Universidade Federal do Rio Grande do Sul
Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

POLIMORFISMO rs1888747 DO GENE *FRMD3*, EXPRESSÃO GÊNICA E PROTEICA: PAPEL NA DOENÇA RENAL DO DIABETES

Dissertação de Mestrado

Marjoriê Piuco Buffon

Porto Alegre, fevereiro de 2015

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Dedico essa dissertação à minha família.

“Ho imparato a sognare,

quando inizi a scoprire

che ogni sogno

ti porta più in là

cavalcando aquiloni,

oltre muri e confini

ho imparato a sognare da là”

Negrita

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Esta dissertação de mestrado faz parte de uma linha de pesquisa do grupo e dá continuidade ao trabalho desenvolvido anteriormente por outra aluna de mestrado. A dissertação é composta de três capítulos. O primeiro um artigo de revisão sobre o tema a ser abordado. O segundo o projeto desenvolvido durante o mestrado. O terceiro é um artigo original, que inclui os dados anteriores do laboratório de estudo de associação do polimorfismo rs1888747 e doença renal do diabetes e os estudos de expressão realizados como parte desta dissertação.

- Artigo de revisão: "*FRMD3* gene: the role in diabetic kidney disease– a narrative review" (a ser submetido à revista Diabetology and Metabolic Syndrome).
- Estudo da expressão gênica e protéica do FRMD3 em células renais humanas
- Artigo original: "rs1888747 polymorphism in the *FRMD3* gene, gene and protein expression: role in diabetic kidney disease" (a ser submetido à revista Diabetology and Metabolic Syndrome).

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ABREVIATURAS

ADA	American Diabetes Association
APOL1	apolipoprotein L1 gene
AU	arbitrary units
BMP	bone morphogenetic protein
CV	coefficient of variation
CKD	chronic kidney disease
DCCT/EDIC	Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications study
DKD	diabetic kidney disease
DM	diabetes mellitus
DM1	diabetes mellitus tipo 1
DM2	diabetes mellitus tipo 2
DRD	doença renal do diabetes
eGFR	estimated glomerular filtration rate
ESRD	end-stage renal disease
EURODIAB	The European Diabetes Prospective Complications Study Group
FinnDiane	Finnish DKD Study
FRMD3	*FERM domain containing 3* gene
GENIE	GEnetics of Nephropathy – an International Effort
GFR	glomerular filtration rate
GoKinD	Genetics of Kidneys in Diabetes
GWAS	genome wide association studies
GWS	genome wide-scan

GWU	George Washington University
HCPA	Hospital de Clínicas de Porto Alegre
HOMF	homeodomain factor
HWE	Hardy-Weinberg equilibrium
IHC	immunohistochemistry
IHC	imunohistoquímica
JDC	Joslin Diabetes Center
KDIGO	Kidney Disease: Improving clinical outcomes
LBMC	Laboratório de Biologia Molecular e Celular
LD	linkage disequilibrium
MYH9	non-muscle myosin heavy chain 9 gene
RTq-PCR	reverse transcriptase quantitative real-time PCR
SNPs	single nucleotide polymorphisms
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TFBS	transcription factor binding site
TFBS	transcription factor binding site
TR-PCRq	transcrição reversa de PCR em tempo real quantitativo
U.K.-R.O.I.	All Ireland-Warren 3-Genetics of Kidneys in Diabetes U.K. and Republic of Ireland
UA	unidades arbitrárias
UAE	urinary albumin excretion
WB	western blot

INTRODUÇÃO

A doença renal do diabetes (DRD) é uma complicação crônica do diabetes mellitus, que já foi considerada uma epidemia mundial. A DRD é responsável por um importante aumento na morbidade e mortalidade [1, 2], além de ser uma das principais causas da doença renal em estágio terminal [3, 4]. Entre pacientes em início de diálise, incidência de DRD é estimada em 26% [1].

A etiologia da DRD é multifatorial, sendo assim, a genética tem um papel importante no seu desenvolvimento. Apesar disso, pouco é conhecido sobre o modo exato de transmissão da doença, que provavelmente seja de origem poligênica, através da interação entre fatores ambientais e genéticos levando ao desenvolvimento da DRD. Até o momento, estudos de *genome wide-scan* (GWS) e pesquisas de genes candidatos tem apresentado resultados heterogêneos [5-8].

Vários estudos genéticos têm sido desenvolvidos a fim de elucidar possíveis fatores genéticos envolvidos nesta doença. Em uma meta-análise de estudos de associação genética e DRD, conduzida por Mooyart et al. [9], foram descritas 21 variantes genéticas significativamente associadas com DRD.

