UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS:

ENDOCRINOLOGIA

GENÉTICA DA PREDISPOSIÇÃO À NEFROPATIA DIABÉTICA EM PACIENTES COM DIABETES MELITO TIPO 2

DISSERTAÇÃO DE MESTRADO

MARIANA PALAZZO CARPENA

Porto Alegre, junho de 2010

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, da Universidade Federal do Rio Grande do Sul (UFRGS) como requisito parcial para obtenção do título de Mestre em Endocrinologia.

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SUMÁRIO

LISTA DE ABREVIATURAS

ACE	*Angiotensin-converting Enzyme*
BMI	*Body Mass Index*
CARS	*Cysteinyl-tRNA Synthetase*
CI	*Confidence Interval*
cM	*Centimorgans*
CKD	*Chronic Kidney Disease*
DCCT/EDIC	*Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications*
DN	*Diabetic Nephropathy*
DM	*Diabetes Mellitus*
DR	*Diabetic Retinopathy*
ENPP1	*Ectoenzyme Nucleotide Pyrophosphate Phosphodiesterase*
ESRD	*End-stage renal Disease*
FABP2	*Intestinal Fatty Acid-binding Protein 2*
FRMD3	*4.1 Protein Ezrin, Radixin, Moesin [FERM] Domain Containing 3*
GFR	*Glomerular Filtration Rate*
GoKind	*Genetics of Kidneys in Diabetes*
GWU	*George Washington University*
GWS	*Genome wide-scan*
h^2	*Heritability*
ID	*Insertion/Deletion*

JDC	*Joslin Diabetes Center*
OR	*Odds ratio*
SNPs	*Single Nucleotide Polymorphisms*
TDT	*Transmission Disequilibrium Test*
UAE	*Urine Albumin Excretion*

INTRODUÇÃO

O Diabetes Melito (DM) é caracterizado por hiperglicemia crônica resultante de defeitos na secreção de insulina, na ação da insulina, ou ambos (1). A intensidade e o tempo de exposição à hiperglicemia, a predisposição genética e a presença de outros fatores de risco, como hipertensão e dislipidemia, determinam o aparecimento das complicações crônicas da doença. A doença microvascular leva à neuropatia, retinopatia e nefropatia, enquanto a doença macrovascular se manifesta por aterosclerose em órgãos como coração, cérebro e membros inferiores (2). Com o crescente aumento na prevalência do DM (3), cresce também o número de pacientes com complicações crônicas da doença, incluindo a nefropatia diabética (ND).

Devido à alta prevalência do DM tipo 2, estes pacientes constituem a maior parte dos pacientes diabéticos iniciando em programa de diálise, sendo a ND a causa mais comum de insuficiência renal crônica terminal (IRCT) em vários países (4, 5). Além de sua alta prevalência, a IRCT está associada a um importante aumento da mortalidade, principalmente por causas cardiovasculares (6, 7).

Os principais fatores de risco para a ND são a hiperglicemia e o aumento dos níveis pressóricos. Além destes, a dislipidemia, a obesidade e a disfunção endotelial estão envolvidos na patogênese da ND. Entretanto, nem todos os indivíduos com DM desenvolvem ND. Aqueles que não desenvolvem esta complicação nos primeiros 10 a 15 anos após o início do DM parecem ser protegidos desta complicação (8). Em outras palavras, na presença de fatores ambientais somente os indivíduos geneticamente propensos irão desenvolver a ND.

Grande empenho tem sido realizado na tentativa de identificar os principais genes associados à ND, com resultados ainda muito discrepantes. O modo de

transmissão genética da ND é ainda controverso, mas é possível que seja do tipo poligênica, com alterações em vários loci do DNA, cada uma com um efeito pequeno e aditivo no desenvolvimento da doença.

Essa dissertação reúne uma revisão sobre a genética da ND e um trabalho realizado no Serviço de Endocrinologia do Hospital de Clínicas de Porto Alegre, onde se avaliou a possível associação de nove polimorfismos de troca única (*single nucleotide polymorphism*- SNP) com a ND, em uma população de pacientes com DM tipo 2.

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BASES GENÉTICAS DA NEFROPATIA DIABÉTICA

GENETICS OF DIABETIC NEPHROPATHY

Running title: GENETICS OF DIABETIC NEPHROPATHY

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Resumo

A crescente elevação na prevalência do diabetes mellitus (DM) acarretou em um aumento de suas complicações crônicas, entre elas a nefropatia diabética (ND). Além da elevada prevalência, a ND está associada a importante morbidade e mortalidade principalmente por doenças cardiovasculares. É notória a contribuição genética na patogênese da ND, onde, na presença de fatores ambientais propícios, aqueles indivíduos geneticamente predispostos desenvolverão a doença. Trata-se de uma doença com provável transmissão genética do tipo poligênica e complexa. Duas estratégias principais têm sido utilizadas na busca dos genes associados à ND: a avaliação de genes candidatos e, mais recentemente, a utilização de genoma *wide scan*. Grande empenho tem sido realizado para identificar os principais genes associados à ND, mas os resultados ainda são heterogêneos com diferentes genes apresentando um efeito pequeno em populações específicas. A identificação dos principais genes permitiria prever os indivíduos de maior risco para o desenvolvimento da ND, além de possibilitar um melhor entendimento fisiopatológico da doença.

Descritores

Nefropatia, diabetes mellitus, genética, predisposição genética

Abstract

The increasing prevalence of diabetes mellitus has led to a growing number of chronic complications including diabetic nephropathy (DN). In addition to its high prevalence, DN is associated with high morbidity and mortality especially due to cardiovascular diseases. It is well established that genetic factors play a role in the pathogenesis of DN and genetically susceptible individuals after being exposed to environmental factors can develop it. DN is probably a complex, polygenic disease. Two main strategies have been used to identify genes associated to DN: analysis of candidate genes, and more recently genome-wide scan. Great efforts have been made to identify these main genes, but results are still inconsistent with different genes associated to a small effect on specific populations. The identification of the main genes would allow the detection of those individuals at high risk for DN and better understanding its pathophysiology as well.

