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Genetic heterogeneity in the renin-angiotensin system and the risk of diabetic nephropathy: Association with the angiotensinogen gene, but not with the ACE gene

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Diabetic nephropathy (DN) is a serious complication in a subgroup of patients with insulin dependent diabetes mellitus (IDDM). In order to examine a genetic predisposition to DN we determined the genotypes of the *ACE* gene insertion/deletion polymorphism and the *M235T* variant of angiotensinogen (AGT) gene in 199 blood donors (healthy controls), 55 IDDM patients with DN (median duration of diabetes 28 years, range 6-50) and 44 IDDM patients without DN (median duration 20 years, range 10–46). A statistically significant difference for genotype and allele frequencies was found for the angiotensinogen gene, but not for the ACE gene. The AGT T235 allele frequency was similar in healthy controls and IDDM patients with DN (0.42 vs. 0.45). In IDDM patients without DN the T235 allele frequency was 0.28 (Odds ratio for an additive effect of the T235 variant: 2.10; 95 % CI 1.16–3.81). We conclude that the ACE I/D polymorphism does not provide a risk marker, but that the absence of the AGT T235 allele seems to be associated with a decreased risk for DN in IDDM patients. *J Clin Bas Cardiol 1998; 1: 55–8.*

Keywords: Diabetes mellitus; diabetic nephropathy; renin-angiotensin system; peptidyl-dipeptidase-A-genetics; angiotensinogen-genetics

Diabetic nephropathy (DN) is a serious long term complication of patients with insulin dependent diabetes mellitus (IDDM) leading to end stage renal failure in 30–40 % of patients [1]. Although hypertension and poor metabolic control have been identified as important risk factors for DN [2, 3], 60–70 % of IDDM patients never develop nephropathy despite long duration of diabetes. Reports of familial clustering and an increased risk for DN in diabetic offspring of parents with essential hypertension or cardiovascular diseases suggest genetic determinants of susceptibility to diabetic nephropathy [4–6]. Identification of genes related to DN could provide further insight into pathogenesis and help identify patients at risk earlier. Genes controlling the renin-angiotensin system are promising candidates based on animal models, and in clinical studies a beneficial effect of angiotensin converting enzyme (ACE) inhibitors on the progression of diabetic nephropathy has been postulated [2, 7, 8]. An insertion/deletion *(I/D)* polymorphism within intron 16 of the *ACE* gene controls about 50 % of the inter-individual variability of plasma *ACE* concentrations. Mean plasma *ACE* level in genotype *DD* subjects was about twice that of *II* subjects, with *ID* subjects having intermediate levels [9]. Similarly, among several molecular variants of the angiotensinogen gene (*AGT*) that have been studied, the *T235* (substitution of threonine for methionine at position 235) was associated with higher angiotensinogen plasma levels [10]. Disease association of the *I/D* polymorphism of the *ACE* gene and the *M235T* variant of the angiotensinogen gene are a matter of debate in essential hypertension [10–16], coronary artery disease [17–21], left ventricular hypertrophy [22], pregnancy induced hypertension [23] and progression of IgA nephropathy [24]. We determined these genotypes in IDDM patients to examine their role as risk factors for DN.

Patients and methods

Study population

Fifty-five IDDM patients with DN, and 44 normoalbuminuric IDDM control patients with duration of diabetes of more than 10 years were recruited for this study between January and August 1994 from the University Hospital Zurich, other hospitals in Switzerland, referring physicians in private practice and from the Metabolic Unit, Heinrich Heine University, Düsseldorf, FRG. All individuals selected resided in Switzerland or Germany. Samples from 199 healthy volunteer blood donors (100 M/99 F) were obtained from the Zurich regional blood bank. Diabetic nephropathy (DN) was defined as persistent microalbuminuria (> 30 mg/24 h) or macroalbuminuria by nephelometry, and/or any degree of renal insufficiency due to diabetes. IDDM patients were considered free of DN when no microalbuminuria was detected at repetitive measurements and renal function and urinary analysis were normal after more than 10 years of IDDM. Characteristics of study groups are given in Table 1.

