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PDGF-B gene single-nucleotide polymorphisms are not predictive for disease onset or progression of IgA nephropathy

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glomerulonephritis – growth factors – PDGF – IgA nephropathy – progression

Abstract. Background: Few genetic factors have been identified that determine susceptibility to and progression of IgA-nephropathy (IgAN). Given that IgAN is usually characterized by mesangioproliferative glomerulonephritis and that PDGF-B is of central pathophysiological relevance in this process, we analyzed four single-nucleotide polymorphisms (SNPs) of the PDGF-B gene to evaluate a possible association of these SNPs with disease onset and progression, histological grading and responses to ACE inhibitor (ACEi) therapy. Methods: The total study population consisted of 195 IgAN patients (127 from southern Italy and 68 from northern Germany) and 200 healthy controls (100 from each region). All four SNPs were in Hardy-Weinberg equilibrium and genotype distributions did not differ between patients and controls in either region. Results: SNP distribution in Italian patients reaching end-stage renal disease (n = 45) also was not significantly different from patients maintaining a serum creatinine below 1.2 mg/dl (n = 60) during 5.6 ± 5.5 years of follow-up. Furthermore, we failed to detect significant effects of any SNP on the slope of 1/serum creatinine, proteinuria level or the antiproteinuric response to ACEi. Additionally, particular PDGF-B genotypes did not correlate with histological grading using the Lee classification. Conclusion: We conclude that none of the four PDGF-B SNPs is related to the onset of IgAN in two different populations and that none of them has a major influence on the course of IgAN.

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Introduction

IgA nephropathy (IgAN) is the most common primary glomerulonephritis worldwide. Within the patients coming to medical attention, about 30% progress to end-stage renal failure within 10 – 20 years [D'Amico 2004, Donadio and Grande 2002, Floege and Feehally 2000]. The cause of the disease and the mechanisms contributing to disease progression are incompletely understood [Donadio and Grande 2002, Floege and Feehally 2000]. A genetic component contributing to both manifestation and progression of the disease has long been suspected, based, for example, on HLA associations with the onset or course of IgAN in certain populations [Schena 1995]. More recently, a locus on chromosome 6q22-23 has been identified in patients with familial IgAN [Gharavi et al. 2000] and a syntenic locus was detected in ddY mice that spontaneously develop IgAN [Suzuki et al. 2005]. While the gene(s) associated with familial IgAN still is (are) unknown, an alternative approach to whole genome scanning is to investigate candidate genes, which are known to be involved in the pathogenesis of IgAN or mesangioproliferative glomerulonephritis. Mesangial cell proliferation is a hallmark of IgAN [Floege and Feehally 2000]. Platelet-derived growth factor (PDGF), a pleiotropic cytokine, and in particular the PDGF-B chain appears to play a central role in mediating mesangial cell proliferation and stimulates matrix synthesis [Floege and Johnson 1995]. Increased expression of PDGF-B chain and its receptor has been documented in IgAN [Alpers et al. 1992, 1993, Gesualdo et al. 1991, 1994]. Infusion of recombinant PDGF into rats or transfection of glomeruli with a

PDGF-B cDNA results in typical mesangioproliferative changes [Floege and Johnson 1995]. Furthermore, antagonism of PDGF-B largely prevents the development of renal failure and glomerular as well as tubulointerstitial scaring in rats with mesangioproliferative glomerulonephritis [Ostendorf et al. 2001].

Given the above observations, we asked whether polymorphisms of the PDGF-B gene may predispose to the manifestation of IgAN or modify the course of the disease. Since currently available databases suggest that PDGF-B does not exhibit SNPs in its promotor region, we focused on intronic SNPs, which exhibit a relatively high degree of heterozygosity.

Methods

The study was approved by the local Ethics Committees. Informed consent was given by all participating patients and control subjects.

