S. Fröjdö · L. Sjölin · M. Parkkonen · V.-P. Mäkinen ·
R. Kilpikari · K. Pettersson-Fernholm · C. Forsblom ·
J. Fagerudd · C. Tikellis · M. E. Cooper · M. Wessman ·
P.-H. Groop · FinnDiane Study Group

Polymorphisms in the gene encoding angiotensin I converting enzyme 2 and diabetic nephropathy

Received: 25 April 2005 / Accepted: 2 June 2005 / Published online: 7 October 2005
© Springer-Verlag 2005

Abstract *Aims/hypothesis:* Substantial evidence exists for the involvement of the renin–angiotensin system (RAS) in diabetic nephropathy. Angiotensin I converting enzyme 2 (ACE2), a new component of the RAS, has been implicated in kidney disease, hypertension and cardiac function. Based on this, the aim of the present study was to evaluate whether variations in *ACE2* are associated with diabetic nephropathy. *Materials and methods:* We used a cross-sectional, case–control study design to investigate 823 Finnish type 1 diabetic patients (365 with and 458 without nephropathy). Five single-nucleotide polymorphisms (SNPs) were genotyped using TaqMan technology. Haplotypes were estimated

using PHASE software, and haplotype frequency differences were analysed using a χ^2 -test-based tool. *Results:* None of the *ACE2* polymorphisms was associated with diabetic nephropathy, and this finding was supported by the haplotype analysis. The *ACE2* polymorphisms were not associated with blood pressure, BMI or HbA_{1c}. *Conclusions/interpretation:* In Finnish type 1 diabetic patients, *ACE2* polymorphisms are not associated with diabetic nephropathy or any studied risk factor for this complication. Further studies are necessary to assess a minor effect of *ACE2*.

Keywords ACE2 · Diabetic nephropathy · Haplotype · SNP · Type 1 diabetes

Electronic Supplementary Material Supplementary material is available for this article at <http://dx.doi.org/10.1007/s00125-005-1955-4>.

S. Fröjdö · L. Sjölin · M. Parkkonen · V.-P. Mäkinen ·
R. Kilpikari · K. Pettersson-Fernholm · C. Forsblom ·
J. Fagerudd · M. Wessman · P.-H. Groop (✉)
Folkhälsan Institute of Genetics,
Folkhälsan Research Centre,
Biomedicum, University of Helsinki,
Helsinki, Finland
e-mail: per-henrik.groop@helsinki.fi
Tel.: +35-89-19125459
Fax: +35-89-19125452

S. Fröjdö · L. Sjölin · M. Parkkonen · V.-P. Mäkinen ·
R. Kilpikari · K. Pettersson-Fernholm · C. Forsblom ·
J. Fagerudd · M. Wessman · P.-H. Groop
Department of Medicine,
Division of Nephrology,
Helsinki University Central Hospital,
Helsinki, Finland

C. Tikellis · M. E. Cooper
Baker Medical Research Institute,
Melbourne, Victoria, Australia

M. Wessman
Department of Clinical Chemistry
and the Finnish Genome Centre,
University of Helsinki,
Helsinki, Finland

Abbreviations ACE2: angiotensin I converting enzyme 2 · LD: linkage disequilibrium · RAS: renin–angiotensin system · SNP: single-nucleotide polymorphism

Introduction

Familial clustering of diabetic nephropathy in type 1 and type 2 diabetes suggests genetic predisposition to this complication. An important risk factor is blood pressure, which is largely regulated by the renin–angiotensin system (RAS). The local RAS plays a significant role in kidney function and blood pressure regulation [1]. Blockade of the RAS by ACE inhibitors and angiotensin II receptor blockers provides beneficial effects in terms of prevention of structural and functional changes in the diabetic kidney, in addition to blood-pressure-lowering effects, thus suggesting that the RAS plays a role in diabetic nephropathy [2]. Based on this, several studies have investigated possible associations between polymorphisms in RAS genes and susceptibility to diabetic nephropathy [2].

