Analysis of the intronic single nucleotide polymorphism rs#466452 of the nephrin gene in patients with diabetic nephropathy

RODRIGO GONZÁLEZ^{1,*}, ARES TIRADO¹, LUIS A ROJAS^{1,*}, FRANCISCO J OSSANDÓN^{1,*}, MIRIAM ALVO², CARLOS WOLFF³, DANIELA SEELENFREUND¹, PILAR DURRUTY⁴ and SERGIO LOBOS¹

¹ Laboratorio de Bioquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile.

² Sección de Nefrología, Hospital Clínico de la Universidad de Chile, Santiago, Chile.

³ Departamento de Medicina, Facultad de Medicina, Universidad de Chile, Santiago, Chile.

⁴ Unidad de Diabetes, Facultad de Medicina, Universidad de Chile, Santiago, Chile.

ABSTRACT

We present the analysis of an intronic polymorphism of the nephrin gene and its relationship to the development of diabetic nephropathy in a study of diabetes type 1 and type 2 patients. The frequency of the single nucleotide polymorphism rs#466452 in the nephrin gene was determined in 231 patients and control subjects. The C/T status of the polymorphism was assessed using restriction enzyme digestions and the nephrin transcript from a kidney biopsy was examined. Association between the polymorphism and clinical parameters was evaluated using multivariate correspondence analysis. A bioinformatics analysis of the single nucleotide polymorphism rs#466452 suggested the appearance of a splicing enhancer sequence in intron 24 of the nephrin gene and a modification of proteins that bind to this sequence. However, no change in the splicing of a nephrin transcript from a renal biopsy was found. No association was found between the polymorphism and diabetes or degree of renal damage in diabetes type 1 or 2 patients. The single nucleotide polymorphism rs#466452 of the nephrin gene seems to be neutral in relation to diabetes and the development of diabetic nephropathy, and does not affect the splicing of a nephrin transcript, in spite of a splicing enhancer site.

Words: 198

Key terms: diabetic nephropathy, *NPHS1*, polymorphism, splicing enhancer.

INTRODUCTION

Diabetic nephropathy (DN) is one of the most serious and devastating chronic complications of diabetes (Wolf et al., 2005). There is a genetic basis in the pathogenesis of DN, and several susceptibility genes or specific genetic loci have been identified (Wolf et al., 2005 and Rossing, 2006). One of these is *NPHS1*, which codes for nephrin (OMIM 602716), a protein located at the slit diaphragm of renal glomeruli that plays a crucial role in

the renal filtration barrier of foot processes from podocytes (Ruotsalainen et al., 1999). The protein has 1,241 amino acid residues and is a member of the immunoglobulin family of cell adhesion molecules, containing eight Ig-like modules, one transmembrane domain and one fibronectin III-like module. The nephrin gene maps to chromosome 19q13.1 (GeneID: 4868; Locus NM_004646 on the reverse strand), spans 25,324 bp, contains 29 exons and the processed length is 3,726 bp (Kestila et al., 1998). Mutations in the nephrin gene are

Corresponding author: Sergio Lobos, Laboratorio de Bioquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile. Vicuña Mackenna 20, Santiago de Chile, Chile. e-mail: slobos@uchile.cl, Fax: 56-2-222-7900, Phone: 56-2-9781677.

Received: October 8, 2008 In Revised form: December 30, 2008 Accepted: January 12, 2009

the causative disorder of Finnish-type congenital nephrotic syndrome (Lenkkeri et al., 1999) and at least 60 disease-causing mutations of this gene have been identified and result in a lack of functional protein, some leading to massive proteinuria *in utero* (Beltcheva et al., 2001; Liu et al., 2001). Exonic polymorphisms in the nephrin gene have been associated with diverse acquired proteinuric kidney diseases and failure at the glomerular slit diaphragm (Kim et al., 2002; Koop et al., 2003). DN is clinically characterized by proteinuria and progressive renal insufficiency and diagnosed through the measurement of albuminuria (Durruty, 2003). The presence of micro-albuminuria (>30 mg/24 hrs) indicates renal damage and is associated with functional changes in the filtration barrier. In DN, nephrin mRNA and nephrin protein production are significantly reduced compared to those in non-diabetic subjects (Koop et al., 2003; Toyoda et al., 2004; Benigni et al., 2004).