Study group

A total of 127 randomly selected patients with primary biopsy-proven IgAN were analyzed retrospectively. All patients resided in Southern Italy and were of Caucasian origin. 100 probands with normal urine analysis from the same region served as controls. In addition to this Italian population, we studied a second, independent population of 68 IgAN patients originating from northern Germany. These again were compared with 100 healthy German controls from the same region. The observation period was 5.6 ± 5.5 years starting from renal biopsy until the last follow-up with a minimum follow-up of 2 years.

The inclusion criteria of the study were a histologically proven diagnosis, lack of evidence for a secondary form of IgAN or systemic disease, availability of genomic DNA for adequate genotyping of the PDGF-B gene single nucleotide polymorphisms (SNPs) and sufficient baseline and follow-up data (see below).

The following demographic and clinical data were obtained for patients with IgAN at the time of biopsy: gender, age, body weight, serum creatinine (mg/dl), proteinuria (g/d), histological grading using Lee's classification [Lee et al. 2005] and treatment with ACEi. Creatinine clearance (ml/min) was calculated using the Cockroft-Gault formula. The course of renal function was classified as "stable" (i.e. serum creatinine at follow-up equal or below 1.2 mg/dl), "deteriorated" (serum creatinine ≥ 1.2 mg/dl) and end-stage renal disease requiring renal replacement therapy. Follow-up was defined as the time from renal biopsy and the time of obtaining the last clinical information. Minimum follow-up was 2 years. Antiproteinuric response to ACEi therapy was defined as patients with an at least 25% decrease of 24-h proteinuria at the time of the last follow-up, whereas non-responders were defined as patients with a lesser reduction, stable values of proteinuria or an increase of proteinuria despite current ACE inhibitor therapy at the time of the last follow-up.

Single nucleotide polymorphisms

Intronic single nucleotide polymorphisms rs2040399, rs2285094, rs2267406 and rs2285097 of the PDGF-B gene were selected from publicly accessible databases (www.ncbi.nlm.nih.gov/SNP). The four selected SNPs all had average estimated heterozygosity values above 0.3 (see Table 1 for details). Similar heterozygosity rates were observed in the healthy control populations.

PDGF-B genotyping

Genomic DNA was isolated from peripheral blood leukocytes according to a standard salting-out method (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). The identification of the 4 SNPs was carried out using PCR followed by restriction fragment length polymorphism analysis (RFLP) (for details see Table 1).

4 µl containing 80 ng of genomic DNA were added to 20 µl of amplification buffer containing 9.8 µl H_2O, 2.5 µl PCR buffer, 2.5 µl $MgCl_2$, 4 µl dNTPs, 0.2 µl Taq DNA polymerase and 1 µl forward and 1 µl reverse primer. PCR was run for 40 cycles using the following temperature profile: denaturation at

Table 1. Primers and restriction endonucleases used for the 4 investigated SNPs.

SNP	Average estimated hetero-zygosity	Forward primers	Reverse primers	Poly-morphism	Restriction site
rs2040399	0.38	5'-AAACAAGGGCAAGGG CT-3'	5'-GGTAAACAGGGG TCCGACTT-3'	C/G	*HpHI* C-allele 34-87-150 G–allele 121-150
rs2285094	0.49	5'-CCATGTGCTGACCACT TCAT-3'	5'-GCCACATAACAGG TCTGGCT-3'	A/G	*AciI* G-allele 78-193 A-allele 271
rs2267406	0.35	5'-CTCAGAAGAGAGACC ACGCC-3'	5'-GCCTCCTGGCAG AATTAGAA-3'	C/T	*AluI* T-allele 127-145 C-allele 272
rs2285097	0.49	5'-ATTGGTTGCAGTCAGC TGGT-3'	5'-CAGTACTGGACAC AGAGCCG-3'	A/G	*AluI* G-allele 146-145 A-allele 291