Keywords

Nephropathy, diabetes mellitus, genetics, genetic predisposition

INTRODUCTION

Diabetes mellitus (DM) is a set of metabolic disorders with different etiologies characterized by hyperglycemia resulting from defects in insulin secretion and/or action. It was estimated 171 million cases of DM worldwide in 2000, and it is expected to increase to 366 million cases in 2030 (1). At the same time there has been a growth in the number of patients who develop chronic complications of DM, including diabetic nephropathy (DN).

Chronic kidney disease (CKD) is defined as renal damage characterized by structural or functional abnormalities of the kidneys, or glomerular filtration rate (GFR) <60 ml/min/1.73 m^2, with or without renal damage, over a period of at least three months, regardless of its causes (2). DN is characterized by a set of diabetic pathophysiological changes, which begin with glomerular hyperfiltration and renal hypertrophy, and then progress to proteinuria and GFR reduction. Based on the levels of urine albumin excretion, in a didactic manner, DN has two phases: incipient nephropathy or microalbuminuria phase with urine albumin excretion (UAE) between 20–199 μg/min (or 30–300 mg/24h); and clinical nephropathy or proteinuria phase with UAE >199 μg/min (>300 mg/24h) or proteinuria ≥500 mg/24h. Microalbuminuria is considered a risk factor for DN progression (3).

About 20 to 30% of patients with type 1 and type 2 DM develop DN; however, a smaller proportion of patients with type 2 DM will progress to end-stage renal disease (ESRD). Due to its high prevalence, the majority of patients requiring dialysis are type 2 DM. DN is the most common cause of ESRD in several countries (4, 5) but not all diabetic individuals will develop this complication. Those who do not develop DN in the first 15 years after disease onset seem to be genetically protected (6). Many environmental factors have been established as contributing to the development of DN

while the role of others has yet to be clearly understood (7). It is known that factors such as hyperglycemia, arterial hypertension and/or dyslipidemia play a role in the development of DN in genetically predisposed individuals only.

The rates of hospitalization for all causes are about three times higher in patients with CKD than in those who do not have the disease (8). According to Pagano et al., patients with type 2 DM with DN and peripheral arterial disease are 1.2 to 1.3 times more likely to be hospitalized (9). Besides its high disease burden, ESRD is associated with increased mortality, mainly due to cardiovascular causes (10). Reduced renal function is by itself an indicator of high mortality. Other concomitant risk factors such as hypertension and autonomic neuropathy can contribute to cardiovascular diseases (10). Even patients with DN initially characterized by microalbuminuria have already an increased risk for cardiovascular diseases and higher mortality (11). According to accumulating evidence, the risk of developing DN and cardiovascular disease starts when UAE values are still within normal ranges (12). Some authors consider albuminuria a major risk factor for cardiovascular events rather than just a simple marker of DN progression (13) as some patients develop fatal cardiovascular events even before showing reduced renal function. In the metropolitan area of Porto Alegre, southern Brazil, 25% of new patients requiring dialysis are due to DM, and they show high mortality rate during the first two years on dialysis (14).

It has not yet been clearly established the mechanism by which some diabetic patients will progress towards loss of renal function and require dialysis while others will maintain normal renal function. Research has focused on seeking potential genetic alterations associated to CKD and ESRD. In fact, genetic evidence has been found in case-control association and linkage studies, and more recently using genome-wide scan

(GWS). These studies support the assumption that onset, progression, and severity of DN can be in part attributed to genetic factors (15).

The identification of genes associated with DN will allow recognizing those individuals who are at high risk of developing this complication. It will also allow a better understanding of the mechanisms and progress of DN. Earlier and more aggressive therapies could be provided to high-risk individuals and thus reduce the associated high disease burden and mortality. Advances in pharmacogenetic research may help treatment choices by selecting renoprotective drugs according individual haplotypes (16).

The present review discusses the main information available in the literature that reaffirms the importance of genetic factors in DN. Some findings of our recent genetic studies are also summarized.

GENETIC TRANSMISSION MODELS

The genetic transmission mode of DN is still controversial. Theoretically, as in other diseases, it might occur in three distinct forms, which would lead to the development of DN (6, 17).

- Monogenic form: mutations in a gene with a dominant role;
- Oligogenic form: mutations/polymorphisms in a few genes would contribute in an independent and cumulative manner to increased susceptibility;
- Polygenic form: alterations in many DNA loci, and each would have a small and cumulative effect on DN development.

Considering that DN is a multifactorial disease, the mode of transmission is likely polygenic, and genetic interaction with other environmental factors and clinical

data, such as duration of the DM, arterial hypertension, dyslipidemia, smoking would lead to the development of DN. Familial aggregation studies do not allow to clearly establish a mode of transmission, but they provide evidence supporting that this is a polygenic complex disease (6, 17). On the other hand, studies using segregation analysis (18, 19) suggest that familial aggregation of DN is due mainly to the action of a main genetic locus. This effect would be consistent with a monogenic or oligogenic form, where few genes would have greater effect on the phenotype.

Since several genes may be involved in the development of DN, and many of them have not yet been identified, it is not possible to be completely sure about the exact heritability pattern in most cases.

STRATEGIES FOR IDENTIFICATION OF GENES ASSOCIATED WITH DIABETIC NEPHROPATHY

Genes that confer susceptibility to DN can be sought in different ways. A widely established method is the candidate gene approach. The search for candidate genes includes the study of polymorphisms in one or more genes potentially involved in the pathogenesis of the disease. This approach is useful even when the influence of a gene on disease development is small (17).

Candidate genes are often analyzed in case-control studies by comparing the frequency of polymorphisms/mutations in candidate genes among patients with and without the disease. This is an appropriate study for investigating complex genetic transmission, and it is especially useful in situations where the genetic influence is relatively low, and disease-related alleles are common in a population (17). However, this approach is very sensitive to population stratification, which may lead to spurious associations. In the light of that it has been proposed that these studies should include a

large sample to obtain very small p-values and be based on well-established a priori assumption. This approach has allowed to describing many polymorphisms associated with DN; however, the study results have been inconsistent. One of the most studied alterations is insertion/deletion on angiotensin-converting enzyme (ACE) gene. A meta-analysis have showed that D allele is associated with high risk of DN (20).