Missense mutations or non-synonymous polymorphisms produce changes in protein structure. However, apparently silent polymorphisms and point mutations that do not seem to affect protein structure, can also alter gene function (Kimchi-Sarfaty et al., 2007). As well, polymorphisms that modulate pre-mRNA processing are gaining importance, since at least 15% of point mutations are related to human genetic disease caused by RNA splicing defects (Wang et al., 2005). Mutations affecting pre-mRNA processing can be located in introns or exons, resulting in exon skipping or creating new splice sites (Baralle and Baralle, 2005). It is of interest to examine polymorphisms that might appear neutral for translation, but may alter sequences inactivating regulatory elements that participate in pre-mRNA processing.

To date a total of 148 SNPs have been described in the human nephrin gene in the SNP database at NCBI, which correspond to 10 non-synonymous single nucleotide polymorphisms (SNPs), 6 synonymous, one frameshift, one 3'-untranslated and 130 intronic SNPs; the FastSNP server also identifies 141 SNPs (10 non-synonymous and 6 synonymous SNPs) in transcript

ENST00000378910 from *NPHS1*. Polymorphisms of the nephrin gene might be associated with DN, however the FinnDiane study (Pettersson-Fernholm et al., 2003) of three non-synonymous polymorphisms found no involvement of these particular coding regions of the *NPHS1* gene in the pathogenesis of DN in a large Finnish population of type 1 diabetic patients. Nonetheless, a study of Japanese patients (the Funagata study) showed that two silent and one intronic polymorphism of the nephrin gene are associated with impaired glucose tolerance in type 2 diabetes, suggesting that these variations may have a functional influence on nephrin expression in the pancreas (Daimon et al., 2006). This study underscores the importance of examining apparently silent polymorphisms that may result in unexpected functional variants or deleterious effects on gene expression.

The bioinformatic web tool PupaSuite (<http://pupasuite.bioinfo.cipf.es/>) (former PupasView) examines SNPs with potential pathological effects, to allow a rational strategy to select markers for disease susceptibility (Conde et al., 2005). To our knowledge, no study involving diabetic nephropathy and an intronic SNP of the nephrin gene has been reported. The aim of this work was to examine the association of a nephrin polymorphism (SNP rs#466452, located in intron 24) with diabetes and DN. For this purpose we analyzed Chilean patients with diabetes type 1 and type 2, to determine if the frequency of this SNP differs in diabetic patients and control individuals. We also examined if this specific intronic polymorphism of *NPHS1* alters the processing of the nephrin transcript.

MATERIALS AND METHODS

Bioinformatics approach to select a suitable SNP

We analyzed all intronic SNPs described for the human nephrin gene transcript ENST00000378910 with no known function according to the FastSNP server. Analysis

of putative exon splicing enhancer (ESE) elements was performed using ESEFinder (<http://rulai.cshl.edu/tools/ESE/>) and RescueESE (<http://genes.mit.edu/burgelab/rescue-e-se>) bioinformatics web tools. Using this approach we selected the SNP rs#466452 of the nephrin gene, which suggested the appearance of an ESE site (RescueESE) and a change in SR (Serine-Arginine rich) proteins that bind to the putative ESE element (ESEFinder) (Cartegni et al., 2003). SNP rs#466452 corresponded to the only SNP that generated a combined change of both a new ESE site and a modification in the number and type of SR proteins that bind to this ESE (Figure 1).

Subjects

We screened a total of DNA samples from 231 individuals (98 male, 133 female) from the Chilean population (with mixed Hispanic/Caucasian descent and a low percentage of Amerindian background, as 90 % of the subjects in this study have 2 Spanish family names), in agreement with the general structure of the population in

Santiago (Valenzuela et al., 1987). Of these, 168 were diabetic patients (75 male and 93 female) and 63 control subjects (23 male and 40 female). Blood of patients with diabetes type 1 and 2 was sampled from the Diabetes Unit of the San Juan de Dios Hospital, Santiago, Chile. Non-diabetic patients with proteinuric nephropathies were recruited from the Nephrology Section of the Clinical Hospital of the University of Chile.