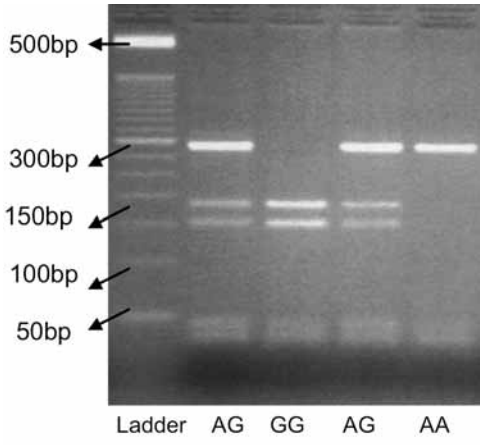

Figure 1. Example of an RFLP of SNP rs2285079 Alu/site GG allele 146-145 bp, AA allele 291bp and AG allele.

94 °C for 5 minutes, annealing at 58 °C for 30 seconds, extension at 72 °C for 90 seconds followed by a single final extension step at 72 °C for 10 minutes. The products were digested with the appropriate restriction endonucleases (Table 1). The resulting products were separated by gel electrophoresis in 3.0% agarose gels, containing 0.5 μg/ml ethidium bromide.

Allelic and genotypic frequencies were obtained by direct counting. The distribution of the genotypes in the collectives was checked for deviations from Hardy-Weinberg equilibrium.

Statistical analysis

Statistical comparison of allelic and genotypic frequencies (significance level $p < 0.05$) between patients with IgAN and healthy controls or patients with stable renal function and ESRD as well as of data on histological grading and antiproteinuric responses to ACE inhibitor therapy was carried out by χ^2 test and Fisher's exact test. Statistical analysis of disease progression (defined as GFR loss over time) was done by 1-way ANOVA test ($p < 0.05$) with post hoc Keuls analysis using the SPSS software package.

Results

Comparison of genotypes in Italian and German IgAN patients and controls

The distribution of the genotypes of the four SNPs was in Hardy-Weinberg equilibrium in both populations.

As shown in Table 2, no statistically significant differences of the distribution of genotypes were noted in the comparison of patients and controls using the χ^2 test for rs2040399, rs2285094, rs2267406 and rs2285097 in both the Italian and German populations. Similarly, allele frequencies were not significantly different (data not shown).

Table 2. Comparison of genotypes in all investigated IgAN and healthy controls performed by χ^2 test and Fisher's exact test. Numbers in brackets indicate relative frequencies of genotypes.

Italian patients						
Polymorphism			Genotype			
			CC	CG	GG	
rs2040399	Controls	n = 99	45 (0.45)	47 (0.47)	7 (0.07)	p = 0.14
	IgAN total	n = 127	44 (0.35)	66 (0.52)	17 (0.13)	
			AA	AG	GG	
rs2285094	Controls	n = 99	33 (0.33)	56 (0.57)	10 (0.08)	p = 0.61
	IgAN total	n = 127	36 (0.28)	74 (0.58)	17 (0.13)	
			CC	CT	TT	
rs2267406	Controls	n = 99	66 (0.66)	31 (0.31)	2 (0.02)	p = 0.42
	IgAN total	n = 127	74 (0.58)	49 (0.39)	4 (0.03)	
			AA	AG	GG	
rs2285097	Controls	n = 99	25 (0.25)	56 (0.57)	18 (0.18)	p = 0.71
	IgAN total	n = 127	37 (0.29)	65 (0.51)	25 (0.2)	

German patients						
Polymorphism			Genotype			
			CC	CG	GG	
rs2040399	Controls	n = 100	45 (0.45)	46 (0.46)	9 (0.09)	p = 0.46
	IGAN	n = 68	31 (0.46)	27 (0.40)	10 (0.15)	
			AA	AG	GG	
rs2285094	Controls	n = 100	22 (0.22)	61 (0.61)	17 (0.17)	p = 0.11
	IGAN	n = 68	13 (0.19)	34 (0.5)	21 (0.31)	
			CC	CT	TT	
rs2267406	Controls	n = 100	59 (0.59)	33 (0.33)	8 (0.08)	p = 0.32
	IGAN	n = 68	39 (0.57)	27 (0.4)	2 (0.03)	
			AA	AG	GG	
rs2285097	Controls	n = 100	23 (0.23)	58 (0.58)	19 (0.19)	p = 0.45
	IGAN	n = 68	12 (0.18)	38 (0.56)	18 (0.26)	