Another approach used to analyze candidate genes is the transmission disequilibrium test (TDT). This approach is not influenced by population stratification, but information is required about the individuals studied and their parents and only heterozygote parents are informative. The frequency of transmission of risk allele is compared to the expected 50%. Its main limitation is having access to the individual and their parents especially for type 2 DM that has a late onset in life. In a case-control study we demonstrated that the ectoenzyme nucleotide pyrophosphate phosphodiesterase (ENPP1) gene is associated with early development of ERSD in patients with type 1 DM (21), and using the TDT approach we confirmed this association was not due to population stratification (21). Araki et al. used the same approach to demonstrate that the APOE-epsilon 2 allele is associated with DN in type 1 diabetic individuals (22).

More recently candidate genes are being tested in prospective studies. This study design is less prone to survival bias than case control-studies but they are expensive and time-consuming. Alternatively, the authors are studying cohorts that have been followed up over a long period of time (23). The limitation of these studies is that they are not specifically designed to address a genetic effect of a specific gene.

The use of microarrays has enabled fast, accurate analysis of a large number of candidate genes and to perform GWS based on single nucleotide polymorphisms (SNPs). GWS can identify chromosomal regions that contain genes potentially involved

in the genesis of a disease under study. Panels of microsatellite markers or SNPs, at ~10 centimorgans (cM) intervals throughout the genome, are genotyped in several generations of families of patients with DM, affected or not with DN. The markers that most commonly occur in family members affected with DN indicate the location of a functional variant associated with the disease and is in linkage disequilibrium with the marker. Given the difficulty of finding large families with many members affected with DN, an alternative approach is to compare the observed and expected frequency of the markers in pairs of diabetic siblings concordant and discordant for DN. A major advantage over the candidate gene approach is that this approach can detect chromosomal regions containing genes that were not previously known to be implicated in the pathogenesis of DN. However, it has the disadvantage of only detecting genes that have a moderate or large effect (24). Using GWS we identified three polymorphisms located on chromosomes 9q and 11p associated with DN in two different populations of patients with type 1 DM (25).

EVIDENCE FOR GENETIC PREDISPOSITION TO DN

Familial Aggregation of Diabetic Nephropathy

Studies of familial aggregation have showed that some families are predisposed to DN (26-28). Studies on siblings with type 1 or type 2 DM have reported that DN in one of the siblings is associated to around 3- to 4-fold increase in the risk of DN in the other sibling (29, 30).

There appears to be a genetic inheritance contributing to the development of CKD. Forsblom et al. showed that the heritability (h^2) of UAE rate is approximately 30% when analyzing non-diabetic children of type 2 diabetic individuals (31). This finding was corroborated by another study, which found that after adjustment for covariables such as sex, age, obesity and DM, approximately 30% of the variability of albumin-creatinine rate was due to genetic factors (32).

The magnitude of the familial association cannot be attributed only to exposure to similar risk factors, suggesting there is a genetic component (33).

Genes Associated with Diabetic Nephropathy and Different Phenotypes

Although proteinuria and loss of renal function often occur concomitantly, there is evidence of different genetic predispositions for each condition (15). This can explain why some patients may have persistent proteinuria without progressing to loss of renal function (34) and other patients have loss of renal function without proteinuria or microalbuminuria (15, 35).

In genetic association studies, it is very important to clearly define the phenotype of interest. Most studies on heritability use albuminuria or proteinuria as a DN marker. In fact, loss of renal function measured by GFR is strongly associated with increased UAE. However, it is important to point out that a significant proportion of patients with DM develop loss of renal function but they maintain normoalbuminuria (35-37) and, conversely, some patients with clinical proteinuria maintain a stable GFR over the years (38).

UAE and GFR reduction are both genetically determined, but apparently independently (15). Langefeld et al. evaluated 310 families comprising 662 patients

with type 2 DM and found an h^2 of 0.35 for UAE, and of 0.69 for GFR (39). These findings were similar to those described in other studies reporting an h^2 of creatinine depuration of 0.63 among mono- and dizygotic twins (40).

Studies of Candidate Genes

One approach to identify genes associated to DN is the study of candidate genes. There are many studies of candidate genes for DN but the results are inconsistent (Table 1). The choice of the gene to be studied depends on knowledge concerning its actions in DN pathophysiology such as those involving blood pressure control, severity of proteinuria or insulin resistance (41). Below we present our experience with this approach.

Type 2 DM patients with microalbuminuria have high serum levels of circulating fatty acids (42) compared to normoalbuminuric patients. Intestinal absorption of long-chain fatty acids is controlled by the intestinal fatty acid-binding protein 2 (FABP2). Thus, alterations in the gene that codifies the FABP2 can be candidates indicating predisposition to DN. A54T polymorphism (rs1799883) in *FABP2* is associated with altered protein conformation, leading to greater affinity of the FABP2 protein for intestinal fatty acids with consequent serum increase. We genotyped this polymorphism in 1042 Brazilian patients with type 2 DM. An association of the T allele was found at different stages of DN (Figure 1) (43). This association was replicated in an independent sample of 483 white American subjects with type 2 DM (43).

Several studies analyzed the insertion/deletion (I/D) polymorphism in the ACE gene but the results about its association with DN in patients with type 1 and type 2 DM were inconsistent, possibly due to racial differences of the populations studied (20).

Besides, several studies used longer duration of DM as an inclusion criterion, which might predispose to survival bias, insofar as the possible genes associated with DN could also be associated with increased mortality. Therefore, we chose to investigate a potential association between I/D polymorphism in the ACE gene and the development of DN in 982 Brazilian patients with type 2 DM, taking into account the duration of their disease. In patients with 10 years or less of disease, having an allele D (DD/ID) resulted in an odds ratio (OR) of 2.66 (95% CI 1.12–6.58, p=0.015) for incipient DN and 3.19 (95% CI 1.18–9.30, p=0.012) for overt DN. On the other hand, in patients with longer disease duration, no increased risk for DN was seen associated with allele D (44).

Gene candidates for insulin resistance can also be considered DN candidates since insulin resistance is a common characteristic of patients with type 1 and type 2 DM who present increased UAE (45, 46). The polymorphism in *ENPP1* gene, previously known as *PC-1*, was found to be associated with insulin resistance (47). Based on this finding, a case-control study was conducted to assess the association between advanced DN and K121Q polymorphism (rs1805101) in the *ENPP1* gene of patients with type 1 DM. The proportion of patients with the variant Q was 21.5% in the control group, 31.5% in those with proteinuria, and 32.2% in those with ESRD (p=0.012). In a stratified analysis for DM duration (<24 or ≥24 years of DM), the risk of early onset of ESRD for patients with the variant Q was 2.3 times greater than for those without the variant Q (95% CI 1.2–4.4) and it was not associated with late onset ESRD (21). This association was confirmed using TDT, which showed that this association was not due to population stratification (21).