Table 1: Clinical characteristics of IDDM study patients according to renal status

	IDDM with nephropathy	Normoalbuminuric IDDM
No. of patients	55	44
Sex	39 M/16 F	23 M/21 F
Median Age (range) *years*	42 (23–66)	35 (20 –74)
Median Age at onset of diabetes (range) *years*	13 (2–48)	15 (3–39)
Median duration of diabetes (range) *years*	28 (6–50)	20 (10–46)
Microalbuminuria (n)	0	–
Overt proteinuria (n)	6	–
End-stage renal disease (n)	49[a]	–
History of hypertension (n)	47	6
Median age at onset of hypertension (range) *years*	34 (9–54)	52 (43–62)
Mean (SD) HbA_{1c} %	7.8 (1.6)	7.5 (1.1)

[a] Among 49 patients with end-stage renal disease 32 had received a renal transplant, 9 were on haemodialysis and 8 were on continuous ambulatory peritoneal dialysis.

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DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp^R Blood Kit (Quiagen). *ACE* genotype was determined by PCR amplification of genomic DNA with primers 5' CTGGAGACCACTCCCATCC TTTCT 3' and 5' GATGTGGCCATCACATTCGTCAGAT 3' as described [25]. PCR products were separated by electrophoresis on 2 % agarose gels and identified by ethidium bromide staining. Rarely, weak bands were confirmed by Southern blots which were hybridised with a ^{32}P-labelled 190 bp PCR product *D* of the *ACE* gene. Because of reports of potential mistyping of *ID* genotypes as *DD* using these conditions [26], samples typed as *DD* were repeated in a PCR in 5 % dimethylsulphoxide (DMSO) and in a PCR using a third, insertion-specific primer 5' TTTGAGACGGAGTC TCGCTC 3' and raising the annealing temperature to 61 ° C for the insertion-specific amplification. In all the samples tested by these two additional PCRs, *DD* genotypes were confirmed and no mistyping had occurred. Detection of *AGT* variant: A PCR with primer 5' CAGGGTGCTGTCCACA CTGGACCCC 3' and a modified primer 5' CCGTTT GTGCAGGGCCTGGCTCTCT 3' on the noncoding strand that introduces an Asp I half site into the amplified product was used as described by Russ et al. [27]. After an initial denaturation step at 90 ° C for 3 min, 10 cycles of 94 ° C, 68 ° C and 72 ° C (for 1 min each) were followed by 30 cycles of 90 ° C for 30 s, 68 ° C for 1 min, and 72 ° C for 30 s. An aliquot of 4 μl of the PCR product was digested with Asp I (Boehringer) at 37 ° C overnight, subjected to electrophoresis in a 2.5 % agarose gel and visualised by ethidium bromide staining. The alleles coding for M235 and T235 were detected as bands corresponding to fragment sizes of 165 bp, and 141 bp respectively.

Statistical analysis

Frequencies of the alleles and genotypes among the case and control patients and blood donors were counted and were compared by the χ-square test with the values predicted under the assumption of a Hardy-Weinberg equilibrium in the sample. Genotype frequencies were compared with 3 x 2 contingency tables (2 df), allele frequencies were compared with 2 x 2 contingency tables (1 df). Odds ratios were calculated as a measure of the association of the genotype with the phenotype of diabetic nephropathy using the Mantel-Haenszel test. For each odds ratio 95 % confidence intervals were calculated.

Results

Characteristics of patient study groups are given in Table 1. Median age (p=0.16), median age at onset of diabetes (p=0.53) and glycaemic control as assessed by HbA_{1c} levels (p=0.18) were similar in patients with and without DN. Median duration of diabetes in IDDM patients without DN was slightly shorter than in those with DN (median 20, range 10–46 years vs. 28, range 6–50 years, respectively). In IDDM patients without DN the minimal duration of diabetes was 10 years and there were only 5 of 44 patients with IDDM of less than 15 years. Not surprisingly arterial hypertension was more prevalent in the DN group. Onset of hypertension in DN patients was significantly earlier than in normoalbuminuric controls ($p < 0.01$). The definition of DN in our protocol included patients with persistent microalbuminuria as well as overt proteinuria and/or any degree of renal insufficiency due to IDDM. However, there was no patient in the group with DN who presented with persistent microalbuminuria only. All had either overt proteinuria or even more advanced stages of DN.