Effects of PDGF-B polymorphisms on disease progression in IgAN patients

Within the Italian IgAN patients we identified a subgroup with serum creatinine values remaining below ≤ 1.2 mg/dl during a mean follow-up of 5.6 ± 5.5 years and a second subgroup of patients with end-stage renal disease. Using the χ^2 test no statistically significant difference was found for any of the four SNPs between these two groups (Table 3, data for alleles not shown) suggesting no

Table 3. Comparison of genotypes in 60 Italian IgAN patients with stable renal function (serum creatinine (sc) ≤ 1.2 mg/dl) and 45 patients with ESRD performed by χ^2 test and Fisher's exact test. Numbers in brackets indicate relative frequencies of genotypes.

Polymorphism		Genotype			
		CC	CG	GG	
rs2040399	stable	17 (0.28)	33 (0.55)	10 (0.16)	p = 0.15
	ESRD	21 (0.47)	19 (0.42)	5 (0.11)	
		AA	AG	GG	
rs2285094	stable	18 (0.3)	34 (0.57)	8 (0.13)	p = 0.94
	ESRD	14 (0.31)	26 (0.58)	5 (0.11)	
		CC	CT	TT	
rs2267406	stable	35 (0.58)	22 (0.37)	3 (0.05)	p = 0.6
	ESRD	24 (0.53)	20 (0.44)	1 (0.02)	
		AA	AG	GG	
rs2285097	stable	18 (0.3)	31 (0.52)	11 (0.18)	p = 0.91
	ESRD	12 (0.27)	25 (0.56)	8 (0.18)	

relevant association between the analyzed SNPs and final outcome of IgAN. Analogous subgroups within the German patient population were too small to yield meaningful data.

In addition to final outcome, we also analyzed the role of the four SNPs in disease progression of Italian IgAN patients using the slope of creatinine clearance over time. Using 1-way ANOVA test with post hoc analysis again no statistically significant difference for either SNP was detectable (Figure 2). Results also did not change when patients with a GFR loss of more than 25 ml/min/year were excluded, since these patients might have suffered from acute or acute-on-chronic renal failure (data not shown). These data indicate that neither of the four polymorphisms of the PDGF-B gene appears to have a major influence on the disease progression in patients with IgAN.

Effects of PDGF-B polymorphisms on the antiproteinuric response to ACE inhibitor therapy in patients with IgAN

Proteinuria was determined at the time of renal biopsy and at the last follow-up. No significant association of either SNP with the extent of proteinuria was detected (data not shown). Out of the total of 127 Italian IgAN patients, 59 patients received treatment with an ACE inhibitor. This later subgroup was divided into two groups, i.e. responders (n = 22) and non-responders (n = 37) to ACEi therapy. Again, no statistically significant difference for either of the four SNPs was detected between responders and non-responders (data not shown)

Comparison between histological grading according to Lee's classification and genotype distribution of PDGF-B polymorphisms

Finally, Italian IgAN patients were divided into five subgroups based on the histological grading using Lee's classification [Lee et al. 2005]. Since very few patients (five or less) fell into the extreme Groups G1 and G5, these were pooled with the G2 and G4 Groups, respectively. Data analysis revealed that all four SNPs were evenly distributed between histological grades (Figure 3).