Polymorphisms in *GLUT1* gene associated with DN were also examined. GLUT1 is a glucose transporter in the kidneys and it has been associated with early kidney alterations leading to proteinuria. We studied 230 controls (patients with DM1,

disease duration of at least 15 years and normoalbuminuria), and 262 cases (151 patients with persistent proteinuria and 111 with ESRD). The homozygosis for the XbaI(-) allele was associated with a discrete increased risk for DN when compared to other genotypes combined [OR 1.83 (95% CI 1.01–3.33)]. A significant difference in genotype distribution among cases and controls was seen for the enhancer-2 SNP1 (p=0.036). There was an excess of genotype AA among cases (10.7%) compared to controls (4.8%). These homozygous individuals presented an increased risk for DN compared to AG and GG genotypes combined [OR 2.38 (95% CI 1.16–4.90)] (48).

Other studies analyzed different genes and did not find an association with DN (Table 1). One of these studies showed that, when patients were stratified for smoking, allele T (p22phox C242T polymorphism; rs4673) was more frequently seen in smokers with ESRD or persistent proteinuria than in normoalbuminuric patients (43% vs. 32%; p=0.045). The multiple logistic regression analysis confirmed that CT and TT genotypes were independently associated with a greater risk of overt DN among smokers (OR= 6.76; 95% CI 1.83–25.02) (49).

Our experience with candidate genes allowed us to identifying some genes that can be related to the development and severity of DN.

Genome-Wide Scan Studies

Recent GWS studies have demonstrated chromosomal regions potentially associated with DN. Table 2 shows the studies using a GWS approach. In DN concordant Pima Indians, a potential association was seen between chromosome 7q and phenotype of DN. The area between two markers (D7S500 and D7S1804), in 144 cM, showed the strongest association, with a LOD score of 2.04. Two other susceptibility

loci were also found in chromosomes 20 (LOD score of 1.83) and 3 (LOD score of 1.48) (50).

Genes located in chromosomes 22q, 5q, and 7q might be involved in the determination of UAE severity in patients with and without DM (51). In a genome-wide association study, regions in chromosomes 19 and 2q were identified as associated with proteinuria and ESRD in patients with type 1 DM (52). A locus in chromosome 1q was associated with ESRD only, while a locus in chromosome 20p was associated with proteinuria only (52). Two independent loci were identified in chromosome 3q, 59 cM from each other, one associated with proteinuria and the other with ESRD (52).

A genome-wide association study with 360,000 SNPs using the microarray Affymetrix 5.0 was recently conducted in two independent cohorts of Caucasians patients with type 1 DM (25). SNPs that were highly significant in the two cohorts were selected for further analyses. Eleven SNPs located at 4 loci were closely associated with DN ($p<1 \times 10^{-5}$) (Table 3). Some of these associations found in the cross-sectional study were confirmed in a prospective sample of the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC). Three of the 11 initial SNPs had their associated confirmed, two were borderline and the remaining did not show a significant association with the development of DN (proteinuria or CKD) (25) (Table 4).

Other authors reported an association of different chromosomal regions using GWS approach to search for DN related genes (Table 2) (53-56).

CONCLUSION AND PERSPECTIVES

In conclusion, clinical and epidemiological studies have evidenced a genetic component of DN. However, no specific gene has been able to explain most DN cases yet. The results of studies that identified genes or genome regions associated with DN were quite inconsistent. The lack of more consistent results is probably due to different factors. Most genetic studies have been performed in selected populations but they are heterogeneous between them. It should also be pointed out that an isolated candidate gene is sought when various genes are probably involved and possibly interlinked. Joint efforts are essential to achieve robust findings in the study of genetics of DN. In the light of remarkable advances in this area of study, we hope that in the near future patients at high risk for developing DN could be identified and benefited with earlier specific therapies. We also expect to see a consequent reduction in disease burden and mortality due to this serious complication. New pharmacogenomic developments will contribute to better treatment choices for DN and, more importantly, will help preventing it based on an individual's genetic characteristics.

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Table 1 – Candidate genes for diabetic nephropathy

AUTHOR	GENE	POLYMORPHISM	OR (95% CI)	p-value	CASE DEFINITIONS
Canani et al., 2002 (21)	ENPP1/PC-1	K121Q (rs1805101)	2.3 (1.2-4.4)	0.014	Patients with type 1 DM with ESRD
Leitão et al., 2008 (57)	ENPP1/PC-1	K121Q (rs1805101)	1.06 (0.73-1.54)	0.830	Type 2 DM with micro- or macroalbuminuria
Ng et al., 2002 (48)	GLUT1	XbaI enhancer-2	1.83 (1.01-3.33) 2.38 (1.16-4.90)	0.044 0.036	Patients with type 1 DM with persistent proteinuria or ESRD
Santos et al., 2006 (58)	CATALASE	-262C/T (rs35448603)	0.92 (0.63-1.37)	0.776	White patients with type 2 DM with micro- or macroalbuminuria or ESRD
Canani et al., 2005 (44)	ACE	Ins/Del polymorphism	2.66 (1.12-6.58) 3.19 (1.18-9.30)	0.015 0.012	Type 2 DM with less than 24 years of disease: incipient and overt DN, respectively
Santos et al., 2005 (49)	CYBA	p22phox C242T (rs4673)	2.24 (1.15-4.37)	0.045	White patients with type 2 DM, smokers, with micro- or macroalbuminuria or ESRD
Santos et al., 2005 (59)	RAGE	-429C (rs1800625) -374A (rs1800624)	1.31 (0.62-2.76) 0.77 (0.40-1.45)	0.720 0.270	703 Brazilians with type 2 DM (520 Caucasians and 183 of African ascendance with micro- or macroalbuminuria or ESRD
Canani et al., 2005 (43)	FABP-2	A54T (rs1799883)	2.4 (1.1-5.4)	0.005	Type 2 DM with microalbuminuria or persistent proteinuria or ESRD
Caramori, et al., 2003 (60)	PPARγ2	Pro12Ala (rs1801282)	0.465 (0.229-0.945)	0.034	Type 2 DM with CKD and normoalbuminuria