The study population consisted of 67 diabetes type 1 and 101 diabetes type 2 patients, 25 non-diabetic patients with proteinuric nephropathies and a control group of 38 healthy individuals. Phenotypic characteristics of patients and control subjects are summarized in Table I. Subjects with BMI kg/m² over 35, individuals aged over 80, minors and pregnant women were excluded. In all subjects clinical and biochemical parameters were measured.

Informed consent was obtained from all participants specifically stating the use of blood samples for DNA extraction and analysis of the nephrin gene. The study protocol followed the principles of the

Fig. 1: A SNP (rs#466452) in intron 24 of the human nephrin gene produces a putative change in the processing of the *NPHS1* gene.

A) Creation of a putative ESE site in SNP rs#466452 according to the RescueESE server.

B) Change of binding of SR proteins due to SNP rs#466452, as indicated by bioinformatics analysis using the server ESEFinder: disappearance of binding of the SF2/ASF protein and the additional binding of the SRp40 protein.

Declaration of Helsinki and approval was given by the ethics committee from the San Juan de Dios Hospital (*Servicio de Salud Metropolitano-Occidente*).

Diabetic patients were classified as normo-, micro- or macro-albuminuric according to their albumin excretion rate (AER). Normo-albuminuric patients were defined by albuminuria of less than 30 mg/g in at least two of three morning urine samples taken during a period of less than 6 months. The inclusion criteria for diabetic patients with micro-albuminuria and macro-albuminuria were persistent albuminuria defined by an AER between 30 and 300 mg/g and over 300 mg/g under the same conditions, respectively. Albuminuria (A) is expressed in relation to excreted urinary creatinine (C), where $A/C = \text{mg albuminuria} / \text{g creatininuria}$.

Measurements were performed in the absence of urinary tract infections, fever or menstrual period using a Bayer-DCA 2000 system based on monoclonal antibodies with a sensitivity and specificity of over 90% and an intra-assay variation of less than 6.6%. Patients receiving angiotensin converting enzyme inhibitors (IACE) continued their treatment.

Genotyping

Peripheral blood was collected from patients and DNA extracted from 200 μL of blood leukocyte samples using the QIAmp DNA blood minikit (QIAGEN). Yields of 60 μg of pure DNA ($\text{DO}_{260 \text{ nm}/280 \text{ nm}}$ 1.6-1.9) were regularly obtained.

A 244 bp amplicon of the nephrin gene including the entire exon 24 and 77 bp of intron 24, where SNP rs#466452 is located, was used to screen all DNA samples. Primers were FW1 5'-ACAGCCTGTTGTC TGGGATTCACT-3' and REV1 5'-GACCT TCAGTATGCAGCAACCACA-3'.

The PCR fragment containing the SNP rs#466452 was analyzed using the Nebcutter V2.0 webtool (Vincze et al., 2003), selecting the Ava I restriction enzyme for detection of the polymorphism and genotyping. The amplified fragment contains two Ava I restriction sites, where one site corresponds to the base change of the SNP creating the loss

of the restriction site. The second site does not correspond to a polymorphic site and is therefore useful as a positive internal control of all genotyping assays. Consequently, the absence of a restriction fragment cannot be attributed to factors such as enzyme efficiency and digestion time. A diagram illustrating the restriction sites is shown in Figure 2. Polymorphisms were verified by direct sequencing at Macrogen Inc, Korea.

RNA extraction and RT-PCR

The source of RNA for analysis of nephrin transcripts was a kidney biopsy from a 70-year-old man with diabetes type 2 and macro-albuminuria subjected to radical right nephrectomy. Histology showed two renal adenocarcinomas-one on the upper and one on the lower pole-with involvement of the renal capsule, but without infiltration of the vessels, urether, nodes or perirenal fat. No metastasis was found. RNA was extracted from 0.5 g of fresh renal tissue of normal appearance using the SV total RNA isolation kit (Promega).

RT-PCR was performed using ImPromII reverse transcriptase (Promega) with 1 mg of RNA and primer Nef2REV (5'-TCTTCACCTGTGAAACCTCGGGAA-3'). PCR reactions were performed using primers FW2 (5'-TACAAGATACAGGGT CTGGCTGCT-3') and REV3 (5'-ATTCG TTCCTGACTCGGTCCTCTT-3') to obtain a 350 bp amplicon, that was cloned (TOPO-TA cloning kit, Invitrogen) and sequenced.

