Original Article

The Influence of Two *megsin* Polymorphisms on the Progression of IgA Nephropathy

(IgA nephropathy / gene polymorphisms / *megsin*)

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Abstract. The clinical course of chronic renal diseases and their progression to ESRD is highly variable. The strongest predictors of poor outcome of IgAN involve hypertension, severe proteinuria and elevated serum creatinine level. Different candidate gene polymorphisms have been advocated as possible modulators of the progression of IgAN. Megsin belongs to the serpin superfamily and was mapped to chromosome 18q21.3. Megsin plays a role in the regulation of a wide variety of processes in mesangial cells, such as matrix metabolism, cell proliferation, and apoptosis. Overexpression of Megsin might lead to mesangial dysfunction, and impair degradation of the mesangial matrix and disposal of immune complexes. The expression of Megsin is upregulated in a variety of glomerular diseases with mesangial injury in humans and in animal models. We investigated a possible association of two C2093T, C2180T polymorphisms of the *megsin* gene with the progression of IgAN towards ESRD, as well as the haplotype reconstruction of *megsin* gene polymorphisms and clinical manifestation of IgAN. We examined a group of 197 pts with histologically proven IGAN (84 pts with normal renal function, 113 pts with progressive renal insufficiency); as a control group we used 61 genetically unrelated healthy subjects. DNA samples from collected blood were genotyped for two single-nucleotide polymorphisms of *megsin* C2093T, C2180T by means of PCR with defined primers, electrophoresis on 2% agarose gel, UV light visualization and direct sequencing. The *megsin* genotype distribution showed no differences among the groups of IgAN with normal renal function, progressive renal insufficiency and the control group. According to haplotype analysis, the TT haplotype (defined as T-2093, T-2180 alleles) was substantially more frequent in pts with IgAN and normal renal function (Table 1, P = 0.025; Table 3, P = 0.062). Pts in the progressive group showed significantly higher levels of 24-h UP (3.53 ± 2.80 vs 2.06 ± 2.06, P = 0.042; Table 10), diastolic blood pressure (92.89 ± 15.66 vs 84.93 ± 10.43, P = 0.047; Table 10) and almost significantly systolic blood pressure (150.79 ± 32.88 vs 135.21 ± 14.88, P = 0.058; Table 10). We confirmed the negative prognostic influence of hypertension and proteinuria on the progression of IgAN in Czech pts. We found out that the TT haplotype (defined as T-2093, T-2180 alleles) could play a protective role in the progression of IgAN. In our Czech population, we excluded the negative influence of the 2093C-2180T haplotype, which was proposed by Chinese studies.

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Abbreviations: BP – blood pressure, ESRD – end-stage renal disease, IgA – immunoglobulin A, IgAN – IgA nephropathy, IgA-CICs – IgA-containing immune complexes, *megsin* – mesangial cell-specific gene with homology to serpin, PCR – polymerase chain reaction, pts – patients, S-Cr – serum creatinine, serpin – serine protease inhibitor, SNPs – single-nucleotide gene polymorphisms, UP – proteinuria, UTR – untranslated region.

Introduction

IgA nephropathy (IgAN) is a mesangial proliferative glomerulonephritis characterized by diffuse mesangial deposition of immunoglobulin A (IgA, mainly IgA 1) or IgA-containing immune complexes (IgA-CICs) and may progress to end-stage renal disease (ESRD) in 15–40 % of pts in the course of twenty years.

