer (using a common reverse primer 5’AGGCAGTTTCTCTGGAGG3’ and

Subjects and methods

Patients

A diagnosis of sporadic IgAN was assigned to 138 patients (98 males and 40 females), who had no relatives affected by the same disease, as documented by a detailed anamnestic investigation. These patients were originally from different regions of continental Italy, most of them were living in the north. Forty-four IgAN patients who had been less than 18 years old at onset, and had creatinine clearance (CrCl) values <50 ml/min/1.73 m² and proteinuria ≥1 but <3.5 g/1.73 m²/day were identified as group IGAN1.

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the two specific forward primers wild type (WT) 5’CCAA-GATGGGCGTGATGG and mutant (MT) 5’CCAAGATGGGCGTGATGA respectively for the WT and for the MT alleles), and 1 U of AmpliTaq GOLD DNA polymerase (PE Biosystems) in a thermal cycler 2400 (PE Biosystems). After 35 cycles at 95°C for 30 s, 55°C for 30 s and at 72°C for 30 s, PCR products were detected in a 3% agarose gel.

**Statistical analysis**

Allele frequencies were calculated by direct gene counting and the differences analysed by the $\chi^2$ test using 2×2 contingency tables. The relative risk (RR) was calculated according to the Woolf formula [29], whilst the delta values for linkage disequilibrium were calculated using the software Arlequin 1.1 available for non profit use on the Internet at http://anthropologie.unige.ch/arlequin/.

**Results**

The allele and genotype frequencies of the H/L polymorphism at position −550 were similar in the three subgroups of sporadic IgAN patients and in familial cases of IgAN. No differences were revealed when they were compared with 74 healthy Italian controls (Table 1). Allele L showed a higher genotype and phenotype frequencies than allele H: 89% of the 135 analysed sporadic IgAN patients, 82% of familial IgAN patients and 88% of healthy controls were positive for the L allele, while subjects positive for the H allele in these three groups were 56%, 64% and 59% respectively ($P$, not significant).

The frequencies of alleles Y and X at position −221, as well as the genotype and phenotype frequencies, did not show any differences between the different groups of IgAN sporadic patients (composed by 41 IGAN1, 55 IGAN2 and 39 IGAN3), 22 IgAN familial cases and 74 controls (Table 2). The majority of the analysed patients carried the Y allele (96% of sporadic IgAN patients, 95% of familial IgAN patients), that showed a similar frequency also in healthy control subjects (97%).

The deletion of 6 bp (−328delAAAGAG) at position −328, showed similar allelic and phenotypic frequencies among the 138 sporadic IgAN, 22 familial IgAN and 74 healthy controls (Table 3), even though the phenotype and allele frequencies of the deleted allele was slightly increased in all IgAN groups in respect to the control population. The frequency of the homozygous deleted genotype was also slightly increased in the IgAN patients. This genotype was significantly more frequent in familial IgAN than in healthy controls (0.14 and 0.03, respectively, $P = 0.0427$; RR: 5.684; 95% confidence limits: 0.88–36.5). This difference, however, was no longer significant.

**Table 1.** Frequencies of H/L polymorphisms at position −550 of the promoter region of MBL2 in patients with sporadic and familial IgAN

<table>
<thead>
<tr>
<th>Frequencies</th>
<th>Sporadic IgAN</th>
<th>Familial IgAN</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 41)</td>
<td>(n = 55)</td>
<td>(n = 135)</td>
</tr>
<tr>
<td>Allele H</td>
<td>31 (38%)</td>
<td>34 (31%)</td>
<td>91 (34%)</td>
</tr>
<tr>
<td>L</td>
<td>51 (62%)</td>
<td>76 (69%)</td>
<td>179 (66%)</td>
</tr>
<tr>
<td>Genotype H/H</td>
<td>7 (17%)</td>
<td>4 (7%)</td>
<td>15 (11%)</td>
</tr>
<tr>
<td>H/L</td>
<td>17 (41%)</td>
<td>26 (47%)</td>
<td>61 (45%)</td>
</tr>
<tr>
<td>L/L</td>
<td>17 (41%)</td>
<td>25 (45%)</td>
<td>59 (44%)</td>
</tr>
<tr>
<td>Phenotype H</td>
<td>24 (59%)</td>
<td>30 (54%)</td>
<td>76 (56%)</td>
</tr>
<tr>
<td>L</td>
<td>34 (83%)</td>
<td>51 (93%)</td>
<td>120 (89%)</td>
</tr>
</tbody>
</table>