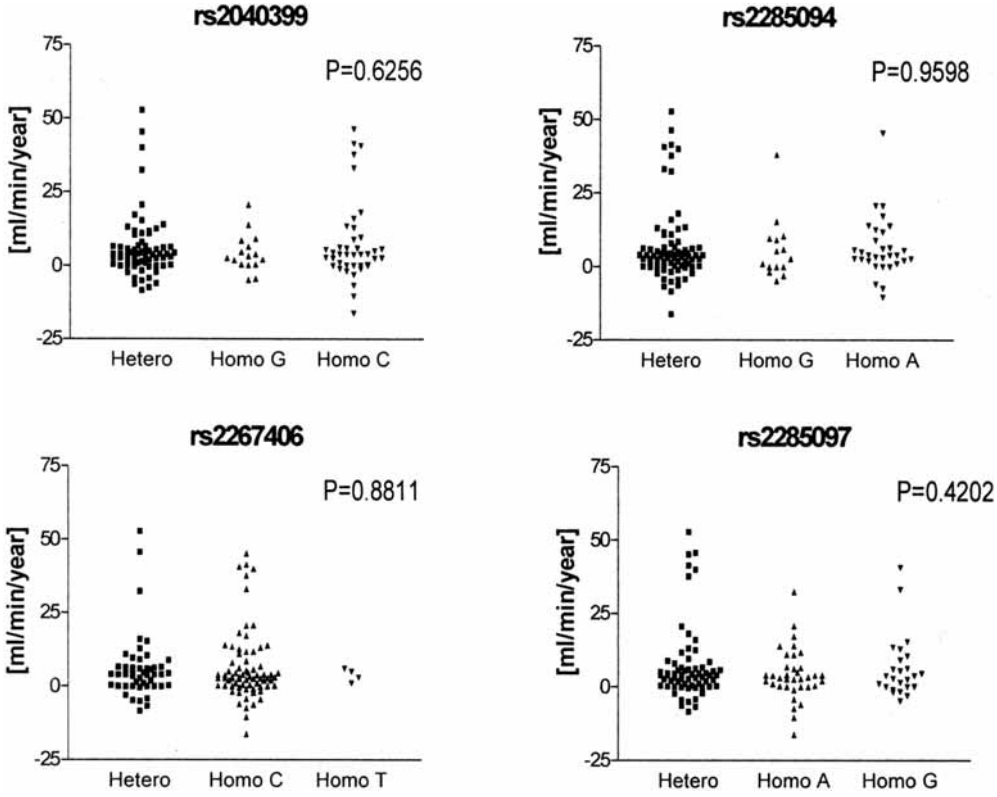

Figure 2. Italian IgAN patients: comparison of GFR loss during the follow-up period with the various SNPs by 1-way ANOVA. Individual values are depicted.

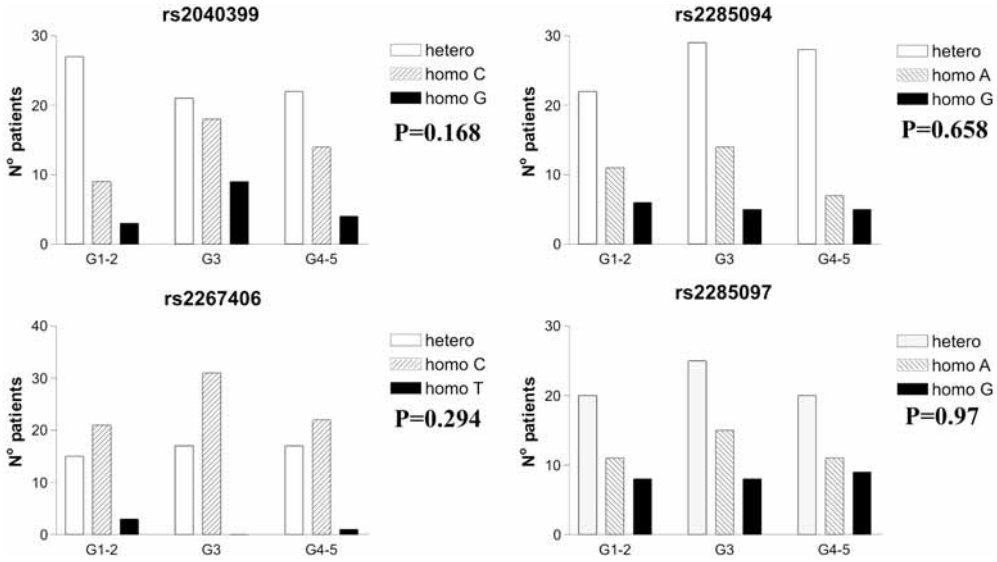

Figure 3. Italian IgAN patients: comparison of histological grading by Lee's classification with genotype distribution performed by χ^2 test and Fishers exact test. G1 + G2: n = 39, G3: n = 48, G4 + G5: n = 40.