Strong predictors of progression of IgAN include arterial hypertension, severe proteinuria, elevated serum creatinine level, as well as histological signs of glomerular sclerosis and interstitial fibrosis. Recently, hyperli-

poproteinemia and hyperuricaemia have been implicated in the progression of IgAN. However, different candidate gene polymorphisms, affecting mainly the onset/development of arterial hypertension, have been suggested as possible modulators of the progression of IgAN towards ESRD (Syrjanen et al., 2000a; Narita et al., 2002, 2003a, b; Song et al., 2003).

megsin, mesangial cell-specific gene with homology to serpin, belongs to the serpin (serine protease inhibitor) superfamily. *megsin* plays a role in the regulation of a wide variety of processes in mesangial cells, such as matrix metabolism, cell proliferation, and apoptosis. Inagi et al. (Inagi et al., 2002a, 2003) speculate that overexpression of *megsin* might impair the degradation of mesangial matrix, lead to disposal of immune complexes and mesangial dysfunction. *megsin* was mapped to chromosome 18q21.3 (Inagi et al., 2002b, 2003). The expression of *megsin* is upregulated in diseases with mesangial proliferation and extracellular matrix expansion (Bachmann et al., 1995; Kruithof et al., 1995; Bird et al., 1998) in human and in animal models. *megsin* therefore represents a good candidate for playing a role in the pathogenesis of IgAN.

Xia et al. (2006) found out an association of the *megsin* 2093C-2180T haplotype at the 3' untranslated region (UTR) with the severity and progression of IgAN, even though a previous case-control study of polymorphism C2093T did not demonstrate a significant association with the disease progression (Szelestei et al., 2000).

We decided to investigate a possible association of two single-nucleotide gene polymorphisms (SNPs) of *megsin*, i.e. C2093T and C2180T, with the progression of IgAN towards ESRD, as well as the haplotype reconstruction of *megsin* gene polymorphisms and clinical manifestation of IgAN.

Material and Methods

We examined a group of 197 patients (pts) with histologically proven IgAN, and as a control group we used 61 genetically unrelated healthy subjects. The pts were divided into a stable group (84 pts) defined by renal function within normal ranges, and a progressive group (113 pts) defined by an increase of the serum creatinine level over 50 % during the period of two years or less between the time of renal biopsy and the latest follow-up (Tables 1, 2).

Informed consent was obtained from all pts included. The pts with the diagnosis of Henoch-Schonlein purpura as well as with liver cirrhosis were not included into the study.

Genomic DNA was isolated from peripheral-blood lymphocytes by the salting-out procedure.

C2093T was genotyped by PCR amplification of a 256-bp fragment in the 3' UTR of the gene. The primer sequences were: sense: 5'-TTG TTG ACC TAT GAA GAT TTT AGA-3', antisense: 5'-AAC TGC CAA CAG TTA AAA GA-3'. The reaction mixture (50 µl of PCR product) contained 5× PCR buffer, 3 mmol/l $MgCl_2$, 1 µl of deoxynucleotide triphosphates (dNTPs), 1.2 unit Taq DNA polymerase (MBI), 1 µl of each primer, 1 µl of genomic DNA, and water. PCR conditions were following: initial denaturation at 94° C for 5 min, followed by 30 cycles of denaturation, annealing (50° C for both alleles of C2093T polymorphism) and extension. A final extension was performed at 72° C for 5 min. The PCR products were digested with restriction endonuclease *Hae*III, electrophoresed on 2% agarose gel with ethidium bromide and visualized under ultraviolet light. The 2093C allele produces 114- and 142-bp fragments; the T allele has no digestion site. The genotyping results were also confirmed by direct sequencing.

C2180T was genotyped by PCR amplification with the following primers: sense: 5'- TTG TTG ACC TAT GAA GAT TTT AGA-3' and antisense: 5'- TTC TTT ATT CTG ATA AAT AGA GGA A -3'. The PCR products were sequenced in an ABI PRISM 310 Genetic Analyzer with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Clinical parameters

Clinical data were collected by retrospective examination of the case records (in 80 % of all pts, the data of other pts were not available) and included age, duration of follow-up, serum creatinine (S-Cr), 24-h urinary protein (UP), systolic and diastolic blood pressure (BP), serum cholesterol, serum triglycerides and serum uricaemia at the time of renal biopsy.