The discovery of angiotensin I converting enzyme 2 (ACE2), an ACE homologue, increased the complexity of the RAS and attracted immediate interest. ACE2 is similar to ACE in that it is a membrane-associated and secreted enzyme that is predominantly expressed in the endotheli-

um. At least 12 peptides that are significantly hydrolysed by ACE2 have been identified, including angiotensin I and II [3]. *ACE2* is located on chromosome Xp22. It consists of 18 exons, which encode an 805-amino acid protein that shares 42% homology with ACE in the catalytic domain. *ACE2* is highly expressed in kidney, testis and heart and has been implicated in hypertension and cardiovascular disease [3]. Interestingly, *ACE2* production is decreased in rat diabetic kidney [4] and is altered in human kidney disease such that neo-expression of *ACE2* occurs in glomerular and peritubular capillary endothelium [5]. The only study published to date on *ACE2* polymorphisms and essential hypertension reported no association [6]. Whether *ACE2* polymorphisms influence susceptibility to diabetic nephropathy is not known. The aim of this case-control study was to determine whether single-nucleotide polymorphisms (SNPs) in human *ACE2* are associated with diabetic nephropathy in type 1 diabetes.

Subjects and methods

Subjects In total, 823 carefully characterised type 1 diabetic patients (Table 1) from the Finnish Diabetic Nephropathy Study (FinnDiane) were investigated. Patients were required to have onset of diabetes before 35 years of age, permanent insulin treatment initiated within 1 year of diabetes diagnosis and a fasting C-peptide level <0.3 nmol/l. Demographic data and blood and urine samples for determination of HbA_{1c}, lipids and AER were collected. Renal status was based on the AER values in at least two of three consecutive overnight or 24-h urine collections; patients were classified as having no diabetic nephropathy (AER <20 µg/min or <30 mg per 24 h) or diabetic nephropathy (AER >200 µg/min or >300 mg per 24 h). Patients with end-stage renal disease were not included. Patients without diabetic nephropathy were required to have had a diabetes duration of >15 years to ensure normal renal status. Informed written consent was obtained from all patients, and the local ethics committee of each participating centre approved the study protocol, which was conducted in accordance with the principles of the Declaration of Helsinki.

Methods The SNPs for *ACE2* were determined from DNA isolated from whole blood using phenol extraction or a PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). The SNPs were chosen from public databases and were required to be evenly distributed over the gene. The following SNPs were investigated in the present study (listed 5'–3'): rs2285666, rs2048684, rs879922, rs714205 and rs5978731. Even if these are not tagSNPs, they capture 15 of the 17 SNPs in HapMap with a minor allele frequency of over 0.05, and thus most of the common variants across the gene (Fig. 1). The fact that five SNPs can capture most of the information across 40 kb is probably explained by the regions of high linkage disequilibrium (LD) on the X-chromosome. The promoter region contains several repeats that are problematic for PCR; thus, no SNP was successfully genotyped in this region. Genotyping of the following SNPs in the promoter regions failed: rs4646112, rs4646114 and rs12009805. Genotyping was performed using TaqMan chemistry with ABI Prism 7000 and 7900HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). This genotyping method is based on fluorogenic 5'-nuclease allelic discrimination chemistry, with no post-PCR processing steps necessary, making it more reliable and safe to use [7]. Furthermore, genotyping quality was ensured by using 2% internal controls in each run, for which we demanded 100% accuracy. The success rate with this system is >99%.

Statistical analyses Statistical analyses were performed using SPSS, Version 11.5 for Windows (SPSS, Chicago, IL, USA). Associations with categorical variables were analysed using the χ^2 -test; associations with continuous variables were investigated with ANOVA. Mantel-Haenszel statistics were used to combine male and female *p* values. A *p* value of less than 0.05 was considered significant. For LD analysis we used Linkage Disequilibrium Analyzer 1.0 (available at <http://www.chgb.org.cn/lda/lda.htm>, last accessed in August 2005) [8]. PHASE, Version 2.0.2 (available at <http://www.stat.washington.edu/stephens/software.html>, last accessed in August 2005) [9] was used to estimate haplotypes, which were further analysed with a specially designed C-language-based program that finds

Table 1 Clinical characteristics of the patients

Parameter	Patients without diabetic nephropathy		Patients with diabetic nephropathy	
	Men	Women	Men	Women
<i>n</i>	183	275	221	144
Age (years)	43.1±10.5	42.7±9.7	41.1±9.0	36.6±8.2
Diabetes duration (years)	27.3 (19–53)	28.2 (19–53)	27.1 (14–41)	26.5 (13–40)
Diabetes duration to diabetic nephropathy (years)			18 (5–39)	17 (6–32)
BMI (kg/m ²)	25.2±2.7	24.7±3.1	25.8±4.0	25.7±3.7
Systolic blood pressure (mm Hg)	134±14	131±17	145±19	142±20
Diastolic blood pressure (mm Hg)	79±9	77±8	85±10	82±10
HbA _{1c} (%)	8.1±1.1	8.1±1.3	8.9±1.5	9.2±1.7
AER (mg per 24 h) ^a	8 (3–76)	7 (1–85)	719 (10–8,348)	406 (4–6,069)