Pezzolesi et al. [10] conduziram um estudo de GWS em caucasianos com diabetes mellitus tipo 1 e encontraram 13 SNPs associados com DRD. As maiores associações foram identificadas em variantes localizadas entre regiões cromossomais distintas, entre elas a região que inclui o gene *FRMD3* no cromossomo 9. Na tentativa de replicar o estudo citado anteriormente, porém em japoneses com diabetes mellitus tipo 2, Maeda et al. [11] não encontraram associação nas variantes gênicas localizadas no gene *FRMD3*.

O gene *FRMD3* é um forte candidato selecionado a partir de estudos de GWS, e faz parte de uma família de proteínas envolvidas na função do citoesqueleto, com o

objetivo de dar forma e integridade celular. FRMD3 é expresso em ovários adultos, músculo esquelético fetal, cérebro e timo [12], assim como em células tubulares renais e podócitos [10, 13].

Alguns polimorfismos de um único nucleotídeo localizados no gene *FRMD3* têm sido descritos associados à DRD em diferentes etnias. Entretanto, existem controvérsias e mais estudos devem ser realizados. O *FRMD3* é um gene relativamente novo, e os dados na literatura envolvendo sua expressão, tanto gênica como protéica são escassos. Mais estudos são necessários para entender melhor o papel dos polimorfismos e suas possíveis influências na expressão deste gene e tradução em suas respectivas proteínas.

Em um estudo prévio, realizado no Serviço de Endocrinologia pela mestre Mariana Carpena (2010), no qual foram avaliados diversos polimorfismos, foi possível mostrar a possível relação entre um dos polimorfismos (rs1888747) no gene *FRMD3* e a DRD. A partir deste achado surgiu o interesse de estudar se o polimorfismo rs1888747 está de alguma forma influenciando a expressão gênica ou protéica do FRMD3.

A presente dissertação apresentará uma revisão sobre o papel do gene *FRMD3* na DRD, assim como os dados originais resultantes do estudo de expressão gênica e protéica, e ao final, um artigo original que inclui os dados apresentados em soma aos achados gerados pelo estudo desenvolvido pela Mestre Mariana Carpena.

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CAPÍTULO I

Artigo de revisão

“*FRMD3* GENE: THE ROLE IN DIABETIC KIDNEY DISEASE– REVIEW”

“GENE *FRMD3*: PAPEL NA DOENÇA RENAL DO DIABETES – UMA REVISÃO NARRATIVA”

FRMD3 **GENE: THE ROLE IN DIABETIC KIDNEY DISEASE– A NARRATIVE REVIEW**

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Role of *FRMD3* gene in diabetic kidney disease – a narrative review

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SUMMARY

Diabetic kidney disease (DKD) is a chronic complication of diabetes mellitus, which is considered worldwide epidemic. Several studies have been developed in order to elucidate possible genetic factors involved in this disease. The *FRMD3* gene, a strong candidate selected from genome wide association studies (GWAS), encodes a structural protein (4.1O) involved in maintaining cell shape and integrity. Some single nucleotide polymorphisms (SNPs) located in *FRMD3* have been associated with DKD in different ethnicities. Despite these findings, there is still controversy and more studies should be conducted. The aim of this narrative review is to summarize the evidence regarding the role of *FRMD3* in DKD.

INTRODUCTION

Diabetes mellitus (DM) is a complex, chronic illness involving a state of hyperglycemia [1]. Depending on the intensity and duration of exposure to hyperglycemia, structural lesions may occur in the vascular endothelium and nervous tissue, causing damage, dysfunction in multiple organs and tissues, and chronic complications [2]. One such complication is diabetic kidney disease (DKD), in which there is increased urine excretion of proteins, predominantly albumin [3]. The exact mechanism that determines which patients with DM would or would not progress to renal failure and dialysis has yet to be elucidated. A great effort has been devoted to the study of candidate genes related to DKD development and progression.

DIABETIC KIDNEY DISEASE

DKD, which was initially described as a glomerular disease characterized by proteinuria, seems to be much more than that. Chronic kidney disease (CKD) is defined as renal damage resulting from structural or functional abnormalities of the kidneys, or glomerular filtration rate (GFR) <90 ml/min/1.73m² with or without kidney damage over a period of time equal to or greater than three months [4]. DKD affects around 25-30% of individuals with DM and is responsible for over a third of new cases of individuals with renal disease starting dialysis [5, 6]. In 25% of patients with DM, increased urinary albumin excretion (UAE) is observed [7]. Furthermore, low GFR has been also reported in a substantial proportion of normoalbuminuric subjects [7-10]. As a result, the American Diabetes Association (ADA) recommended DKD assessment based on UAE and estimated GFR (eGFR) since 2006 [11, 12]. More recently, ADA has suggested that albuminuria be classified as normal UAE or increased UAE [12], and although the terms microalbuminuria and macroalbuminuria are still widely used, the latest guidelines recommend against their use [13].