Statistical analyses

Allele frequencies of the C2093T, C2180T *megsin* polymorphisms were estimated by the allele counting method, and the Hardy-Weinberg equilibrium was tested. The χ^2 test was used to compare *megsin* genotype distribution in the pts and in the controls, and the frequencies of different genotypes between the IgAN sta-

Table 1. Haplotype frequencies in stable and control groups

Groups	2093T-2180C	2093C-2180T	2093T-2180T*
IgAN - stable group	47.6	45.8	6.0
control group	54.1	44.2	0.9
P value	0.2763	0.7901	0.0246

Table 2. Haplotype frequencies in progressive and control groups

Groups	2093T-2180C	2093C-2180T	2093T-2180T	2093C-2180C
IgAN – progressive group	49.5	46.4	2.3	1.8
control group	54.1	44.2	0.8	0.8
P value	0.4164	0.6937	0.3391	0.4841

ble and progressive groups. Haplotype frequencies were estimated by the Maximum Likelihood Method (Haploview, version 3.32) in pts and controls. Differences of haplotype frequencies between pts and controls, and between the IgAN stable and progressive group were calculated by using the χ^2 test (STATISTICA version 7.1). Clinical parameters were compared by means of *t*-test.

Results

We investigated an association of two single-nucleotide gene polymorphisms of *megsin* with the progression of IgAN towards ESRD.

We compared the frequencies of different genotypes between the stable and progressive IgAN groups. The *megsin* genotype distribution showed no differences among the groups of IgAN with normal renal function (1. C2093T – 20.2 % CC, 52.4 % TC, 27.4 % TT; 2. C2180T – 23.8 % CC, 48.8 % TC, 27.4 % TT), progressive renal insufficiency (1. C2093T – 25.7 % CC, 45.1 % TC, 29.2 % TT; 2. C2180T – 28.6 % CC, 44.6 % TC, 26.8 % TT) and control group (1. C2093T – 16.4 % CC, 57.4 % TC, 26.2 % TT; 2. C2180T – 27.9 % CC, 54.1 % TC, 18 % TT).

Table 3. Haplotype frequencies in stable and progressive IgAN groups

Groups	2093T-2180C	2093C-2180T	2093T-2180T*	2093C-2180C
Progressive (%)	49.4	46.4	2.3	1.8
Stable (%)	47.6	45.8	6.0	0.6
P value	NS	NS	$\chi2 = 3.484$, P = 0.062	NS

* NS – not significant

Table 4. Haplotype frequencies in hypertensive and non-hypertensive groups

Groups	2093T-2180C	2093C-2180T	2093T-2180T
Hypertensive (%)	43.8	48.5	6.2
Non-hypertensive (%)	51.0	43.8	5.2
P value	NS	NS	NS

Table 5. Haplotype frequencies in groups with high or low proteinuria

24-h UP (g/d)	2093C-2180T	2093T-2180C	2093T-2180T
24-h UP ≥ 2 (%)	47.8	44.9	5.8
24-h UP < 2 (%)	46.3	47.5	6.2
P value	NS	NS	NS

The distribution of *megsin* genotypes did not differ among IgAN with normal renal function, IgAN with progressive renal insufficiency, and control group (Tables 6, 7).

Haplotype analysis

We calculated the linkage disequilibrium values among the groups with two *megsin* polymorphisms analysed, and the haplotypes were reconstructed by the Maximum Likelihood Method.

None of these studied haplotypes was significantly associated with IgAN (Table 8), but the TT haplotype occurrence (defined as T-2093, T-2180 alleles) was substantially more frequent in pts with IgAN and normal renal function than in the control group (Table 1, P = 0.025) or in comparison with the progressive group (Table 3, P = 0.062; considerably close to the level of significance). Haplotype frequencies did not differ among hypertensive and non-hypertensive groups, and in groups with high or low proteinuria (Table 4, 5).

Finally, we compared the clinical parameters (in 80 % of pts in total) between the progressive group and the stable group (Table 9, 10).