Statistical and correspondence analyses

Analyses were carried out using XLSTAT statistical package. Allele frequencies were analyzed by two-tailed Fisher's exact test by the MedCalc program; p values less than 0.05 were considered statistically significant.

The Correspondence Analysis (COA) (Fellenberg et al., 2001) is an explorative computational method for the study of associations between variables; it reveals the main axes of the high-dimensional space produced by the simultaneous inclusion of multiple conditions and their respective experiments/samples. The dimension reduction enables to project the data in 2 or 3

dimensions of maximum variance, which shows that two or more variables will be associated if they appear near each other in the plot. This analysis was performed using The Institute for Genomic Research MultiExperiment Viewer program (TIGR MeV) (<http://www.tm4.org/>) (Saeed et al., 2003), with default settings to search for associations between the variables analyzed and the different groups of patients by their distance in a virtual space. Diabetes type 1 and type 2 patients along with healthy control samples were analyzed and clinically relevant features for each subject were added to the data matrix as binary data (0 for absence, 1 for presence of the feature). Included features were presence of SNP#466452 mutation, homozygosity of SNP#466452 mutation, female sex, BMI over 25, albuminuria equal to or higher than 30 mg/24 h, creatinine equal or higher than 1, glycosylated haemoglobin A1c equal or higher than 6, use of inhibitors of

angiotensin converting enzyme (IECA), presence of hypertension and dyslipidemia. Only subjects with at least 8 of 10 clinical parameters available (144 individuals) were included.

RESULTS

SNP analysis with Ava I

A combined analysis of all intronic SNPs of the nephrin gene by the Rescue ESE and, ESEfinder using the FASTSNP server, which acts as a web wrapper, found that of a total of 74 intronic SNPs, 31 had no assigned function. Of these, 3 involved the appearance of a putative ESE motif and 12 indicated a change in binding of SR proteins. However, of all SNPs analysed, only SNP rs#466452 predicted a new ESE motif, the disappearance of binding of the SF2/ASF protein and additional binding of

Fig. 2: Detection of allelic variants of SNP rs#466452 with restriction enzyme Ava I

Diagram showing Ava I restriction sites in 244 bp sequence amplified with primers FW1 and REV1. The 51 bp fragment serves as an internal control of digestion with Ava I. The “C” allele is part of the palindrome of the second restriction site, generating a 149 bp fragment. The “T” allele creates a loss of this restriction site producing a 193 bp fragment.

Lower panel shows restriction fragments separated by electrophoresis on 3% agarose gels and stained with ethidium bromide. Lane 1 shows the amplified fragment of 244 bp without digestion (ND = Non-digested control). All samples digested with the Ava I enzyme generate the 51 bp fragment, although this fragment and the 44 bp digestion product are not seen on the gel. Lanes 2, 5, 6 and 7 show the restriction pattern of heterozygous individuals. Lanes 3, 4 and 8 correspond to samples of homozygous CC subjects and lane 9 corresponds to the sample of a homozygous TT individual.

a SRp40 protein, according to the output of both servers (Figure 1). This SNP is located 36 nt from the 5' end of intron 24, which corresponds to the position 20,230 bp from the ATG (HGVS Names [NM_004646.1: c.3286+36C>T] [NT_011109.15: g.8590727G>A]).

Restriction enzyme analysis of the amplified fragment showed that an *Ava* I site disappears when the allele "T" of the SNP rs#466452 is present. DNA samples of all subjects were amplified and digested with *Ava* I as described. A representative result is shown in Figure 2. To verify these results, 40 samples including all 3 genotypes were subjected to automatic sequencing, confirming restriction results in all cases.

Of the 231 subjects analysed by digestion with the *Ava* I restriction enzyme, 93 (0.403) were homozygous for the CC genotype, 98 (0.424) were heterozygous and 40 (0.173) were homozygous for the TT genotype of SNP rs#466452 (Table I). The distribution of SNP rs#466452

according to diagnostic groups showed no significant differences between healthy controls and diabetic patients.

