Role of genetic polymorphisms in factor H and MBL genes in Tunisian patients with immunoglobulin A nephropathy

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Abstract: The molecular mechanisms of IgA nephropathy (IgAN) remain poorly understood. Several different polymorphic genes have been investigated in order to demonstrate their possible association with this disease. It is evident that mainly alternative and lectin pathways complement activation and play an important role in renal injury of IgAN. This study was conducted to determine eventual deficiencies of factor H in the SCR20 gene region and to look for a possible association between the polymorphism (+54) exon 1 of the MBL gene and the predisposition in Tunisian patients with IgAN. We then evaluated the effects of these FH mutations and/or this MBL polymorphism on nephropathy susceptibility and progression. Polymorphism A/B (+54) in the exon1 of the MBL gene and analysis within the C-terminal domain of the protein SCR20 in the exon 22 of the factor H (FH) gene were conducted in 36 sporadic IgAN Tunisian patients and 117 age and gender matched healthy subjects recruited from blood donors, by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and direct sequencing respectively. The analysis of the Gly54Asp (+54) mutation of the MBL gene according to the criteria of gravity of the IgAN reveals that the patients with genotype AB present more frequently with end-stage renal disease (ESRD) compared with those of genotype AA [OR: 8, CI (1.74–54.49), P = 0.019]. Moreover, the variant allele B was statistically more frequent than the allele A in patients with an association with initial arterial high blood pressure, ESRD and class V of the Haas classification compared to those without this association (P = 0.009). The direct sequencing of exon 22 (SCR 20) of FH gene did not reveal any abnormal mutational deficiency for this factor in all patients and controls. The data did not support the hypothesis that FH is a susceptibility factor for the IgAN. However the data did show there was an association between AB (+54) exon1 MBL genotype and severe sporadic forms of this disease in Tunisian patients. Because of the small number of subjects studied, a much larger cohort of IgAN patients with varying severity of the disease and its progression would seem necessary to confirm these findings.

Keywords: IgA nephropathy, genetics, factor H, MBL polymorphism, susceptibility

Introduction

Immunoglobulin A nephropathy (IgAN) is the most prevalent form of primary glomerulonephritis and is an important cause of end-stage renal disease requiring renal transplantation. The molecular mechanisms of IgAN remain poorly understood. The environmental and genetic factors together with various inflammatory mediators were involved in the pathogenesis of IgAN.¹

The mannose-binding lectin (MBL) gene is located on the long arm of chromosome 10 at 10q11.2-q21 and contains 4 exons. Several functional polymorphisms of the MBL gene are localized in the region of the promoter and the exon 1 have been described.
The presence of any of mutant allele results in a significant reduction of serum MBL concentrations and have been reported,\textsuperscript{2–5} in particular codon 54 mutation of the MBL gene, has been found to be associated with recurrent infections and autoimmune diseases.\textsuperscript{6–8} MBL is a C-type lectin that has a high affinity to mannose. This protein takes part in natural immunity and it is known to activate C4 and C2 without C1 component, which is called “lectin pathway”.

The factor H (FH) is a 155 Kd soluble multiple-domain glycoprotein that it is abundant in human plasma. It regulates the alternative pathway (AP) activation of the complement system and it is a key molecular component of immune defense. The gene encoding FH is localized on the long arm of chromosome1 at 1q32, a locus called RCA (regulators of complement activation)\textsuperscript{7} which contains genes encoding different regulatory proteins of complement activation. Primary sequence analysis of FH revealed a tandem array of 20 homologous units, called short consensus repeats (SCRs),\textsuperscript{10} each about 60 amino acid residues long. Conserved amino acid residues in the SCR unit include four cysteines that form disulfide bonds in a Cys1–Cys3 and Cys2–Cys4 linkage. These disulfide bridges are important in maintaining the characteristic structure of the SCR module.\textsuperscript{11–13} Factor H controls amplification via the AP both in fluid phase and selectively on self-surfaces, thereby helping to direct complement toward its target: foreign or unwanted cells or particles. Via unknown mechanisms, FH competes with factor B for binding to C3b, accelerates the decay of C3 convertase and acts as a cofactor for factor I-mediated cleavage of C3b.\textsuperscript{14,16} Low-resolution structural studies suggest a “beads-on-a-string” arrangement of SCRs within an elongated FH molecule that may bend back on itself. The C3b-binding sites have been mapped to SCRs 1–4 and SCRs 19–20. The C-terminal C3b-binding site additionally binds polyanions on certain cellular surfaces, conferring to them resistance to damage as a consequence of AP activation.\textsuperscript{17,18}