DM = diabetes mellitus; DN = diabetic nephropathy; ESRD = end-stage renal disease; OR = odds ratio; CI = confidence interval

Table 2 – Genome-Wide Scan Studies for Genes Associated with Diabetic Nephropathy

Author	Population Studied	Regions Associated	Phenotype
Imperatore et al., 1998 (50)	Pima Indians- 98 siblings with DM2, concordant for ND	7q, 3 and 20	Albuminuria or proteinuria
Vardarli et al., 2002 (53)	125 patients with DM2	18q22.3-23	Albuminuria
Tanaka et al., 2003 (54)	Japanese with DM2 grouped into cases (with DR and DN) and controls (with DR and without DN)	16q13	Albuminuria
Bowden et al., 2004 (55)	266 siblings with DM2, African-Americans, concordant for DN	3q, 7p and 18q	CKD
Krolewski et al., 2006 (51)	59 Caucasian families, 1 African-American, and 3 Hispanic. Members with and without DM2	22q, 5q and 7q	Albuminuria
Iyengar et al., 2007 (56)	1,227 subjects from 378 families with DM1 or DM2, concordant or discordant for DN	2q14.1, 7q21.1 and 15q26.3 7q21.3, 10p15.3, 14q23.1 and 18q22.3	Albuminuria Proteinuria or ESRD
Rogus et al., 2008 (52)	Sibling concordant for DM1 and discordant for ND	19q, 2q and 3q 1q 20p	Proteinuria and ESRD ESRD Proteinuria
Pezzolesi et al., 2009 (25)	Type 1 DM patients with DN (cases) and without DN (controls)	7p- CPVL/CHN2 9q - locus FRMD3 11p - locus CARS 13q	Proteinuria and ESRD

DM= diabetes mellitus; DN= diabetic nephropathy; DR= diabetic retinopathy; ESRD= end-stage renal disease

Table 3 – Single Nucleotide Polymorphisms Associated with Diabetic Nephropathy in a Population of GoKinD

					Frequencies of risk alleles for cases and controls					
					GWU* GoKinD			JDC** GoKinD		
SNP	**Chromosome**	**Position (Mb)**	**Nearest gene**	**Risk Allele**	**Controls**	**Cases**	**p-value**	**Controls**	**Cases**	**p-value**
N					413	379		472	441	
rs39059	7p	29.2	CPVL/CHN2	A(G)	0.61	0.69	8.8×10^{-4}	0.60	0.67	1.7×10^{-3}
rs39075	7p	29.2	CPVL/CHN2	G(A)	0.57	0.66	2.0×10^{-4}	0.57	0.64	8.2×10^{-4}
rs1888747	9q	85.3	FRMD3	G(C)	0.68	0.73	3.6×10^{-3}	0.66	0.74	4.4×10^{-5}
rs10868025	9q	85.4	FRMD3	A(G)	0.59	0.66	1.9×10^{-3}	0.56	0.66	7.2×10^{-5}
rs739401	11p	3.0	CARS	C(T)	0.46	0.54	4.7×10^{-4}	0.49	0.55	3.6×10^{-3}
rs451041	11p	3.0	CARS	A(G)	0.46	0.54	6.9×10^{-4}	0.48	0.56	1.3×10^{-3}
rs1041466	13q	109.0	No gene	G(A)	0.39	0.47	3.6×10^{-3}	0.43	0.51	2.7×10^{-4}
rs1411766/rs17412858	13q	109.1	No gene	A(G) G(A)	0.31	0.39	8.5×10^{-4}	0.32	0.40	6.4×10^{-4}
rs6492208/rs2391777	13q	109.1	No gene	T(C) G(A)	0.55	0.62	8.7×10^{-3}	0.56	0.65	1.9×10^{-4}
rs7989848	13q	109.1	No gene	A(G)	0.49	0.56	2.0×10^{-3}	0.50	0.57	1.1×10^{-3}
rs9521445	13q	109.1	No gene	A(C)	0.47	0.54	2.1×10^{-3}	0.47	0.55	4.2×10^{-4}

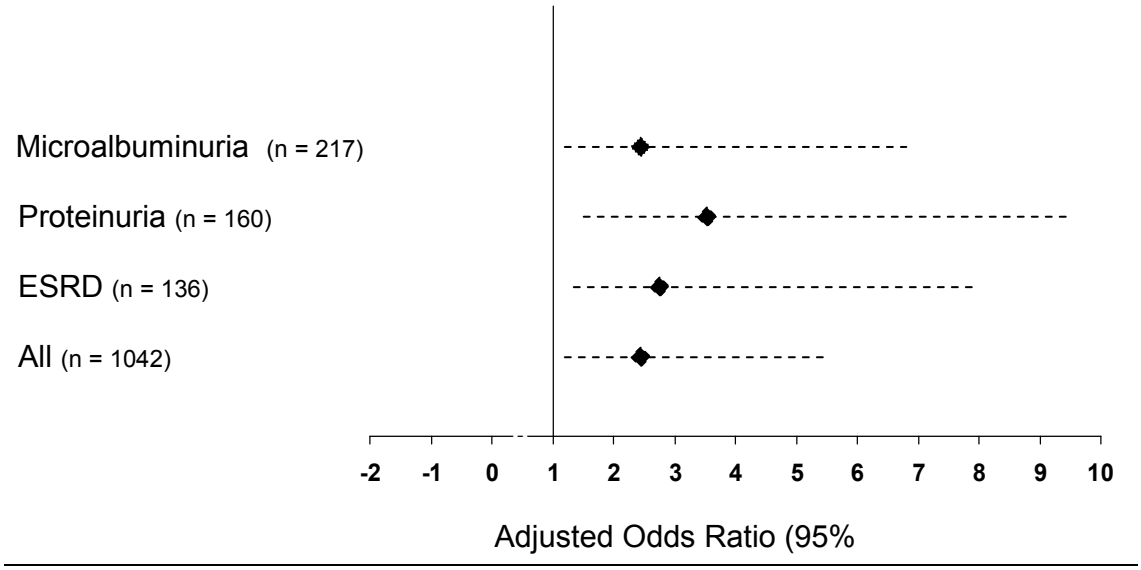
* GWU – George Washington University

** JDC – Joslin Diabetes Center

Table 4 – Single Nucleotide Polymorphisms Associated with Diabetic Nephropathy in a Population of DCCT/EDIC