SNP rs#466452 and DN

To assess whether there is an association with DN or proteinuric nephropathy, we examined the frequency of SNP rs#466452 in normo-albuminuric patients compared to diabetic patients with incipient or clinical nephropathy (albuminuria >30 mg/g) and a group of 25 non-diabetic patients with diverse proteinuric nephropathies. No association was found with the degree of renal damage, either in diabetes type 1 or type 2 patients, as the distribution of genotypes was not significantly different among these groups. Statistical analysis also indicated that all groups were in Hardy-Weinberg equilibrium (Table I).

Data in Table I show that the severity of renal damage of diabetic patients is associated with the duration of diabetes, age and hypertension. However, as patients exhibited similar levels of HbA1c, we did

TABLE I

Clinical, laboratory and genetic data

Groups	N	Men/women (n/n)	Age (years)	DM (years)	BMI (Kg/m ²)	Hypertension (%)	Use of LACE (%)	HbA1c** (%)	Albuminuria (mg/g)	Creatinine (mg/dl)	Dyslipidemia (%)	CC*†	CT*†	TT*†
DM1 total	67	30/37										0.388	0.403	0.209
DM1 normo-alb.	32	10 / 22	38.9±14.0	17.6±9.9	24.8±3.3	19	32	8.5±1.9	12.2±7.3	0.9±0.2	10	0.500	0.281	0.219
DM1 micro-alb.	21	11 / 10	39.1±13.5	18.8±12.0	25.6±4.7	38	50	9.1±1.7	135.3±67.4	1.8±0.3	19	0.286	0.476	0.238
DM1 macro-alb	14	9 / 5	49.2±14.5	32.7±13.0	24.0±3.3	50	38	9.3±2.2	422.6±194.0	1.3±0.3	27	0.357	0.500	0.143
DM2 total	101	45/46										0.386	0.446	0.168
DM2 normo-alb	35	11 / 24	55.5±12.2	10.8±7.4	29.0±5.2	35	58	8.2±1.9	14.0±7.8	0.9±0.3	33	0.486	0.429	0.086
DM2 micro-alb	38	22 / 16	59.6±14.7	14.0±11.6	29.6±5.3	38	36	8.4±2.5	129.7±79.4	1.5±0.9	47	0.316	0.447	0.237
DM2 macro-alb	28	12 / 16	63.4±8.8	19.1±6.8	29.5±3.0	86	89	8.2±2.1	556.5±262.8	1.3±0.7	59	0.357	0.464	0.178
Non-diab nephroph.	25	8 / 17	44.9±12.7	---	28.7±4.6	63	86	---	394.2±435.3*	2.7±1.9	73	0.660	0.320	0.120
Healthy controls	38	15 / 23	43.9±14.3	---	23.8±2.5	0	0	---	8.9±5.7	0.8±0.1	0	0.342	0.500	0.158
Subjects total	231	98/133										0.404	0.424	0.173

Data are expressed as mean ± standard deviation.

* CC, CT and TT indicate relative frequencies of genotypes

† Expressed as proteinuria in mg/L (not albuminuria)

** Evaluated at enrolment of the patient

t test

① p<0.05

② p<0.01

③ NS

Fisher's exact test

③ NS

⑤ p<0.05

⑥ p<0.01

‡ not significant when comparing healthy controls to DM1 and/or DM2 patients

not find an association between renal damage and the degree of metabolic control (glycemia).

Since the duration of diabetes is an important factor in the development of DN, we further investigated the relationship between this polymorphism, the degree of albuminuria and years of diabetes in type 1 diabetic patients. The genotypes between normo-albuminuric patients with more than 15 years of diabetes closely matched the genotypes found in patients with diabetes type 1 and renal damage after this period of time. Therefore no association was detected between SNP rs#466452 and absence of DN after more than 15 years of disease in patients with type 1 diabetes. Conversely, association between the development of kidney damage in less than 10 years and SNP rs#466452 genotypes could not be evaluated, as the number of affected patients was not amenable to statistical analysis.

Correspondence Analysis

The location of SNP rs#466452 in the periphery (Figure 3) suggests that it does not have a significant influence on the development of DN and can be considered a neutral change. The distance of the SNPs from clinical features and the main cloud representing type 1 and type 2 diabetic patients, points to a lack of significant association between the polymorphism and clinical parameters and diabetes as a whole. Additionally, SNP rs#466452 homozygosity is even more distantly located compared with SNP rs#466452 heterozygosity, confirming its lack of association. Glycosylated haemoglobin A1c, BMI, dyslipidemia, hypertension, use of IECA (a side effect of hypertension), albuminuria, and creatinine are more associated with diabetes, as indicated by their co-localization in the middle of the patient's cloud, than SNP rs#466452 polymorphism and sex (Figure 3).