Despite the high prevalence and severity of DKD, not all individuals with DM will develop this disease. The cumulative incidence of persistent albuminuria (microalbuminuria) in patients with type 1 diabetes (T1DM) has been reported as 12.6% over 7.3 years according to The European Diabetes Prospective Complications Study Group (EURODIAB) [14] and as 33% in an 18-year study carried out in Denmark [15]. It seems that T1DM patients who do not develop DKD 10-15 years after diagnosis are protected from this complication [16]. Furthermore, some subjects with elevated albuminuria will progress to a more severe albumin profile and GFR loss, while others will remain stable or even return to normoalbuminuria [17, 18]. Thus, it is believed that the presence of risk factors such as hyperglycemia and hypertension will lead to DKD in genetically predisposed individuals.

THE GENETICS OF DIABETIC KIDNEY DISEASE

Familial clustering of DKD, a multifactorial disease, has been reported, supporting the hypothesis that genetic factors are involved in its pathogenesis [19, 20]. However, all the efforts to identify a gene with a major effect have been disappointing, possibly because several genes are involved, acting synergistically or additively [21]. For instance, some genes might be related to increased albuminuria, and others to GFR decline [22, 23].

Pezzolesi et al. [24] conducted a genome wide association study (GWAS) in Caucasians with T1DM and found 13 SNPs (single nucleotide polymorphisms) associated with DKD. The most significant associations were identified in variants located in genes located in four chromosomal regions: *CHN2/CPVL* on chromosome 7, *FRMD3* on chromosome 9, *CARS* on chromosome 11, and a locus near the *IRS2* on chromosome 13. The strongest association was found with the *FRMD3* gene (*4.1 protein ezrin, radixin, moesin [FERM] domain containing 3*). Nevertheless, Maeda et

al. [25] analyzed polymorphic variants of these gene [24] in Japanese type 2 diabetes mellitus (T2DM) individuals and did not find any association with DKD.

Regardless of extensive evidence of genetic susceptibility to DKD, the identification of susceptibility genes and their variants has had limited success [26, 27]. Mooyart *et al*. [28] conducted a meta-analysis of genetic association studies focusing on DKD and found that 21 genetic variants were significantly associated with DKD [28], two of them in the *FRMD3* gene. Other variants were in or near the following genes: *ACE, AKR1B1*, *APOC1, APOE, EPO, NOS3*, *HSPG2, VEGFA*, *CARS*, *UNC13B, CPVL, CHN2, GREM1*, and others. Additional variants were detected in subgroup analyses: ELMO1 (Asians), CCR5 (Asians), and CNDP1 (T2DM). However, these associations were not successfully confirmed by Williams et al. [29].

The inconsistencies in the results of genetic association studies in complex diseases could be due to small sample sizes or incorrect associations conducting to false positive results [30-33]. Therefore, independent replication of positive associations remains essential to avoid spurious associations.

FRMD3 GENE

The *FERM domain containing 3* gene (*FRMD3*) (ID: 257019) is located on chromosome 9, in chromosome band q21.32. It has 14 coding exons spanning 2,282 base pairs [34] (figure 1).

FRMD3 encodes protein 4.1O, a structural protein that is part of the protein 4.1 family [35]. Members of this family act as cytoskeletal proteins, maintaining cell shape and integrity in a variety of cell types, including rat nephron cells [36, 37]. However, the role of protein 4.1O was not elucidated yet [38]. It contains a FERM domain, which contributes to cell integrity by interacting with transmembrane proteins and actin

filaments [38, 39]. FRMD3 is expressed in adult ovaries, fetal skeletal muscle, brain, thymus, and human podocytes [35].

The 4.1 protein family comprises a group of proteins including 4.1R, 4.1G (general type), 4.1B (brain type), and 4.1N (neuron type) [40]. Erythrocyte protein 4.1 (4.1R) is a multifunctional protein essential for maintaining erythrocyte shape, mechanical properties of the membrane, deformability, and stability through interactions with spectrin and actin skeleton network [40]. The FERM domain (F, *4.1*; E, ezrin; R, radixin; M, moesin) [38] is an N-terminal 30-kDa domain containing binding sites for cytoplasmatic tails of integral membrane proteins such as band 3 (integral membrane protein involved in spectrin-actin interaction) [41, 42], glycophorin C (erythroid membrane proteins) [43], CD44 (transmembrane glycoprotein found in erythroid as well as non-erythroid cells) [44], p55 (abundantly palmitoylated phosphoprotein of the erythroid membrane) [43] and calmodulin (a highly conserved calcium-binding protein, ubiquitously distributed in eukaryotic cells, known to affect a plethora of biological functions) [40]. An internal 8-10 kDa domain contains the spectrin-actin binding activity that is necessary for membrane stability [45], and the 22-24 kDa C-terminal domain has been reported to bind immunophilin FKBP13 (membrane-associated protein thought to function as an endoplasmic reticulum chaperone) [46] and nuclear mitotic apparatus protein (NuMA) [47].