Values are presented as mean±SD or median (range)

^aSome patients with previously abnormal AER had responded to antihypertensive treatment and showed a reduction in AER to/towards normal values at the time of investigation. The AER values in the table represent a measurement from a single 24-h urine collection

Fig. 1 LD pattern of SNPs with a minor allele frequency >0.05 in *ACE2* (calculated using HapMap), with results presented as r^2 values (*white*=1, $0 < \text{grey} < 1$, *black*=1). HapMap tagSNPs are marked with an *asterisk*, and the SNPs genotyped in the present study are enclosed in a box. Fifteen of 17 SNPs with an $r^2 > 0.8$ were captured. Three SNPs (rs5978731, rs714205 and rs2285666) were not in HapMap and have been added to this figure.

all haplotypes in the material and tests their statistical significance using the χ^2 -test. A power calculation was performed using the Genetic Power Calculator, case-control for discrete traits (available at <http://www.statgen.iop.kcl.ac.uk/gpc/>, last accessed in August 2005) [10]. In order to calculate power, the following assumptions were made:

disease prevalence 0.003; marker allele frequency 0.3; D' with disease allele 0.9; high-risk allele frequency 0.3; genotype AA relative risk 2.3; genotype Aa relative risk 1.0001. Using these assumptions, 365 cases and 456 control subjects would give a power of 80% with a type I error rate of 0.05.

Table 2 *ACE2* genotype frequencies in male and female patients according to renal status

			rs2285666 C/T		rs2048684 A/C		rs879922 C/G		rs714205 C/G		rs5978731 A/C	
			<i>n</i>	Frequency	<i>n</i>	Frequency	<i>n</i>	Frequency	<i>n</i>	Frequency	<i>n</i>	Frequency
Patients with diabetic nephropathy	Men	1	173	0.790	70	0.320	69	0.315	179	0.817	69	0.315
		2	46	0.210	149	0.680	150	0.685	40	0.183	150	0.685
	Women	11	99	0.697	15	0.104	15	0.104	105	0.734	15	0.104
		12	35	0.246	63	0.438	57	0.396	33	0.231	55	0.382
Patients without diabetic nephropathy	Men	22	8	0.056	66	0.458	72	0.500	5	0.035	74	0.514
		1	139	0.777	51	0.283	42	0.232	146	0.830	42	0.233
	Women	2	40	0.223	129	0.717	139	0.768	30	0.170	138	0.767
		11	179	0.653	27	0.099	24	0.087	198	0.744	23	0.084
		12	85	0.310	111	0.408	113	0.411	61	0.229	114	0.416
		22	10	0.036	134	0.493	138	0.502	7	0.026	137	0.500
	<i>p</i> value (males) ^a		0.746		0.432		0.065		0.752		0.070	
	<i>p</i> value (females) ^b		0.296		0.799		0.842		0.882		0.693	
	Combined <i>p</i> value ^c		0.595		0.352		0.193		0.631		0.257	

Genotypes according to SNP: rs2285666: 1=C, 2=T; rs2048684: 1=A, 2=C; rs879922: 1=C, 2=G; rs714205: 1=C, 2=G; rs5978731: 1=A, 2=C

^aComparing male patients with and without diabetic nephropathy

^bComparing female patients with and without diabetic nephropathy

^cCombined *p* values for male and female patients' allele frequencies were calculated using Mantel-Haenszel statistics

Results

Since *ACE2* is located on the X-chromosome, men and women were analysed separately. Genotypes were in Hardy–Weinberg equilibrium among the women. Pairwise LD (r^2 and D') was calculated using the genotypes of the women, with $r^2 > 0.8$ detected only between rs5978731 and rs879922 ($r^2 = 0.98$, $D' = 0.99$). Neither in men nor women did the allele frequencies differ significantly between patients with and those without diabetic nephropathy (Table 2). Combined analysis of male and female patients using Mantel–Haenszel statistics did not show any significant differences in allele frequencies; neither the alleles nor the genotypes were associated with blood pressure, BMI or HbA_{1c}. No association was found using haplotype analysis (data not shown).