SNP	Chromosome	Position (Mb)	Nearest Gene	Risk allele	Allele frequency	p-value (unicaudal)	*Hazard ratios*
rs39075	7p	29.2	CPVL/CHN2	G	0.60	NS	0.85
rs1888746	9q	85.3	FRMD3	C	0.70	0.02	1.33
rs13289150	9q	85.4	FRMD3	A	0.62	0.05	1.23
rs451041	11p	3.0	CARS	A	0.51	0.01	1.32
rs1041466	13q	109.0	No gene	G	0.47	0.11	1.22
rs1411766	13q	109.1	No gene	A	0.36	0.11	1.17
rs6492208	13q	109.1	No gene	T	0.61	NS	0.90
rs7989848	13q	109.1	No gene	A	0.53	NS	0.93

Figure 1



Legend

Figure 1: Odds ratio (♦) with 95% confidence intervals (dotted line) for the presence of A54T polymorphism in *FABP2* at the different stages of renal involvement adjusted for diabetes duration, body mass index, arterial hypertension, HbA1c, and cholesterol levels. ESRD = end-stage renal disease.

FRMD3 Polymorphism: Possible Role in Diabetic Nephropathy

Short title: FRMD3 polymorphism in diabetic nephropathy

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Abstract

Objective- There is strong evidence that diabetic nephropathy (DN) aggregates in families and that genetic factors contribute to the development of the disease. In a recent study, 13 single nucleotide polymorphisms (SNPs) were found to be associated with DN in American patients with type 1 diabetes. Based on these findings, we carried out a case-control study to evaluate the possible association between these SNPs and DN in Brazilian patients with type 2 diabetes.

Research Design and Methods – 1,098 white patients with type 2 diabetes were evaluated. Cases (n=718) included patients with established microalbuminuria (n=323) and with macroalbuminuria or on renal replacement therapy (n=395). Controls (n=380) included patients with type 2 diabetes diagnosed for at least five years, without renal disease. Of the 13 polymorphisms positively associated with DN in type 1 diabetic patients, nine were chosen to be evaluated in this study: rs1888747, rs9521445, rs39075, rs451041, rs1041466, rs7956328, rs11186286, rs1411766 and rs6492208.

Results - The frequency of allele C of the rs1888747 polymorphism was lower in cases compared to controls (0.27 *vs*. 0.31), but with borderline statistical significance (p=0.06). The presence of this allele in homozygosis was associated with reduced risk of DN (RC=0.6, 95%CI 0.3-0.9; p=0.022).

Conclusions – Replication of the association between SNP rs1888747 and DN in a different population is strong evidence that this association is not by chance. This polymorphism is located at FRMD3 gene which is expressed in human kidney. It can be a new candidate gene for DN.

INTRODUCTION

Diabetic nephropathy (DN) has become the major cause of end stage renal disease (ESRD) in developed countries (1, 2). Among patients starting dialysis, DN incidence is estimated to be 26% (3). Besides its high prevalence, DN causes an important increase in morbidity and mortality, mainly resulting from cardiovascular diseases (3, 4). Therefore, the prevalence of diabetes in dialysis is only around 15% (5).

It is well settled that DN aggregates in families (6-8), which strongly suggests that genetic factors contribute to the development of DN. Meanwhile, little is known about the mode of transmission, which is probably of a polygenic nature, with the interaction between genetic and environmental factors leading to the development of DN. Until this moment, genome wide-scan (GWS) studies and research on candidate genes have reached heterogeneous results (9-12).

A GWS study with 360,000 single nucleotide polymorphisms (SNPs) has recently identified 11 SNPs associated with DN in two independent populations of type 1 diabetic patients (GoKinD Study) (13), comprising a total of 1,705 patients. Confirmation of implicated SNPs was also sought in 132 of 1,304 participants of the Diabetes Control and Complications Trial (DCCT) / Epidemiology of Diabetes Interventions and Complications (EDIC) study, a long-term prospective investigation of the development of diabetes-associated complications (14-16). Of the 11 SNPs, two were associated with DN in all these three North American populations of type 1 diabetic patients.

The aim of this study was to investigate the association of SNPs found to be associated with DN in type 1 diabetes in an independent population of type 2 diabetic patients and to evaluate the effect of these SNPs on less severe stage of DN, defined as

microalbuminuria. The rationale for using these SNPs is the assumption that patients with type 1 and type 2 diabetes share common genes related to DN.

RESEARCH DESIGN AND METHODS

A case-control study was conducted with 1,098 white type 2 diabetic patients selected from an ongoing cross-sectional study in the state of Rio Grande do Sul, Brazil. Patient recruitment began in 2002. Type 2 diabetes was defined according to World Health Organization criteria (17): diagnosis of diabetes after the age of 35 years, no use of insulin during the first year after diagnosis and no episodes of diabetic ketoacidosis. Control patients were those with known diabetes duration of at least 5 years and normoalbuminuria. Cases were divided into three categories: microalbuminuria, macroalbuminuria and ESRD for those attending dialysis clinics. Stage of DN was defined based on two out of three albuminuria measurements: <30 mg/24h for normoalbuminuria, 30-300 mg/24h for microalbuminuria and >300 mg/24h for macroalbuminuria. The study included six dialysis centers: Hospital de Clínicas de Porto Alegre, Grupo Hospitalar Nossa Senhora da Conceição, Centro de Diálise e Transplante, Hospital Centenário, Hospital Ernesto Dorneles and Centro de Diálise e Transplantes/Vila Nova. Control patients and DN patients on conservative treatment were recruited during their routine medical visits at Hospital de Clínicas de Porto Alegre. Patients with other causes of albuminuria or renal diseases other than DN were excluded.