Mutations and polymorphisms in the regulator of complement activation, FH, have been linked to atypical hemolytic uremic syndrome,\textsuperscript{19} membranoproliferative glomerulonephritis and recurrent membranococcal meningitis. The majority of these mutations are heterozygous and cause either single amino acid exchanges or premature translation interruption within the C-terminal domain of the protein SCR20.\textsuperscript{20}

This study was conducted to determine eventual deficiencies of factor H in SCR20 gene region and to look for a possible association between the single nucleotide polymorphism (SNP) (+54) exon 1 of the gene MBL; and the predisposition in Tunisian patients with IgAN and to evaluate the effects of these FH mutations and/or this MBL polymorphism on this nephropathy susceptibility and progression.

Material and methods

Patients

A diagnostic of sporadic IgAN was assigned to 36 Tunisian patients (28 males and 8 females) by renal biopsy in the department of Nephrology, Charles Nicolle Hospital. The mean age was 35 ± 14 years and the mean disease duration at the time of the study was 5.54 ± 3 years. None of the patients had either clinical or laboratory evidence of Henoch-Schoenlein purpura, liver or systemic disease. Patients were included in the study between August 2004 and March 2008. From all patients, clinical and laboratory data were obtained retrospectively. The criteria of severity of the disease were estimated for all patients; Haas histological classification, the presence of an initial arterial high blood pressure (HAP), as well as the renal function.

The glomerular filtration rate was estimated using the Cockcroft–Gault equation for creatinine clearance (eGFR). The renal function was considered normal when the (eGFR) was >60 mL minute/1.73 m\textsuperscript{2}. The chronic renal failure (CRF) stage 1 to 5 was defined as follow as:

- **Stage 1**: normal or relatively high eGFR (>90 mL/minute/1.73 m\textsuperscript{2}) but presence of kidney damage (including abnormalities in blood or urine test or imaging studies).
- **Stage 2**: mild reduction in eGFR (60–89 mL/minute/1.73 m\textsuperscript{2}) with kidney damage.
- **Stage 3**: moderate reduction in eGFR (30–59 mL/minute/1.73 m\textsuperscript{2}).
- **Stage 4**: severe reduction in eGFR (15–29 mL/minute/1.73 m\textsuperscript{2}) and preparation for renal replacement therapy.
- **Stage 5**: established kidney failure (ESRD) (GFR < 15 mL/minute/1.73 m\textsuperscript{2}) required permanent renal replacement therapy.

The Haas histological classification was defined as: subclass I, minimal or no mesangial hypercellularity, without glomerular sclerosis; subclass II, focal and segmental glomerular sclerosis without active cellular proliferation; subclass III, focal proliferative GN; and subclass IV, diffuse proliferative GN; and subclass V, any biopsy showing > or = 40% globally sclerotic glomeruli and/or > or = 40% estimated cortical tubular atrophy or loss.

Controls

As a control group, we studied 117 age and gender matched healthy subjects recruited from the blood donors of the same area as the patients.
The study was approved by the local ethics committee and informed consent was obtained from all subjects.

Methods

Complement assays

Freshly drawn ethylene diamine tetraacetic acid (EDTA) plasma samples were obtained from all patients.

Functional CH50 levels were measured by the method of Mayer and expressed in units CH50/mL.

Functional alternative pathway activity (AP50) was measured in agarose plates, using 0.5% guinea pig erythrocytes suspended in 1% agarose made with veronal buffered saline, pH:7.4, 0.01M ethylene glycol tetraacetic acid (EGTA) and 0.005 M MgCl2. Results were expressed as a percentage of mean values obtained with reference plasma prepared from 100 healthy blood donors (normal range, 100% ± 30%).

