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SHORT COMMUNICATION

Association analysis of podocyte slit diaphragm genes as candidates for diabetic nephropathy

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Abstract
Aims/hypothesis The slit diaphragm is an adhesion and signalling protein complex linking the interdigitating podocyte foot processes in the kidney glomerulus, and mutations in slit diaphragm-associated genes result in severe proteinuria. Here we report a genetic association analysis of four slit diaphragm genes, *LRRC7*, *KIRREL*, *NPHS2* and *ACTN4*, in a Finnish diabetic nephropathy cohort.
Materials and methods A total of 40 single nucleotide polymorphisms (SNPs) were genotyped in 1103 patients with type 1 diabetes. The patients were classified according to their renal status, and the genotype data were analysed in a cross-sectional case–control setting. To confirm positive associations, four SNPs were genotyped in 1,025 additional patients with type 1 diabetes.
Results No associations with diabetic nephropathy were observed for any of the analysed SNPs. The SNPs were not associated with the time from the onset of diabetes to the diagnosis of nephropathy or with glomerular filtration rate or AER as quantitative variables. In a sex-specific sub-analysis, the variants rs979972 and rs749701 in the first intron of *ACTN4* were nominally associated with diabetic nephropathy in females, with odds ratios of 1.81 (95% CI 1.18–2.79, p=0.007) and 1.93 (95% CI 1.26–2.96, p=0.003) respectively.
Conclusions/interpretation Our study has not found any evidence that common variants in *LRRC7*, *KIRREL*, *NPHS2* and *ACTN4* contribute to susceptibility to diabetic nephropathy in Finnish patients with type 1 diabetes.

Keywords Diabetic nephropathy · Proteinuria · Podocytes · Molecular genetics · Glomerular filtration barrier

Abbreviations

ESRD	end-stage renal disease
MAF	minor allele frequency
SNP	single nucleotide polymorphism

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) requiring dialysis or renal transplantation, but the predisposing factors and pathogenetic mechanisms of diabetic nephropathy have remained elusive. Family studies have supported a genetic compo-

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nent in the development of the complication [1]. The podocyte foot processes and their interposed slit diaphragms form the outermost layer of the glomerular capillary wall and are responsible for the ultrafiltration of primary urine. Mutations in genes encoding the slit diaphragm lead to rare monogenic forms of proteinuria with variable age of onset and disease severity [2]. Defects in the podocin gene (*NPHS2*) manifest soon after birth and are characterised by uncontrolled, steroid-resistant proteinuria [3]. Mice lacking the gene encoding the nephrin-like protein NEPH1 (*Kirrel*) develop massive proteinuria and foot process effacement [4]. Mutations in the *ACTN4* gene, which encodes alpha actinin 4, account for the familial form of focal segmental glomerulosclerosis with later onset of the disease [5]. Densin-180 (*LRRC7*) was originally identified from synaptic structures in the brain and has recently been reported as a slit diaphragm-associated protein [6]. The slit diaphragm genes can be considered excellent candidate genes for a more common form of proteinuria, diabetic nephropathy. Here, we present a genetic association analysis of *LRRC7*, *KIRREL*, *NPHS2* and *ACTN4* in Finnish patients with type 1 diabetes.

Materials and methods

Participants Two sets of Finnish patients with type 1 diabetes were selected for the cross-sectional study (Table 1). Type 1 diabetes was defined as an age of onset <35 years, permanent insulin treatment initiated within 1 year of diagnosis, and a fasting C-peptide level below 0.3 nmol/l. Demographic data and blood and urine samples were collected for the determination of HbA_{1c}, AER and serum creatinine level. The study protocol was approved by the ethics committees of the participating centres and followed the principles of the Declaration of Helsinki. Informed consent was obtained from all patients.