Pts in the progressive group showed significantly higher levels of 24-h UP (3.53 ± 2.80 vs 2.06 ± 2.06, P = 0.042; Table 10), higher diastolic blood pressure (92.89 ± 15.66 vs 84.93 ± 10.43, P = 0.047; Table 10) and almost significantly higher systolic blood pressure (150.79

Table 6. Genotype frequencies of C2093T polymorphism of the megsin gene among IgAN patients

Groups	T/T	T/C	C/C
stable (%; N = 84)	27.4	52.4	20.2
progressive (%; N = 113)	29.2	45.1	25.7
control (%; N = 61)	26.2	57.4	16.4

Table 7. Genotype frequencies of C2180T polymorphism of the megsin gene among IgAN patients

Groups	T/T	T/C	C/C
stable (%; N = 84)	27.4	48.8	23.8
progressive (%; N = 113)	26.8	44.6	28.6
control (%; N = 61)	18.0	54.1	27.9

Table 8. Haplotype frequencies in stable, progressive and control groups

Groups	2093T-2180C	2093C-2180T	2093T-2180T	2093C-2180C
IgAN pts	48.7	46.2	3.9	1.3
control group	54.1	44.2	0.9	0.9
P value	0.2982	0.7072	0.097	0.696

± 32.88 vs 135.21 ± 14.88, P = 0.058; Table 10). No statistically significant differences were observed in the other clinical parameters tested between the two groups.

Discussion

IgAN, a mesangial proliferative glomerulonephritis, belongs to the most common nephropaties.

Many gene polymorphisms have been reported to be associated with the development and/or progression of IgAN (Syrjanen et al., 2000a; Kim et al., 2001; Goto et al., 2002; Narita et al., 2002, 2003a, b; Song et al., 2003).

Mesangial cells play an important role in maintaining the structure and function of the glomerulus and in the pathogenesis of glomerular diseases such as chronic glomerulonephritides.

Characterization of the mesangium-specific gene and understanding the exact pathogenic mechanisms at the molecular level are critical for developing an effective treatment for renal disease.

Inagi et al. (2001) obtained the gene profile of cultured human mesangial cells and identified genes specifically or abundantly expressed in mesangial cells. Among these genes they found out *megsin*, which was mapped to chromosome 18q21.3 (20-kb long and having eight exons).

Megsin, protein of 380 amino acids, is a member of the serpin (serine protease inhibitor) superfamily, which includes SCCA and PAI. More than 200 serpins have been identified, and they are divided into 16 subgroups.

Biological functions of serpins include coagulation, fibrinolysis, extracellular matrix metabolism, inflammation, cell differentiation, proliferation, and apoptosis (Bachman et al., 1995; Kruithof et al., 1995; Bird et al., 1998; O'Relly et al., 1999).

megsin is expressed predominantly in mesangial cells (Miyata et al., 1998) and plays a role in the regulation of a wide variety of processes in mesangial cells, such as matrix metabolism, cell proliferation, and apoptosis. Mesangial proteases participate in the degradation of matrix components and immune complexes.

Inagi et al. (2002, 2003a) identified plasmin as one of the possible targets of Megsin, which is able to bind to plasmin and consequently inhibits its protease activity. Inagi et al. (2002a, 2003) speculate that overexpression of *megsin* might lead to mesangial dysfunction and impair degradation of the mesangial matrix. Megsin may also impair the degradation and disposal of immune complexes, change the microenvironment of mesangial cells and affect mesangial cell function and proliferation. Inagi et al. (2002b, 2003) examined *megsin* transgenic mice and detected supraphysiologal amounts of Megsin transcripts in mesangial cells of the whole kidney, and by means of polyclonal antibody confirmed the expression of Megsin at the protein level.