Functional analysis of SNP

To evaluate whether SNP rs#466452 indeed produces a functional ESE, the nephrin

transcript of a patient homozygous for this polymorphism was examined. The nephrin transcript was obtained from renal biopsy tissue, as described. Analysis of the sequence from a 350 bp amplicon which included exons 24 to 26, indicated that splicing was identical to the published consensus sequence at relevant sites, and no change in the processing of the transcript had occurred, suggesting that this putative ESE site is not functional (results not shown).

DISCUSSION

This study was initiated as a screening of several amplicons of the nephrin gene using SSCP-PCR ("Single Strand Conformational Polymorphism-PCR"). Preliminary results of the amplicon analyzed in this work showed changes in migration patterns of the samples of some patients. These were further analyzed by automatic sequencing, where the variation was found to be located at the intronic SNP. Given the growing importance attributed to polymorphisms affecting splice sites (Baralle and Baralle, 2005), which are mostly located in introns, we decided to continue the analysis of this particular variant. An interesting feature is that the sampled population presents a frequency of 66.3% of the C allele, which is similar to the frequency described for European populations (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=466452).

Although splicing enhancers have been identified in both exons and introns, exonic splicing enhancers are better characterized and are probably more common. For this reason, the possibility of analyzing a putative motif of an intronic splicing enhancer or silencer was very attractive. Splicing enhancers are recognized and activated by splicing factors known as SR proteins, which also participate in spliceosome assembly (Liu et al., 1998). Individual SR proteins differ with respect to specificity of their RNA-binding domains and seem to show tissue-specific expression patterns, but their redundancy, as well as the ubiquitous nature and degeneracy of

ESE sequences, complicates the understanding of the complex relationship between splicing enhancer sequences and the binding of SR proteins.

There are several *in silico* procedures that are appropriate for evaluating polymorphisms, and some of these are particularly useful for non-coding SNPs (Bhatti et al., 2006). Rescue-ESE was one

of the first available tools to discover candidate sequences involved in splicing processes using a large database of human genes. The analysis of the hexamer sequence that includes SNP rs#466452 produced two changes above threshold, such as the creation of a binding site for SRp40 (Rescue-ESE) and loss of SF2/ASF binding (Rescue-ESE and ESEfinder),

Fig. 3: Correspondence Analysis of clinical features and SNP rs#466452

Samples of Diabetes type 1 and type 2 patients and healthy control individuals were analyzed and clinically relevant features for each subject were added as binary data (0 for absence, 1 for presence of the feature) to the data matrix. Representation of the clinical parameters is indicated as large white dots, while the small dots represent each patient (some overlaps). The small white dots are healthy control subjects, while the small black dots are diabetes type 1 and type 2 patients. The figure accounts for 55% of the Total Inertia (value proportional to the total variance) and shows that both the presence (dot 9, white arrow) and homozygosity (dot 10, black arrow) of SNP rs#466452 are located in the lower right quadrant, distant from the clinical features and from the main cloud representing diabetic patients. The large white dot located in the upper right quadrant (dot 1) represents the female sex and also does not show any strong association. In the upper left quadrant glycosylated haemoglobin A1c (dot 2), BMI (dot 3) and dyslipidemia (dot 4) are located. In the lower left quadrant appear albuminuria (dot 7), creatinine (dot 8), and the closest dots, which represent hypertension (dot 6) and use of IECA (dot 5).

which deserved further investigation (Cartegni et al., 2003). However, these tools were designed for the analysis of exonic sequences, but the functional importance of these same hexameric sequences in introns was an open question that was interesting to address, as has also been discussed by other authors (Pagani and Barelle, 2004).