The nephropathy status of the patients was ascertained, and four classes were generated (Table 1). Normoalbuminuria was defined as an AER less than 30 mg/24 h or 20 μg/min in an overnight urine collection, and the patients were required to have a duration of diabetes longer than 15 years to ensure their renal status. Patients with microalbuminuria had an AER between 30 and 300 mg/24 h or between 20 and 200 μg/min, and patients with macroalbuminuria an AER >300 mg/24 h or >200 μg/min. ESRD patients were either on dialysis or had received a kidney transplant. It was required that AER in at least two out of three consecutive 24 h or overnight urine collections exceeded the threshold for classification. The 24 h AER and serum creatinine values in Table 1 represent the single last central laboratory measurements, and some patients showed values deviating from the values used for the classification at the time of the investigation. Altogether, 32 patients in the normoalbuminuria class (n=1,066) presented with an AER exceeding 30 mg/24 h. The GFR was

Table 1 Clinical characteristics of the patients

	Study sample I			Study sample II		
	Normoalbuminuria (n=459)	Microalbuminuria (n=276)	Macroalbuminuria (n=368)	Normoalbuminuria (n=607)	Macroalbuminuria (n=158)	ESRD (n=260)
Male/female (%)	40/60	59/41	60/40	47/53	54/46	60/40
Age (years)	42.9±10.0	37.1±10.9	39.3±9.0	39.5±12.1	44.5±10.5	44.0±8.0
Diabetes duration (years)	29.0±6.8	25.0±9.4	27.2±6.4	25.3±10.4	34.2±9.4	32.6±7.7
Diabetes duration to diabetic nephropathy (years)	–	–	18.4±6.3	–	23.5±9.7	19.8±7.4
BMI (kg/m^2)	24.9±2.9	25.6±3.6	25.8±3.9	25.0±3.4	25.6±3.8	24.1±3.6
Systolic blood pressure (mmHg)	132±16	136±17	144±19	131±17	146±22	153±23
Diastolic blood pressure (mmHg)	78±9	81±10	84±10	78±10	81±11	86±12
HbA_{1c} (%)	8.1±1.1	8.8±1.4	9.0±1.6	8.3±1.3	9.1±1.5	8.6±1.5
AER (mg/24 h)	7 (1–85)	59 (2–613)	588 (4–8348)	8 (1–101)	426 (11–4609)	–
GFR (ml min^{-1} 1.73 m^{-2})	89.5±18.2	95.5±25.5	64.8±32.0	96.4±24.4	61.3±29.4	–
Serum creatinine (mmol/l)	84 (43–144)	89 (35–194)	127 (20–1278)	83 (20–238)	126 (46–728)	–

Data are mean±SD or median (range)

estimated using the Cockcroft–Gault formula adjusted for body surface area [7].

Power calculations A relative risk of 1.6 and a recessive model of inheritance were assumed, and the prevalence of diabetic nephropathy in type 1 diabetes was set to 20%. The powers attained with minor allele frequencies (MAFs) 0.05, 0.25 and 0.40 were 0.06, 0.64 and 0.98 ($p=0.05$) respectively using the sample size of 1066 individuals with normoalbuminuria and 786 with diabetic nephropathy. Power calculations were performed with the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/).

Markers and genotyping Forty SNPs were genotyped successfully [Table 2; Electronic supplementary material (ESM) Table 1, ESM Fig. 1); we attempted to genotype five other SNPs but either the attempt failed or the SNPs were out of Hardy–Weinberg equilibrium. The SNPs were required to have a MAF >0.05 in the Centre d'Etude du Polymorphisme Humain (CEPH) population. The SNPs captured 73% of SNPs for *NPHS2* and 83% for *ACTN4* according to the HapMap data (pairwise tagging with $r^2>0.8$ using the Tagger program; HapMap Public Release 20, January 2006). For *KIRREL*, we chose evenly distributed SNPs with an interval of 6–14 kb, and for *LRRC7* we chose those with an interval of 5–216 kb. The SNPs were determined from DNA extracted from peripheral blood, and genotyping was performed using the Homogeneous Mass-extend MassArray System (Sequenom, San Diego, CA, USA) or the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Genotyping quality was ensured by using 2% internal controls in each run, for which complete accuracy was demanded. The average genotyping success rate was over 99%.