**Table 2.** Frequencies of X/Y polymorphisms at position −221 of the promoter region of MBL2 in patients with sporadic and familial IgAN

<table>
<thead>
<tr>
<th>Frequencies</th>
<th>Sporadic IgAN</th>
<th>Familial IgAN</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 41)</td>
<td>(n = 55)</td>
<td>(n = 135)</td>
</tr>
<tr>
<td>Allele X</td>
<td>13 (16%)</td>
<td>21 (19%)</td>
<td>50 (18%)</td>
</tr>
<tr>
<td>Y</td>
<td>69 (84%)</td>
<td>89 (81%)</td>
<td>220 (81%)</td>
</tr>
<tr>
<td>Genotype X/X</td>
<td>1 (3%)</td>
<td>1 (2%)</td>
<td>5 (4%)</td>
</tr>
<tr>
<td>X/Y</td>
<td>11 (26%)</td>
<td>19 (34%)</td>
<td>40 (30%)</td>
</tr>
<tr>
<td>Y/Y</td>
<td>29 (71%)</td>
<td>35 (64%)</td>
<td>90 (67%)</td>
</tr>
<tr>
<td>Phenotype X</td>
<td>12 (29%)</td>
<td>20 (36%)</td>
<td>45 (33%)</td>
</tr>
<tr>
<td>Y</td>
<td>40 (97%)</td>
<td>54 (98%)</td>
<td>130 (96%)</td>
</tr>
</tbody>
</table>

Patients with sporadic IgAN were also subdivided in three different groups, according to the progression of the disease.
after the correction of the probabilities level for the number of comparisons made.

Finally, the genotype and phenotype frequencies of the WT and the MT alleles at codon 54 did not show any differences between the different groups of patients and controls (Table 4). In fact, the Gly54Asp phenotype was typed in 31% of 138 IgAN sporadic patients, 24% of 21 analysed familial cases, and 25% of 74 healthy controls.

Genotype frequencies for polymorphisms in the promoter region and in the first exon of the MBL2 gene agree with the Hardy-Weinberg equilibrium. A significant linkage disequilibrium was observed between the deletion at position −328 and the L allele at position −550 in healthy population (Δ = 0.021; P = 0.0421). The same preferential gametic association was also found in IgAN patients.

Discussion

MBL is a C-type lectin that has a high affinity to mannose and N-acetyl glucosamine. This protein, codified by the MBL2 gene, takes part in natural immunity. It is known to activate C4 and C2 without the C1 component, which is called the lectin pathway. At present it is unknown whether this so-called lectin pathway of complement activation plays any role in the pathogenesis of human glomerulonephritis.

Recent studies [18–20] have investigated the presence of MBL and MBL-mediated complement activation in renal glomeruli of IgA nephropathy patients using immunohistochemical methods. In about 20% of patients with IgA nephropathy MBL was detected in the glomerular mesangial area and co-localized with IgA1. Interestingly, MBL-positive patients showed disease characteristics denoting heavier immunological involvement. The C2- and/or C4-positive rate was higher in the MBL-positive group than in the MBL-negative group of IgA nephropathy [18]. Mesangial cell proliferation was also marked in MBL-positive patients [18]. The MBL-positive IgAN patients were younger than the MBL-negative patients and the renal biopsies had been performed at an early stage of the disease [19]. Correlation between glomerular deposition of MBL and renal function showed contrasting results. While one study failed to show any significant correlation [19], another study demonstrated that MBL-positive patients had a reduced CrCl
and a higher urinary protein excretion in comparison with MBL-negative patients [18].