All patients underwent an evaluation that included a standardized questionnaire and physical examination, as previously described (18). Fasting blood samples were also collected for laboratory and molecular analysis. Briefly, information was collected about age, age at diabetes diagnosis, drug treatment and smoking. Height and weight

(without shoes, wearing light clothes) were measured, and body mass index (BMI, kg/m^2) was calculated. For patients on dialysis, the mean of three weights measured after dialysis sessions was used. Hypertension was defined as blood pressure ≥140/90 mmHg or use of any antihypertensive medication. Office blood pressure was defined based on the mean of two measurements in the sitting position using a standard mercury sphygmomanometer (phases I and V of Korotkoff). Retinopathy was assessed by fundus examination after mydriasis by an ophthalmologist and graded as absent, non-proliferative or proliferative diabetic retinopathy.

Laboratory analysis. Fasting plasma glucose was determined by a glucose oxidase method and HbA1c by an ion-exchange high performance liquid chromatography procedure (Merck-Hitachi L-9100 Glycated Hemoglobin Analyzer, Tokyo, Japan) with inter- and intra-assay CV of 2.4% and 0.5%, respectively (reference interval 4.1 to 6.0%) (19). Serum creatinine was determined by the Jaffé reaction (20). Triglycerides and cholesterol levels were measured by enzymatic methods. LDL-cholesterol was calculated using the Friedewald equation. Urinary albumin excretion (UAE) was measured in 24h-urine samples by immunoturbidimetry (Bayer, TarryTown, NY, USA), with intra- and interassay coefficients of variation of 4.5% and 11%, respectively (21). Patients interrupted the use of angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists for at least one week before having their albuminuria measured.

Molecular analysis. DNA was extracted from peripheral blood leukocytes by a standardized salting-out procedure. Seven out of eleven SNPs initially associated with DN in type 1 DM (14) were chosen to be included in the present study (rs1888747, rs9521445, rs39075, rs451041, rs1411766, rs6492208, rs1041466). Not all eleven SNPs were genotyped because some were in linkage disequilibrium: rs1888747 and rs10868025 ($r^2 = 0.81$), both located at chromosome 9q, so that only rs1888747 was

genotyped; rs39059 and rs39075, located at chromosome 7p29.2, were in partial linkage disequilibrium (r^2 >0.96), and rs39075 was chosen for analysis for having a stronger association with DN (14); rs451041 and rs9521445 were chosen instead of rs739401 and rs7989848. Two additional SNPs (rs11186286 and rs7956328) were included because preliminary analysis of the type 1 diabetes study data indicated an association with DN, even though this association was not confirmed and the results were not presented in the original manuscript (14). To define linkage disequilibrium for SNPs, the International HapMap Project (www.hapmap.org) was searched. All polymorphisms were determined using primers and probes contained in the Human Custom TaqMan Genotyping Assay 40x (Assays-By-Design Service, Applied Biosystems, Foster City, CA; USA). The reactions were conducted in a 96-well plate, in a 5 µl total reaction volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Applied Biosystems), and Custom TaqMan Genotyping Assay 1x. The plates were then positioned in a real-time PCR thermal cycler (7500 Fast Real PCR System; Applied Biosystems) and heated for 10 minutes at 95°C, followed by 40-50 cycles at 95°C for 15 seconds and 60-62°C for 1 minute. Fluorescence data files from each plate were analyzed using automated allele-calling software (SDS 2.1; Applied Biosystems). Genotyping success was more than 95%, with a calculated error rate based on PCR duplicates of 0.01%.

Statistical analysis. Two comparisons were performed: controls vs. cases, where cases were defined as any degree of DN; and controls vs. DN stages. Data were analyzed assuming autosomal dominant or recessive models to check which analyses would fit best. Hardy-Weinberg equilibrium was calculated for all SNPs using allele frequencies and the chi-square (χ^2) test. Continuous data are presented as means ± SD. Categorical data are expressed as number of cases and percent of individuals affected. Variables

without normal distribution were log-transformed. Chi-square, Student's t test or one-way analysis of variance (ANOVA) were used to compare clinical and laboratory characteristics of the groups. The Tukey Test was used for post-hoc multiple comparisons. Multiple logistic regression analysis was used to evaluate the independence of possible SNP association with DN. The magnitude of the association was estimated using odds ratios (ORs) with 95% confidence interval (95% CI). Since this is a confirmatory study, when the direction of the associations was known *a priori*, a one-sided P value was adopted (P <0.05).

RESULTS

We evaluated 1,098 patients, 718 (65.4%) with DN (323 microalbuminuria, 395 macroalbuminura/ESRD). Patients with DN had higher blood pressure, more diabetic retinopathy and a worse lipid profile compared to the control group (Table 1). Both groups had similar HbA1c levels.

All SNPs were in Hardy-Weinberg equilibrium (P >0.05) (Table 2). Of the 9 SNPs studied, only rs1888747 was significantly associated with DN (CC/CG/GG = cases vs. controls = 6.8% / 41.5% / 51.7% vs. 10.8% / 40.0% / 49.2%, P = 0.037). Minor allele frequencies were 0.31 and 0.27 in controls and cases, respectively (P = 0.06). The strongest association was observed assuming a recessive model (CC vs. CG/GG, OR 0.57 95% CI 0.37-0.88, P = 0.008). This association persisted (OR 0.52 CI 0.39 - 0.90, P= 0.021) in the multivariate analysis controlled for diabetes duration, gender, systolic blood pressure and triglycerides**.** None of the other SNPs were associated with DN in this sample of type 2 diabetic patients.

Table 3 shows the genotype distribution of SNP rs1888747 according to renal status (normo-, micro- or macro/ESRD). The same pattern was observed, with a

decrease in homozygous genotypes for the minor allele in subjects with microalbuminuria or macroalbuminuria/ESRD compared to controls. Minor allele frequency was also lower in individuals with microalbuminuria or macroalbuminuria/ESRD compared to controls. Genotype and allele frequency was similar in cases and controls for all other SNPs.

DISCUSSION

DN is probably a complex trait disorder resulting from the interaction between environmental and genetic factors. So far, a specific gene has not been found to explain most DN cases, possibly because more than one gene is involved in the pathogenesis of DN. GWS studies can identify unknown chromosomal regions that may be involved in the pathogenesis of DN. However, due to multiple testing, replication of the findings in distinct populations is essential to confirm the associations observed in such studies.

In the present study, we were able to replicate the association of SNP rs1888747 with DN in patients with type 2 diabetes. The homozygous C allele conferred protection against the development of DN. This result is encouraging, since it corroborates a previous finding (14) in a distinct population. In the present study, a subgroup with microalbuminuria was also included. This group was not evaluated in the original study of type 1 subjects (14). Neither allele frequency nor genotype distribution was different from those of the control group.