Discussion

In recent years, a very large number of studies has assessed polymorphisms of potential candidate genes in patients with IgAN [Bantis et al. 2005, Ohtsubo et al. 2005, Schena et al. 2001, Thibaudin et al. 2004]. In general, these studies are notable for the fact that most have examined a relatively limited number of patients (often less than 100) and that most report a positive association of a particular SNP with disease onset or course,

usually at the 0.05 significance level. Thus, it is likely that a pronounced publication bias does exist, whereby positive but not negative studies tend to be reported. Second, a number of published studies with a significance level of 0.05 may indeed represent chance observations. This may explain why, for example, in the case of the I/D polymorphism of the ACE gene, where a considerable number of studies has been performed, conflicting results emerged and so far no clear conclusion as to the role of this particular polymorphism can be drawn [Schena et al. 2001]. A third problem of many studies on SNPs in IgAN (and other diseases) is that populations from different regions are pooled. This is why we have avoided to mix our Northern German patients with Southern Italian patients. Despite the fact that all are of Causasian origin, genetic differences of the populations are likely and IgAN, for example, is notable for the fact that more cases of familial IgAN appear to exist in Southern Italy as compared to Northern Germany [Schena et al. 2002] (F.P. Schena and J. Floege, unpublished observations). This assumption is further strengthened by the recent observation that even German and French patient populations with IgAN appear to differ with respect to the influence of polymorphisms of the CCR5 chemokine receptor [Berthoux et al. 2006].

The key finding of the present study was that SNPs of the PDGF-B gene, a central mediator of mesangioproliferative glomerulonephritis, are not related to disease onset, progression or histological features of IgAN in Caucasian patients. In view of the above, we feel that it is important to also report such negative findings to exclude further candidate genes that might modulate the onset or course of IgAN.

Our study complements an every growing list of SNPs and other genetic factors that might modify IgAN. Most recently, several studies have reported genetic effects, which are of such high statistical power that they likely do affect the course of IgAN. First, Ohtsubo et al. [2005] starting with a whole genome screening of 465 IgAN patients, identified a G34448A polymorphism in the Ig μ-binding protein-2 gene, which was associated with the development of IgAN in Japanese patients ($p < 0.0005$). Ig μ-binding protein-2 is likely involved in immunoglobulin class switching and, thus, another attractive candidate in the pathogenesis of IgAN. Second, Li et al. [2004] identified two megsin gene polymorphisms (C2093T and C2180T) in 127 Chinese trios, i.e. patients with IgAN and their family members, that again related to the onset of IgAN with a very high statistical probability ($p < 0.005$). Megsin is a serin protease inhibitor, which is predominantly expressed in the mesangium and up-regulated in IgAN [Inagi et al. 2002]. Finally, we reported [Panzer et al. 2005] that a 32bp deletion polymorphism in the CCR5 chemokine receptor gene is associated with a benign course of IgAN in 228 Caucasian IgAN patients, although a very recent French study unexpectedly showed the opposite, i.e. an adverse course in patients with the deletion [Berthoux et al. 2006]. It is likely that with more refined technology, such as genome-wide SNP analysis, more such disease mediators will be identified.

In summary, we have failed to detect any relevant effects of PDGF-B gene polymorphisms on the manifestation, clinical course or histological features in patients with IgAN.

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B. Bicanski and M. Wenderdel contributed equally to the study. This study was supported by the 5th European Framework Programme (QLG1-CT-2000-00464).

Conflict of interest

None declared.

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