Is it conceivable that the putative splicing enhancer site found in intron 24 of the nephrin gene plays a similar role as ESE elements present in exons? The aim of this study was to confront the output of bioinformatics webtools designed to evaluate the functional effects of SNPs with a set of clinical and experimental data. For this purpose a restriction digestion assay was set up to evaluate unambiguously the genotype of each subject for a particular SNP that seemed to produce a distinct effect on the splicing process of the *NPHS1* gene. The appearance of altered splicing could conceivably relate to an enhanced susceptibility for the development of renal complications in diabetic patients, which is in agreement with data indicating lower levels of nephrin transcripts found in DN (Toyoda et al., 2004). However, obtained results indicate that the frequency of SNP rs#466452 was not statistically different in patients with diabetes, patients with non-diabetic proteinuric nephropathies or healthy controls (Table I).

One finding of this study is that bioinformatic tools should be used with caution, basically as a guide, and always contrasted or validated with experimental data. Discrepancies between *in silico* and experimental results are due in part to the fact that the former are supported by databases that start rather precariously, but are constantly updated and therefore improving. A more fundamental reason for discrepancies is that motifs such as the ESE hexamer are generally assayed in the context of reporter mini-genes, which correspond to an *in vitro* context, where the influence of neighbouring sequences may not be thoroughly evaluated and distant sequences are disregarded.

Similarly, Correspondence Analysis suggested that neither the presence nor

homozygosis of this SNP was associated with diabetes or any related clinical feature. Furthermore, the molecular analysis of a nephrin transcript obtained from a renal biopsy of a patient with diabetes type 2 indicated that no change in splicing of this gene had occurred. In conclusion, the results of this study strongly advocate the importance of validating results obtained from bioinformatic analysis and suggest that SNP rs#466452 is neutral with respect to diabetes and the susceptibility of DN.

ACKNOWLEDGEMENTS

The authors would like to thank all subjects who participated in the study. This work was supported by a grant from the *Fondo Nacional de Investigación en Salud FONIS* (#SA-04i2095) from the government of Chile. We thank Matías Gutiérrez for critical review of the manuscript.

REFERENCES

- BARALLE D, BARALLE M (2005) Splicing in action: assessing disease causing sequence changes. *J Med Genet* 42: 737-748
- BHATTI P, CHURCH DM, RUTTER JL, STRUEWING JP, SIGURDSON AJ (2003) Candidate Single Nucleotide Polymorphism using publicly available tools: a guide for epidemiologists. *Am J Epidemiol* 164: 794-804
- BELTCHEVA O, MARTIN P, LENKKERI U, TRYGGVASON K (2001) Mutation spectrum in the nephrin gene (*NPHS1*) in congenital nephrotic syndrome. *Human Mutation* 17: 368-373
- BENIGNI A, GAGLIARDINI E, TOMASONI S, ABBATE M, RUGGENENTI P, KALLURI R, REMUZZI, G (2004) Selective impairment of gene expression and assembly of nephrin in human diabetic nephropathy. *Kidney Int* 65: 2193-2200
- CARTEGNI L, WANG J, ZHU Z, ZHANG MQ, KRAINER AR (2003) ESEfinder: A web resource to identify exonic splicing enhancers. *Nucl Acids Res* 31: 3568-3571
- CONDE L, VAQUERIZAS JM, FERRER-COSTA C, DE LA CRUZ X, OROZCO M, DOPAZO J (2005) PupasView: a visual tool for selecting suitable SNPs, with putative pathological effect in genes, for genotyping purposes. *Nucl Acids Res* 33: W501-505
- DAIMON M, JI, G, OIZUMI T, KIDO T, BABA M, JIMBU Y, KAMEDA W, SUSO S, YAMAGUCHI H, OHNUMA H, MURAMATSU M, KATO T (2006) Association of nephrin gene polymorphisms with type 2 diabetes in a Japanese population: The Funagata study. *Diabetes Care* 29: 1117-1119
- DURRUTY P (2003) Laboratorio en Diabetes Mellitus. In: GARCIA DE LOS RIOS M, DURRUTY P (eds.),