Plasma concentrations of the third (C3) and/or fourth (C4) complement component and Factor B (FB) antigens were measured by nephelometry (Minineph™, The Binding Site, Birmingham, UK). Normal values established with pooled plasma from 100 healthy donors ranged 1.21 ± 0.36, 0.26 ± 0.12 and 0.20 ± 0.11 g/l, respectively. Plasma levels of the C3 and/or C4 below the lower limit of normal ranges, defined as mean ± 1 standard deviation (SD), were taken to indicate hypocomplementemia.

Serum samples were used to quantify FH antigen concentration by radial immunodiffusion (RID) assay using commercially available kits (The Binding Site, Birmingham, UK). Results of the RID method were confirmed by enzyme-linked immunosorbent assay (ELISA) assay, described previously by Dragon-Durey, using a mouse anti-human FH monoclonal antibody (Serotec, Fidlington, UK). Normal ranges were 100% ± 30%, as calculated by the results from 100 individual healthy donors.

Genomic FH DNA sequencing

Analysis of the FH gene was performed using DNA extracted from 10 mL peripheral EDTA blood using the standard salting-out procedure. Genomic DNA was amplified by means of polymerase chain reaction (PCR) using nucleotides flanking each exon.

All patients and controls were analyzed for SCR20 exon 22, using primer designed on published genomic sequences: 5’ CGA ACC TCA TTT TCA CAT CGA3’ (Forward primer) and 5’ AAC CGT TAG TTT TCC TGG ATT TAA 3’ (Reverse primer).

The PCR reactions were performed in 20 ul final volume using 10 pmol of each primer and containing 100 ng of extracted DNA, 0.5 U of Taq polymerase (Promega, USA), 1.5 mM MgCl2 and 0.2 mMol/l dNTP (deoxynucleotide triphosphate). After an initial denaturing time of 5 minutes at 94°C, PCR reactions were run for 30 cycles including 30 seconds at 94°C, 45 seconds at 60°C and 45 seconds at 72°C, with a final extension at 72°C for 7 minutes. PCR products were purified using QiAquick™ PCR purification kit (Qiagen, Hilden, Germany). Direct sequencing of the purified PCR products was then carried out by the dye terminator cycle sequencing method (Applied Biosystems, Courtabeuf, France) using an ABI prism 310 sequencer (Applied Biosystems). Sequence analyses were performed using the Sequencher® software, (Ann Arbor, MI, USA). The number of nucleotides or codons referenced is indicated according to Warwicker and colleagues.

Polymorphism A/B (+54) I of the gene MBL

The Gly54Asp mutation at codon 54 of gene MBL was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

The PCR reactions were performed in 50 ul final volume using 10 pmol of each primer: 5’AGTCGACCAGATT-GTAGGACAGAGA 3’, forward primer and 5’AGGATCCAGGCAGTTTCTCTGGAAGG 3’, reverse primer, and containing 200 ng of extracted DNA, 1 U of Taq polymerase (Promega, USA), 1.5 mM MgCl2 and 0.2 mMol/l dNTP. After an initial denaturing time of 2 min at 94°C and 5 min at 72°C; PCR reactions were run for 35 cycles including 30s at 94°C, 1 min at 55°C and 2 min at 72°C, with a final extension at 72°C for 5 min. The PCR products obtained were 349 bp and were digested by 1 U of the enzyme Banl (Promega, Madison, WI, USA) at 37°C for 2 hours and then subjected to electrophoresis in 3% agarose gel stained with ethidium bromide. The PCR generated two fragments of 260 and 89 bp for A allele and one fragment of 349 bp for B allele.

Statistical analysis

Allelic and genotypic frequencies were evaluated by direct counting. Statistical comparisons were performed, between patients and controls, by Pearson’s chi-square test calculated on 2 × 2 contingency tables. A Fisher exact test was used when an expected cell value was less than 5. P value < 0.05 was considered to be statistically significant. Statistical power was calculated using a web power calculator (http://calculators.stat.ucla.edu/powercalc/). The strength of the association of MBL (+54) genotypes as genetic factor with
the frequency of a certain IgAN symptom was estimated by the calculation of the Odds ratios (OR) (OR > 1: positive association; OR = 1: no influence; OR < 1: protective) and 95% confidence intervals (95% CI), using the same software. The Mann-Whitney U test was used to determine whether there was any significant difference between MBL genotype groups for continuous data.