As mentioned above, different candidate gene polymorphisms have been advocated to be the possible agents of the progression. A large study in Chinese population revealed that the 2093C and 2180T alleles at the 3' UTR of the *megsin* gene confer susceptibility to IgAN (Li et al., 2004). Moreover, a family-based association study showed that the 2093C and 2180T alleles were significantly more often co-transmitted from heterozygous parents to pts (Li et al., 2004). Furthermore, Xia

Table 9. Serum creatinine levels at the time of renal biopsy and at the end of the follow-up

	Stable group	Progressive group	P value
S-Cr, time of RB (μmol/l)	150.25 ± 147.42	198.16 ± 119.18	0.018 (ln)
S-Cr, end of follow-up (μmol/l)	123.19 ± -63.73	576.00 ± 220.56	P < 0.001

S-Cr, serum creatinine; RB, renal biopsy; ln, logarithmic transformation

*Table 10. Clinical data of IgAN patients in stable and progressive groups**

	Stable	Progressive	P value
Follow-up duration (months)	56.97 ± 39.95	55.68±47.90	NS
Age (years)	38.74 ± 13.79	40.05±15.38	NS
S-Cr (μmol/l)	150.25 ± 147.42	198.16±119.18	0.018 (ln)
24-h UP (g/d)	2.06 ± 2.06	3.53±2.80	0.042
Systolic BP (mm Hg)	135.21 ± 14.88	150.79±32.88	0.058
Diastolic BP(mm Hg)	84.93 ± 10.43	92.89±15.66	0.047
S-Chol (mmol/l)	5.59 ± 1.35	5.44±1.90	NS
S-Tg (mmol/l)	1.95 ± 1.00	2.22±2.39	NS
S-uricaemia (μmol/l)	368.09 ± 96.08	384.56±86.77	NS

*Values expressed as mean ± SD. 24-h UP, 24-h urinary protein; BP, blood pressure; S-Chol, cholesterol; S-Tg, triglyceride.

et al. (2006) found out that the 2093C-2180T haplotype at the *megsin* 3' UTR was associated with a more rapid progression of IgAN.

We anticipated to give support to these data from Asian population, but our results did not confirm the influence of C2093T, C2180T *megsin* gene polymorphisms on the progression of IgAN in Czech pts. No obvious effect of these polymorphisms was found in single-gene as well as in haplotype analysis.

Nevertheless, the *megsin* haplotype reconstruction revealed that the TT haplotype (defined as T-2093, T-2180) could be found in a significantly higher frequency in the stable group of IgAN in comparison with its proportion within the progressive group (Table 3). Such a trend could be observed when the distribution of TT haplotype was compared between the stable group and control group (Table 1). This differences in TT haplotype distribution could play in favour for a protective role of TT haplotype in the progression of IgAN.

The protective effect of TT haplotype on the progression of chronic glomerulonephritides, especially IgAN, might be explained by shared interaction of both *megsin* polymorphisms. Single-nucleotide polymorphisms may not transform expression or function of specific proteins so intensely as to produce clinically significant phenotypes in multifactorial disease. Only the conjunctive influence of both *megsin* polymorphisms may exert a protective role in the disease.

Furthermore, this TT haplotype could be related to other polymorphisms or mutations, which could play a pathogenic role in the progression, and the TT haplotype might play a role as a marker of these polymorphisms/mutations – the linkage might be specific just for the Czech population. However, the pathogenesis and influence of *megsin* polymorphisms on the progression of IgAN to ESRD needs to be clarified by further studies including larger cohorts of European pts, taking in consideration a possible difference between the European and Asian population.

Many risk factors of progression of IgAN – such as arterial hypertension, severe proteinuria, elevated serum creatinine level, hyperlipoproteinemia, hyperuricaemia and histological sings of glomerular sclerosis or interstitial fibrosis – are known to be strong indicators of an unfavourable prognosis (Kobayashi et al., 1997; Daniel et al., 2000; Hsu et al., 2000; Syrjanen et al., 2000b; Bartosik et al., 2001; Donadio et al., 2002; Rauta et al., 2002; D'Amico et al., 2004; Lau et al., 2004). In our study, with respect to clinical parameters, we demonstrated that pts with progressive disease presented with higher levels of 24-h UP and blood pressure than pts with a stable course of the disease (Table 10).

Histopathological changes within renal biopsy specimens, which could contribute to a more precise clinical characterization of both IgA groups, could not be evaluated in this study and remain a challenge for future research in this field. The next steps of our research programme are focused on detection of *megsin* transcripts directly in kidneys of IgAN pts to learn more about the function of *megsin*.

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