ACE *I/D* and *AGT M235T* genotype distributions and allele frequencies in blood donors and IDDM patients with and without DN are shown in Table 2. Among the blood donors, the *ACE D* and *I* alleles had frequencies of 0.523 and 0.477, respectively. The frequencies of the *DD*, *II* and *ID* genotypes were virtually identical to those predicted (0.273, 0.228, and 0.499, respectively) by the Hardy-Weinberg equilibrium (χ^2=0.003 (2 df); p=0.99). The frequencies of the *TT, MM* and *MT* genotypes in the healthy controls were also close to those predicted (χ^2=0.007 (2 df); p=0.99). The allele frequency for the T235 variant was 0.42, which is in good agreement with previous reports by others in a Caucasian population [10].

Statistical comparisons between IDDM patients with and without DN are given in the lower part of Table 2. At the *ACE* locus there was no statistically significant difference for *I/D* genotype distribution or allele frequencies between IDDM patients with and without DN or between IDDM patients and blood donors. Re-evaluation of *DD* genotype samples with a different, insertion specific primer did not detect any case of mistyping [26].

In contrast, the angiotensinogen *T235* allele was significantly underrepresented in IDDM patients without DN as compared with those with DN (frequencies 0.45 vs. 0.28; OR 2.10; 95 % CI 1.16–3.81) and healthy controls (frequency 0.42). Correspondingly the angiotensinogen genotype distribution is unbalanced between diabetics with and without DN. Under the hypothesis of an additive effect, the risk of DN conferred by the *T* allele was 2.57 (95 % CI 1.33–4.97; *TT* vs *MT* vs *MM*); when tested for a dominant role (*TT* and *MT* vs. *MM*) it was 2.69 (95 % CI 1.14–6.36), demonstrating a significantly higher risk for those IDDM patients in our study who carry the *T235* allele to develop DN.

Table 2. Frequencies ACE and angiotensinogen genotypes and alleles in IDDM patients with and without nephropathy and blood donors.

	ACE (*I/D*)				**Angiotensinogen *M235T***			
	Genotypes			**Allele frequency**	**Genotypes**			**Allele frequency**
	DD	*ID*	*II*	*D*	*TT*	*MT*	*MM*	*T*
Blood donors (n=199)	58(29%)	92(46%)	49(25%)	0.52	41(21%)	86(43%)	72(36%)	0.42
All IDDM (n=99)	34(34%)	41(41%)	24(24%)	0.55	9(9%)	57(58%)	33(33%)	0.38
IDDM with nephropathy (n=55)	18(33%)	25(45%)	12(22%)	0.55	8(15%)	34(62%)	13(24%)	0.45
Normoalbuminuric IDDM (n=44)	16(36%)	16(36%)	12(27%)	0.55	1(2%)	23(52%)	20(45%)	0.28
	Odds ratio (95% confidence interval)				Odds ratio (95% confidence interval)			
	DD vs. *ID* vs. *II*		*D* vs. *I*		*TT* vs. *MT* vs. *MM*		*T* vs. *M*	
All IDDM vs. Blood donors	1.11 (0.90–1.38)		1.12 (0.79–1.58)		0.92 (0.87–0.97)		0.83 (0.59–1.18)	
IDDM with vs. without nephropathy	1.02 (0.98–1.07)		1.04 (0.59–1.82)		2.57 (1.33–4.97)		2.10 (1.16–3.81)	

Discussion

The present study was initiated to test the role of genetic polymorphism in genes coding for *ACE* and angiotensinogen as potential risk factors for the development of DN. The diagnosis of DN was based on clinical data, including patients with persistent microalbuminuria, overt proteinuria and impaired renal function due to DN. While persistent microalbuminuria is generally recognised as a strong predictive marker for the development of diabetic nephropathy, this has been questioned by some authors based on the lack of specificity [28]. In the present study none of the IDDM patient with DN had isolated microalbuminuria. All had at least overt proteinuria or some degree of renal insufficiency, making an incorrect assignment to the DN group unlikely. On the other hand the over-representation of end stage renal disease in our nephropathy group might select for genotypes important for the progression of kidney disease.

Was the minimum duration of IDDM in patients classified as not having DN long enough to exclude future nephropathy? The vast majority of our normoalbuminuric IDDM patients had been diabetic for more than 15 years. It has been shown that most IDDM patients susceptible for DN will have developed some signs of nephropathy after approximately 15-20 years [1,29]. Although misclassification is still a possibility, we think it is unlikely to have a significant impact on the present results.