Results

Clinical characteristics
At the time of this study, among 36 patients, 12 (33.33%) had severe histological lesions, 24 (66.66%) had an initial arterial high blood pressure (HAP), 14 (38.88%) were in CRF and 10 (27.77%) were in ESRD. Six IgAN patients had an association between three criteria of severity disease: HAP, class V of Haas classification and ESRD.

Complement component assessment
The mean C3 concentration was 1.31 g/L (range 0.90–1.72) and C4 was 0.41 g/L (range 0.26–0.56). Five patients had low C3 levels of 0.90 g/L (0.77, 0.27, 0.80, 0.84 and 0.86 g/L respectively) and eight had low C4 levels (0.24, 0.25, 0.23, 0.24, 0.20, 0.12, 0.22 and 0.17 g/L respectively). None of patients had low factor H level (Table 1).

Polymorphism A/B (+54) exon 1 of the gene MBL
The genotypic and allelic frequencies of SNP (A/B) at the codon 54 of the gene MBL do not show statistically significant differences between the patients and the controls (Table 1). The analysis of this polymorphism, according to the criteria of gravity of the IgAN, reveals that the patients of genotype AB present more frequently as ESRD compared with those of genotype AA [OR: 8, CI (0.90–86.17), P = 0.038]. Moreover, among the 6 patients carrying three severity criteria of IgAN disease (HAP, class V of Haas classification and ESRD), 4 (66.6%) had the genotype A/B. So, the allele B was statistically more frequent than the allele A in patients with this association compared to those without it (P = 0.009) (Table 2). Nevertheless, there was no statistically significant correlation between this variant allele B and low levels of C3 and/or C4 observed in study patients (data not shown).

Genetic HF genotyping
The direct sequencing of exon 22 (SCR 20) gene of this protein did not reveal mutational deficiency abnormalities for this factor in all patients and controls.

Discussion

It is currently established that activating or regulating complement proteins plays a role in the renal injury of the IgAN.26 MBL is the key protein of the activation of the complement by the lectin pathway. This activation originate with the
deposition of MBL in the renal glomerular mesangial area, and co-localized with IgA, in about 20% of IgAN patients. Furthermore, Roos and colleagues found the same deposition in IgAN patients who had severe renal histological lesions and with the severe clinical forms of the disease. Mesangial cell proliferation was also marked in MBL-positive patients. In addition, MBL deficiency and low serum levels are strongly associated with the presence of MBL alleles that encode three different structural variants of MBL polypeptide (codon 52, 54 and 57). Among these variants at the exon1, Gly54Asp is the most important cause in the reduction of the serum MBL levels. Several different polymorphic genes have been investigated in order to demonstrate their possible association with IgAN susceptibility and progression, as HLA-DQ, cytokines which are associated with the inflammatory factor (tumor necrosis factor [TNF], interleukin 1 [IL1]) or Fc α receptor (CD89). These studies have shown conflicting results and it may be postulated that genetic factors vary among different populations. This is in conflict with the reported results by Pirulli and colleagues, because in our study, the analyzed polymorphism of the MBL gene did not appear to be involved in the susceptibility of IgAN. Nevertheless, the analysis of the clinical, histological and biological characteristics of the patients reveals a positive association between the variant allele B and the severe forms of the renal disease with class V histological of Haas, ESRD and an initial HAP.

In conclusion our study has not found evidence to support the hypothesis that FH is a susceptibility factor for the IgAN, and yet does show an association between A/B (+54) exon1 MBL genotype and severe sporadic forms of this disease in Tunisian patients. However, because of the small number of subjects studied, these results must be treated with caution. A much larger cohort of IgAN patients with varying severity of the disease and its progression would seem necessary to confirm these findings.

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Disclosures
The authors report no conflicts of interest relevant to this research.

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