The nearest gene to SNP rs1888747 is FRMD3 (4.1 protein ezrin, radixin, moesin [FERM] domain containing 3), which encodes a structural protein whose function remains unknown. What is known is that this protein is a member of the 4.1 family and that it is a cytoskeletal protein, involved in maintaining cellular shape. The 4.1 protein was originally found to be abundant in human erythrocytes, stabilizing the

spectrin/actin cytoskeleton. It has also been found in a variety of cell types, including mouse nephron (22, 23).

The other SNP found in the GoKinD sample and replicated in DCCT/EDIC subjects was rs451041, located in chromosome 11p. CARS, the nearest gene to rs451041, encodes cysteinyl-tRNA synthetase and is expressed in mesangial and proximal tubule cells. Mutations in this gene have been associated with neurodegenerative diseases and cystinosis (24, 25). However, neither this SNP nor the others originally associated with DN in type 1 patients showed association with DN in patients with type 2 diabetes. Even though this observation needs to be evaluated in other populations, it suggests that different genes might play different roles in the development of DN in type 1 or type 2 diabetes.

One possible limitation of case-control studies of DN is survival bias. However, we do not believe that this had an impact on the present results, because 1) minor allele frequency was similar in mild and severe stages of DN; and 2) data from the prospective DCCT/EDIC cohort (15) suggest the rs1888747 C allele had a faster progression to severe nephropathy.

The evidence to infer susceptibility genes contributing to the pathogenesis of DN remains elusive. Recent advances in genotyping technologies have allowed the identification of several chromosomal regions potentially associated with DN, but the studies carried out so far are too heterogeneous. Lack of statistical power and important differences in terms of the population studied and phenotype analyzed make it difficult to compare studies and build up on results.

In conclusion, the present study replicates the protective effect of SNP rs1888747 against established DN. It also shows that this effect is already present in less advanced stages of DN, such as microalbuminuria.

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Table 1: Clinical characteristics

	Controls n = 380	**Cases** n = 718	**P**
Age (years)	60.4 ± 9.7	59.4 ± 10.8	0.131
Male - n (%)	142 (37.4)	424 (59.0)	<0.001
Duration of diabetes (years)	14.2 ± 7.4	14.9 ± 10.0	0.230
Body mass index (kg/m2)	29.1 ± 5.3	28.8 ± 5.3	0.372
Systolic pressure (mmHg)	140.8 ± 22.7	145.1 ± 23.5	0.004
Diastolic pressure (mmHg)	84.9 ± 12.6	85.3 ± 12.2	0.351
Diabetic retinopathy - n (%)	125 (32.9)	607 (84.5)	<0.001
Smoking n - (%)	65 (17.1)	118 (16.4)	0.841
Fasting plasma glucose (mg/dl)	168.7 ± 63.0	178.0 ± 82.5	0.055
HbA1c (%)	7.31 ± 1.93	7.35 ± 2.12	0.759
Total cholesterol (mg/dl)	207.7 ± 43.2	201.3 ± 50.2	0.035
HDL cholesterol (mg/dl)	46.1 ± 11.5	43.1 ± 12.4	<0.001
Triglycerides (mg/dl)	143 (26-946)	175 (46 -1669)	<0.001
Serum creatinine (mg/dl)	0.89 ± 0.20	2.67 ± 2.94	<0.001

Data are mean ± SD, median (range) or number of cases (%).

Table 2: Genotype distribution of the polymorphisms among cases and controls

		Controls n = 380	Cases n = 718	P
rs1888747	**CC/CG/GG**	10.8 / 40.0 / 49.2	6.8/ 41.5 / 51.7	0.037
rs9521445	**AA/AC/CC**	18.9 / 53.2 / 27.9	22.3 / 48.5 / 29.1	0.146
rs39075	**AA/AG/GG**	17.9 / 49.5 / 32.6	19.4 / 44.4 / 36.2	0.139
rs11186286	**CC/CG/GG**	3.7 / 31.6 / 64.7	3.7 / 31.5 / 64.8	0.499
rs451041	**AA/AG/GG**	23.4 / 48.2 / 28.4	21.3 / 47.1 / 31.6	0.318
rs7956328	**AA/AT/TT**	6.8 / 35.3 / 57.9	5.8 / 39.0 / 55.2	0.220
rs1041466	**GG/GA/AA**	14.2 / 51.1 / 34.7	17.3 / 44.7 / 38.0	0.059
rs1411766	**AA/AG/GG**	11.8 / 35.5 / 57.2	12.7 / 36.8 / 50.6	0.399
rs6492208	**CC/CT/TT**	20.7 / 43.9 / 35.4	19.9 / 45.0 / 35.1	0.461

Data are expressed as %.

Table 3: Genotype distribution of SNP rs1888747 according to renal status

		Renal status			
		Normoalbuminuria	**Microalbuminuria**	**Macro/ESRD**	
	Genotype	**N = 380**	**N = 323**	**N = 395**	**P**
rs1888747	**CC**	41 (10.8)	25 (7.7)	24 (6.3)	0.04
	GC	152 (40.0)	138 (42.7)	160 (40.9)	
	GG	187 (49.2)	160 (49.5)	211 (52.8)	
	C allele	0.31	0.29	0.26	0.02

CONSIDERAÇÕES FINAIS

Grande progresso vem ocorrendo no entendimento da patogênese e dos fatores de risco associados à ND, além de forte evidência da influência genética no desenvolvimento da doença. Na presente dissertação, revisamos os aspectos genéticos da ND e apresentamos um estudo de replicação de polimorfismos.

Demonstramos que o FRMD3 está associado à ND no DM tipo 2, assim como havia sido demonstrado, em estudo prévio, para os pacientes com DM tipo1. Este se torna um novo gene candidato para ND. Estudos de expressão do gene e da proteína permitirão uma melhor caracterização do papel deste gene na ND.

Um melhor entendimento da suscetibilidade genética à ND permitirá o desenvolvimento de novas estratégias terapêuticas com o objetivo de reduzir a incidência desta doença que apresenta correlação direta com as principais causas de mortalidade entre os pacientes com DM.

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