We were unable to demonstrate an association between the *ACE I/D* polymorphism and DN. The distribution of the genotypes and the allele frequency found in healthy controls as well as in IDDM patients with and without DN is in accordance to those described in the original paper by Rigat et al. [9]. The number of studies on the *ACE I/D* polymorphism in IDDM patients with/without DN that were performed simultaneously to ours mirrors the considerable interest in this genetic marker that was raised by recent reports that the *ACE I/D* polymorphism might predict development and course of certain cardiovascular diseases [14, 15, 17–22]. The results in IDDM patients published so far, however, are conflicting. Marre et al. [30] initially reported a lower proportion of the *II* genotype in patients with DN. Our results do not confirm these findings. Although the size of the present study does not completely preclude the possibility of a type-2 error, our results are in good agreement with three similar studies including the two largest from Heidelberg and Gentofte, all unable to demonstrate any association between the *ACE* gene polymorphism and DN [31, 32, 33]. In addition, differences to findings from other centres are more likely to be based on the limited number of patients enrolled in those studies than on ethnical reasons, since all studies were performed in Caucasians and the published *D* allele frequencies in healthy Caucasian control populations did not vary significantly between different centres [9, 31]. In a large sample from Caucasian populations the *DD* genotype frequency was 0.27, which is similar to our value of 0.29 in blood donors [17]. Mistyping of *ID* heterozygotes as false *DD* homozygotes has been reported [26], but it could not explain the differences in the frequency of the *II* genotype observed. The present data do not exclude a pathogenic role of mutations somewhere else in the *ACE* gene, but the *ACE I/D* polymorphism does not seem to be a useful marker to predict DN in IDDM patients.

In contrast, we could demonstrate a marked under-representation of the *T235* allele of the angiotensinogen gene in IDDM patients without DN as compared to those with DN and healthy controls. This suggests that the presence of the genetic variant associated with higher angiotensinogen serum levels might represent a genetic predisposition to DN. Previous studies found the *T235* allele associated with essential hypertension and pregnancy induced hypertension [23, 38]. Recently, Schmidt et al. showed the *T235* allele to be particularly more frequent in those patients with a familial history of hypertension of early onset [11]. Accordingly, hypertension was more prevalent in our DN group and its onset was much earlier than in the few normoalbuminuric controls with hypertension. Furthermore, some studies described a higher incidence of the *T* allele in African Americans and Hispanics as compared with Caucasians, both groups known to carry a higher risk of DN [34–38]. One might assume that the M235T variant is just a substitute marker for essential hypertension and that the under-representation of the *TT* genotype in our normoalbuminuric group reflects the few hypertensive patients in that group. However, the M235T variant has not been linked to essential hypertension in 63 multiplex families leaving doubts about this variant in explaining all essential hypertension [39]. However, the same study demonstrated a linkage of the angiotensinogen locus to essential hypertension. Despite the fact that statistical significance was reached in the present study, we are aware that these results should be interpreted with some caution, especially since the genotype distribution in our patients with DN was not in perfect match with that predicted by the Hardy-Weinberg equilibrium. This is most likely to be because of the rather small size of the study. Clearly, our data need prospective confirmation in a larger population. The fact that we observed similar allele frequencies in healthy controls and IDDM patients with DN further suggests, that the presence of the T235 allele is unlikely to contribute largely to the genetic predisposition for DN. One might speculate, however, that in concert with other genetic and acquired risk factors the presence of this allele might increase the susceptibility for DN. Up to now, there is only one report on the *M235T* variant of the angiotensinogen gene in DN by the Belfast group in abstract form [40]. The authors found the *TT* genotype to be three times more frequent in 98 nephropathic than in 96 normoalbuminuric IDDM patients. However, no data on the genotype distribution in both healthy controls and study groups were reported. Statistical analysis of their data showed an increased risk of DN with the *TT* genotype ($p = 0.02$), underlining the possible risk for DN conferred by this genotype.

In conclusion, we could not confirm any association of the *ACE I/D* gene polymorphism with DN in IDDM patients. This adds further to a growing number of reports that challenges this hypothesis. In contrast, our data suggest a link between the *T235* variant of the angiotensinogen gene and DN. We hypothesise that the angiotensinogen *T235* allele increases the risk for diabetic nephropathy in IDDM patients, whereas the lack of the *T235* variant might function as a protective factor for its development. However, to overcome the selection bias of cross-sectional studies, which may be caused by selective genetically influenced mortality, long-term, prospective studies are needed. Our findings propose the angiotensinogen *T235* allele as a candidate gene for such studies.

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