Single-marker and haplotype analysis Allele and genotype frequencies of SNPs were compared with the χ^2 test. A logistic regression model with diabetic nephropathy as the dependent variable was used to estimate the independent association of SNPs. Sex, duration of diabetes, HbA_{1c} and systolic and diastolic blood pressures were included as covariates. Quantitative traits were evaluated by ANOVA or the non-parametric Kruskal–Wallis test. Haploview version 3.2 was used to determine linkage disequilibrium values and to estimate haplotypes. A p value below 0.05 was considered statistically significant.

Results

Genotype distributions were in Hardy–Weinberg equilibrium. The genotype and allele frequencies of the SNPs were compared between the patients with normoalbuminuria and those with macroalbuminuria in study sample 1. We observed no association for any of the analysed SNPs with macroalbuminuria (Table 2; ESM Table 1). The SNPs were not associated with microalbuminuria either (data not shown). We further compared the estimated haplotype frequencies within the haplotype blocks (confidence intervals), but none of them yielded statistically significant results (data not shown).

The genetic association of the SNPs with kidney function was also assessed using quantitative variables. However, no differences regarding AER, GFR or serum creatinine levels or time from the onset of type 1 diabetes to development of diabetic nephropathy were observed (data not shown).

Stratification by sex yielded nominal evidence of association with macroalbuminuria for four SNPs in *ACTN4* in female participants, and these SNPs were selected for genotyping in the second study sample. In the joint analysis of both study samples, the associations for the SNPs rs979972 (odds ratio 1.81, 95% CI 1.18–2.79, $p=0.007$) and rs749701 (odds ratio 1.93, 95% CI 1.25–2.96, $p=0.003$) remained significant in female patients with diabetes (for additional analyses see ESM Table 2). Similarly, significant associations were not observed in the male patients with diabetes. Notably, the patients with macroalbuminuria and those with ESRD were pooled in this analysis.

Discussion

This study reports a genetic association analysis of four slit diaphragm-associated genes, *LRRC7*, *KIRREL*, *NPHS2* and *ACTN4*, in a Finnish patient cohort with type 1 diabetes and diabetic nephropathy. The analysed genes did not associate with diabetic nephropathy or with any other clinical variable reflecting the kidney filtration function.

No genetic involvement of *KIRREL* or *LRRC7* in human disease has been reported previously to our knowledge, and this study provides no proof of such an involvement either. Furthermore, our results are in agreement with a previous report excluding linkage to diabetic ESRD for the *NPHS2* and *ACTN4* loci in White and African-American populations [8]. The only functional polymorphism studied here, R229Q in the *NPHS2* gene, has been suggested to contribute to susceptibility to microalbuminuria in the general population [9]. However, we could not establish such a relationship between AER and the R229Q variant, or any other variant in the *NPHS2* gene, in patients with type 1 diabetes.

The sex-specific sub-analysis suggested that common variants (rs979972 and rs749701) in the first intron of *ACTN4* may predispose to diabetic nephropathy in female participants. Alpha actinin 4 is not a slit diaphragm protein per se, but is located in the vicinity of the slit diaphragm in

Table 2 Genotyped SNPs in the *LRRC7*, *KIRREL*, *NPHS2* and *ACTN4* genes and their minor allele frequencies (MAF)