- Diabetes Mellitus, 2nd edition, Santiago de Chile, Chile: Arancibia Hnos. pp. 77-86
- FELLENBERG K, HAUSER NC, BRORS B, NEUTZNER A, HOHEISEL JD, VINGRON M (2001) Correspondence analysis applied to microarray data. *Proc Nat Acad Sci USA* 98: 10781-10786
- KESTILA M, LENKKERI U, MANNIKKO M, LAMERDIN J, MCCREADY P, PUTAALA H, RUOTSALAINEN V, MORITA T, NISSINEN M, HERVA R, KASHTAN CE, PELTONEN L, HOLMBERG C, OLSEN A, TRYGGVASON K (1998) Positionally cloned gene for a novel glomerular protein-nephrin-is mutated in congenital nephrotic syndrome. *Molec Cell* 1: 575-582
- KIM BK, HONG HK, KIM JH, LEE HS (2002) Differential expression of nephrin in acquired human proteinuric diseases. *Am J Kidney Dis* 40: 964-973
- KIMCHI-SARFATY C, OH JM, KIM IW, SAUNA ZE, CALCAGNO AM, AMBUDKAR SV, GOTTESMAN MM (2007) A "silent" polymorphism in the MDR1 gene changes substrate specificity. *Science* 315: 525-528
- KOOP K, EIKMANS M, BAELDE HJ, KAWACHI H, DE HEER E, PAUL LC, BRUIJN JA (2003) Expression of podocyte-associated molecules in acquired human kidney diseases. *J Am Soc Nephrol* 14: 2063-2071
- LENKKERI U, MANNIKKO M, MCCREADY P, LAMERDIN J, GRIBOUVAL O, NIAUDET P, ANTIGNAC C, KASHTAN CE, HOLMBERG C, OLSEN A, KESTILÄ M, TRYGGVASON K (1999) Structure of the gene for congenital nephrotic syndrome of the Finnish type (*NPHS1*) and characterization of mutations. *Am J Hum Genet* 64: 51-61
- LIU HX, ZHANG M, KRAINER AR (1998) Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. *Genes Dev* 12: 1998-2012
- LIU L, DONE SC, KHOSHNOODI J, BERTORELLO A, WARTIOVAARA J, BERGGREN P-O, TRYGGVASON K (2001) Defective nephrin trafficking caused by missense mutations in the *NPHS1* gene: insight into the mechanisms of congenital nephrotic syndrome. *Hum Molec Genet* 10: 2637-2644
- PAGANI F, BARELLE FE (2004) Genomic variants in exons and introns: identifying the splicing spoilers. *Nat Rev Genet* 5: 389-396
- PETTERSSON-FERNHOLM K, FORSBLOM C, PEROLA M, GROOP PH (2003) Polymorphisms in the nephrin gene and diabetic nephropathy in type 1 diabetic patients. *Kidney Int* 63: 1205-1210
- ROSSING P (2006) Prediction, progression and prevention of diabetic nephropathy. The Minkowski Lecture. *Diabetologia* 49: 11-19
- RUOTSALAINEN V, LJUNGBERG P, WARTIOVAARA J, LENKKERI U, KESTILÄ M, JALANKO H, HOLMBERG C, TRYGGVASON K (1999) Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Nat Acad Sci USA* 96: 7962-7967
- SAEED AI, SHAROV V, WHITE J, LI J, LIANG W, BHAGABATI N, BRAISTED J, KLAPA M, CURRIER T, THIAGARAJAN M, STURN A, SNUFFIN M, REZANTSEV A, POPOV D, RYLTSOV A, KOSTUKOVICH E, BORISOVSKY I, LIU Z, VINSAVICH A, TRUSH V, QUACKENBUSH J (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 34: 374-378
- TOYODA M, SUZUKI D, UMEZONO T, UEHARA G, MARUYAMA M, HONMA M, SAKAI T, SAKAI H (2004) Expression of human nephrin mRNA in diabetic nephropathy. *Nephrol Dial Transpl* 19: 380-385
- VALENZUELA C, ACUÑA M, HARB Z (1987) Gradiente sociogenética en la población chilena. *Rev Med Chile* 115: 295-299
- VINCZE T, POSFAI J, ROBERTS RJ (2003) NEBcutter: a program to cleave DNA with restriction enzymes. *Nucl Acids Res* 31: 3688-3691
- WANG J, SMITH PJ, KRAINER AR, ZHANG MQ (2005) Distribution of SR protein exonic splicing enhancer motifs in human protein-coding genes. *Nucl Acids Res* 33: 5053-5062
- WOLF G, CHEN S, ZIYADEH FN (2005) From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. *Diabetes* 54: 1626-1634