In a multivariate regression analysis in male patients with renal status (diabetic nephropathy vs no diabetic nephropathy) as a dependent variable, the following variables were significant predictors: age ($B = -0.019$, $SEE = 0.005$, $p = 0.0003$), systolic blood pressure ($B = 0.018$, $SEE = 0.003$, $p < 0.00001$), HbA_{1c} ($B = 0.155$, $SEE = 0.034$, $p < 0.00001$), rs2048684 ($B = 0.041$, $SEE = 0.020$, $p = 0.040$) and rs5978731 ($B = -0.051$, $SEE = 0.020$, $p = 0.012$). Other variables included in the model were BMI, diastolic blood pressure, rs2285666 and rs714205; rs879922 was not included because it provides the same information as rs5978731. Performance of the same analysis in female patients revealed that the same clinical variables were significant in this population, as was rs5978731 ($B = 0.082$, $SEE = 0.041$, $p = 0.045$). These results were no longer significant after correcting for multiple testing.

Discussion

This study indicates that *ACE2* is not associated with diabetic nephropathy in the Finnish population. Furthermore, haplotype analysis, which improves the ability of detecting an association with other variants in the gene, supports this negative finding. *ACE2* polymorphisms were not found to be associated with clinical variables such as HbA_{1c}, lipids, AER or blood pressure. Our results are in agreement with another study that showed no association between *ACE2* polymorphisms and essential hypertension [6]. However, we cannot exclude a possible weak effect of *ACE2* on diabetic nephropathy in this study.

The Finnish population is a relatively isolated population and is therefore a good population in which to study complex diseases such as diabetic nephropathy. A strength of the present study is that it included a patient sample representative of Finnish type 1 diabetic patients with or without diabetic nephropathy. Furthermore, the patients were well-characterised clinically and the genotyping methodology used was robust.

Given that *ACE2* expression is altered not only in the rat diabetic kidney [4] but also in human kidney disease [5], an association between gene variants and diabetic nephropa-

thy could be expected. However, the present study did not detect any such association. One possible explanation is that the changes in expression are secondary to the kidney disease. Another possibility is that the regulatory elements are located long distances upstream or downstream of the transcription unit. Alternatively, there could be a link between diabetic nephropathy and genetic variability in the transcription factors associated with *ACE2* rather than in *ACE2* itself. It is possible that *ACE2* has only a small effect on susceptibility to diabetic nephropathy and we were unable to detect such an effect. A sex difference may also have remained undetected.

In conclusion, the present study provides no evidence for associations between *ACE2* polymorphisms and type 1 diabetic nephropathy in the Finnish population. A possible weak effect of *ACE2* cannot be excluded and further studies are needed to assess a smaller role of *ACE2* in diabetic nephropathy.

Acknowledgements This study was supported by grants from the Folkhälsan Research Foundation, Samfundet Folkhälsan, the Research Funds of the Helsinki University Central Hospital, the Wilhelm and Else Stockmann Foundation, the Sigrid Juselius Foundation, the Academy of Finland (00213 to M. Wessman), the European Commission (contract no. QLG2-CT-2001-01669), the Liv och Hälsa Foundation, and the Juvenile Diabetes Research Foundation. The skilled technical assistance of S. Lindh, A. Sandelin and T. Vesinenaho is gratefully acknowledged. Finally, we warmly thank all the patients who participated in the FinnDiane Study and gratefully acknowledge all the physicians and nurses at each participating centre who assisted in studying the patients (see Electronic supplementary material for a list of participating centres).

References

- Hollenberg NK, Price DA, Fisher NDL et al (2003) Glomerular hemodynamics and the renin–angiotensin system in patients with type 1 diabetes mellitus. *Kidney Int* 63:172–178
- Schrijvers BF, De Vriese AS, Flyvbjerg A (2004) From hyperglycemia to diabetic kidney disease: the role of metabolic, hemodynamic, intracellular factors and growth factors/cytokines. *Endocr Rev* 25:971–1010
- Burrell LM, Johnston CI, Tikellis C, Cooper ME (2004) ACE2, a new regulator of the renin–angiotensin system. *Trends Endocrinol Metab* 15:166–169
- Tikellis C, Johnston CI, Forbes JM et al (2003) Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy. *Hypertension* 41:392–397
- Lely A, Hamming I, van Goor H, Navis GJ (2004) Renal ACE2 expression in human kidney disease. *J Pathol* 204:587–593
- Benjafield AV, Wang WYS, Morris BJ (2004) No association of angiotensin-converting enzyme 2 gene (ACE2) polymorphisms with essential hypertension. *Am J Hypertens* 17:624–628
- Livak KJ (2003) SNP genotyping by the 5'-nuclease reaction. *Methods Mol Biol* 212:129–147
- Ding K, Zhou K, He F, Shen Y (2003) LDA—a java-based linkage disequilibrium analyzer. *Bioinformatics* 19:2147–2148
- Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 68:978–989
- Purcell S, Cherny SS, Sham PC (2003) Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 19:115–149