The analysis of non-IgAN glomerulonephrites also had contrasting results. While MBL deposition was recognized only in IgAN biopsies [18] or in few cases of immune complex-mediated glomerulonephritis, including lupus nephritis and membranous nephropathy [19], another study demonstrated that MBL was present in the glomeruli of patients with glomerulonephritis involving deposition of IgG and activation of the classical pathway of complement. MBL was, in fact, detected in the glomeruli of the majority of patients with lupus nephropathy, membranous nephropathy, membranoproliferative glomerulonephritis and anti-GBM nephritis [20]. Moreover, focal segmental deposits of MBL were also present in focal segmental glomerulosclerosis, amyloidosis and advanced renal fibrosis. Finally, a recent study [30] reported the presence of glomerular deposition of the lectin pathway components, including MBL and MASP-1, on renal biopsies of patients with Henoch–Schönlein purpura (HSP) and of patients with other glomerulonephritis, including IgAN. However, the complement activation via lectin pathway was greater in HSP and in IgAN than in non-IgA glomerulonephritis. Therefore, MBL is suspected to be involved in glomerular complement activation through the lectin pathway, thus inducing glomerular injury that can be seen in IgAN. As oligosaccharide chain alterations such as reduced sialic acid and galactose of IgA1 molecule have been reported in IgAN patients, MBL may bind to the IgA1 molecule via interaction between MBL and the sugar chain. The lectin pathway of complement activation, which is started by the MBL–MASPs complex, may contribute to the development of glomerular injury in a significant number of cases with IgAN. However, MBL may also bind to agalactosyl oligosaccharides of IgG that terminate in N-acetylglucosamine. The extent to which the lectin pathway of complement contributes to overall complement activation in the glomeruli remains unknown, but it may be not exclusive of IgAN.

As polymorphic variants of the promoter of MBL2 gene regulate the transcription of the gene and the level of the protein in the serum [23,31], it may be hypothesized that they may be responsible for the differences in MBL deposition as demonstrated by immunohistochemical studies. In addition, 95 MBL plays a crucial role in the host defence against various pathogens [21], its relation to infection may be associated with the pathogenesis of IgAN. The DNA analysis of MBL polymorphisms in IgAN patients, which has been performed in our study for the first time to our knowledge, gave us the opportunity to disclose new insight on the pathogenesis of IgAN and to characterize possible genetic markers of disease susceptibility and progression. In order to facilitate this aim, we included IgAN familial patients in our study, in whom genetics factors involved in the pathogenesis of the disease were expected to be more relevant than in sporadic cases.

The results of the present study, however, failed to demonstrate a clear association between IgAN and polymorphic regions of the promoter and of the first exon of MBL2. In IgAN familial cases we showed an increased frequency of the homozygous genotype for the 6 bp deletion at position −328. This genotype seems to be slightly increased in patients with sporadic IgAN. The influence of this deletion on MBL basal levels should be analysed. It should recognized, however, that the deleted allele at position −328 is in linkage disequilibrium with the L allele at position −550, which confers a lower level of serum protein. Nevertheless, our results could be influenced by the low number of familial IgAN patients examined.

Finally, the polymorphism Gly54Asp, that in the Caucasian population is the most important cause of reduction of MBL blood levels by 5–10 times in heterozygous individuals [25], does not confer any susceptibility or resistance to IgAN, nor influence the progression of the disease.

In conclusion, several data suggest the possibility that the complement cascade is initiated through the lectin pathway in certain forms of glomerulopathy characterized by diffuse mesangial deposition of IgA. However, the susceptibility or the progression of IgAN seems to be independent of the polymorphisms of the MBL2 gene, although the relationship between the variability of the MBL deposition in biopsies and MBL2 polymorphisms should be matter of further study.

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