SNP[a]	MAF[b] (%)		Adjusted odds ratio[c] (95% CI)	*p* value
	Normoalbuminuria	Macroalbuminuria		
LRRC7 (chromosome 1)				
rs765795	0.32	0.33	1.00 (0.58–1.73)	0.999
rs10889850	0.31	0.30	1.06 (0.60–1.88)	0.833
rs1361494	0.38	0.40	1.19 (0.73–1.92)	0.485
rs2421306	0.44	0.41	0.71 (0.44–1.14)	0.157
rs659181	0.30	0.31	1.29 (0.73–2.30)	0.386
KIRREL (chromosome 1)				
rs4246539	0.16	0.17	1.20 (0.42–3.43)	0.731
rs6656063	0.40	0.41	0.96 (0.59–1.55)	0.854
rs11580742	0.16	0.14	0.80 (0.31–2.11)	0.655
rs6666443	0.08	0.07	1.82 (0.33–10.09)	0.493
rs6661149	0.07	0.06	1.12 (0.15–8.28)	0.915
rs6686246	0.13	0.10	0.54 (0.15–1.98)	0.356
rs7527735	0.28	0.28	1.02 (0.56–1.85)	0.961
rs1925032	0.21	0.19	0.53 (0.20–1.37)	0.188
rs7368400	0.48	0.46	0.93 (0.59–1.46)	0.743
rs12033891	0.16	0.16	0.56 (0.18–1.76)	0.317
rs7367384	0.47	0.51	1.52 (0.95–2.42)	0.080
rs874844	0.45	0.42	0.70 (0.44–1.13)	0.143
rs17421546	0.04	0.04	0.75 (0.41–1.39)	0.364
NPHS2 (chromosome 1)				
rs11585517	0.30	0.32	1.08 (0.62–1.89)	0.794
rs1410586	0.13	0.12	0.61 (0.07–5.80)	0.668
rs10913815	0.28	0.28	1.17 (0.63–2.15)	0.620
rs1410589	0.33	0.34	1.29 (0.75–2.21)	0.351
rs2274622	0.23	0.24	1.01 (0.50–2.09)	0.987
rs2274625	0.19	0.16	0.32 (0.09–1.12)	0.075
rs2274626	0.31	0.33	0.94 (0.55–1.61)	0.811
R229Q[d]	0.05	0.06	1.47 (0.88–2.46)	0.138
rs6698089	0.05	0.05	1.09 (0.63–1.87)	0.765
rs6657893	0.24	0.22	0.69 (0.33–1.46)	0.329
rs3738423	0.12	0.11	1.16 (0.80–1.70)	0.441
rs3829795	0.36	0.32	0.82 (0.63–1.06)	0.128
ACTN4 (chromosome 19)				
rs979972	0.49	0.52	1.41 (0.90–2.21)	0.136
rs888995	0.12	0.13	1.11 (0.36–3.39)	0.853
rs6508813	0.25	0.27	1.48 (0.81–2.70)	0.206
rs973009	0.10	0.09	2.88 (0.41–20.28)	0.288
rs2112650	0.17	0.17	0.71 (0.29–1.70)	0.437
rs4802744	0.20	0.22	1.27 (0.58–2.80)	0.556
rs749701	0.41	0.44	1.37 (0.87–2.16)	0.174
rs749702	0.12	0.12	1.02 (0.33–3.17)	0.979
rs2086148	0.03	0.02	0.77 (0.37–1.61)	0.492
rs1060186	0.27	0.24	1.48 (0.81–2.71)	0.207

The genetic association of the SNPs with macroalbuminuria was assessed with a regression model adjusted for sex, duration of diabetes, HbA_{1c} and blood pressure

[a] Reference identification number (rs) according to the HapMap database (Release 20, January 2006)

[b] The minor allele as denoted as in ESM Table 1

[c] Patients homozygous for the minor allele (2/2) were compared with patients homozygous for the major allele (1/1)

[d] The non-synonymous R229Q SNP in *NPHS2* characterised by Tsukaguchi et al. [13] was included

the cytoskeleton of the podocyte foot process [5]. Interestingly, the alpha actinin 4 mRNA is underexpressed in the glomeruli of patients with microalbuminuria compared with patients with normal AER [10]. In addition, a combination of glucose and advanced glycation end-products reduced the expression of alpha actinin-4 at both the protein and the

mRNA level in podocytes in vitro [11]. Whether or not the observed variation in *ACTN4* represents a true association remains to be determined in replication studies in other populations.

The analysed patients represented a carefully characterised study sample with a genetically homogeneous background from the Finnish population, which has the highest incidence rate of type 1 diabetes in the world [12], and the genotyping and statistical methods used in this study represent the state of the art. The SNP coverage can be considered sufficient to detect haplotype variation in the *NPHS2* and *ACTN4* genes. It is, however, of note that the five analysed SNPs in the *LRRC7* gene do not necessarily capture all possible causative variants. It is also important to note that, with the present study design, we cannot exclude the existence of rare variants in some cases of diabetic nephropathy.

In conclusion, this study does not provide evidence for a genetic association of *LRRC7*, *KIRREL*, *NPHS2* or *ACTN4* with diabetic nephropathy in Finnish patients with type 1 diabetes.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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