In the study of Pezzolesi et al. [24], FRMD3 expression was increased in proximal renal tubular human cells. Expression data for *FRMD3* and its coexpressed transcripts suggest that these genes are linked to early progression of DKD [48].

It has been hypothesized that the 9q21.32 locus contributes to glomerular injury early in the pathogenesis of DKD [49]. More recently, the rs1888747's risk allele was shown to generate a transcription factor binding site (TFBS) in a module that is

shared by multiple members of the bone morphogenetic protein (BMP) signaling pathway, which has previously been implicated in the development of DKD [48, 50]. Regarding these findings, new pathways are being discovered to explain the role of *FRMD3* in DKD physiopathology.

VARIANTS IN THE *FRMD3* GENE AND DIABETIC KIDNEY DISEASE

Research has been developed to investigate the association of variants in the *FRMD3* gene and risk of DKD. The main variants studied so far are briefly described in Table 1.

The first published study regarding variants in *FRMD3* was a GWAS with T1DM patients from the GoKinD (Genetics of Kidneys in Diabetes) collection [24]. Variant rs10868025 had the strongest association with DKD (OR = 1.45, P = $5.0X10^{-7}$) [24]. The rs10868025 polymorphism, which involves a G for A nucleotide substitution, is located on the long arm of chromosome 9 at position 85.4, near the 5' end of *FRMD3* [24]. The frequency of the risk allele was 0.66/0.56 (cases/controls) in the Joslin Diabetes Center study (JDC study) and 0.66/0.59 in the George Washington University study (GWU study) [24]. Two *FRMD3* SNPs associated with DKD in the GoKinD collection (rs1888746 and rs13289150) were also associated with the development of severe nephropathy in the DCCT/EDIC (Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications) follow-up study [24].

In a meta-analysis, involving three studies including 1,052 cases and 2,057 controls, the rs10868025 variant was associated with lower risk of DKD in European T1DM subjects (OR 0.72 [95% CI 0.64–0.81) [28], whereas in a study with T2DM subjects [25], no significant differences between the *rs10868025* variants and DKD were detected in four independent Japanese populations. The frequency of the G allele for cases *vs.* controls in the four populations studied was: 0.74 *vs.* 0.73, 0.72 *vs.* 0.73,

0.67 *vs.* 0.73, and 0.70 *vs.* 0.73 (P > 0.25 in all cases). The same findings were replicated in the Chinese population [51], for which no association between *FRMD3* SNPs and DKD was observed (P = 0.249).

The *rs1888747* polymorphism is intergenic, located on the long arm of chromosome 9 at position 85.3, near the promoter region of the gene [52]. It involves a C for G nucleotide substitution. In the study developed by Pezzolesi et al. [24], a relevant association of polymorphism rs1888747 was found with DKD (P = 6.3 $x10^{-7}$) in Caucasians with T1DM. The frequency of the risk allele (G) was 0.68/0.73 for controls/cases in the GWU GoKinD and 0.66/0.74 for controls/cases in the JDC GoKinD [24]. These findings were strengthened by a meta-analysis including three studies analyzing rs1888747 in a total of 1,052 cases and 2,057 controls [28] confirming an association of rs1888747 with DKD (OR 0.74 P 0.602 [95% CI 0.65-0.83] [28]. However, in Japanese individuals with T2DM, no associations between rs1888747 and DKD were demonstrated in four different groups [25]. The frequency of the risk G allele for cases/controls was 0.81/0.80, 0.80/0.80, 0.70/0.82 and 0.77/0.81 (P > 0.05).

In 2011, Freedman et al. [53] identified a potential interaction between *FRMD3* and the APOL1 (apolipoprotein L1 gene) - MYH9 (non-muscle myosin heavy chain 9 gene) gene region on chromosome 22 contributing to DKD susceptibility in African Americans. Nevertheless, initially no association was found between *FRMD3* SNPs (rs942280 and others, Table 1) and T2DM before adjusting for variants on chromosome 20. The results revealed an approximate 25–30% increase in DKD risk with multiple *FRMD3* SNPs in subjects not homozygous for the *MYH9* risk haplotypes (or APOL1 risk variants).

One year later, Williams et al. [29] formed the GEnetics of Nephropathy – an International Effort (GENIE) consortium to examine previously reported genetic

associations with DKD in T1DM observed in the GoKinD collection [24]. The samples were provided by the All Ireland-Warren 3-Genetics of Kidneys in Diabetes U.K. and Republic of Ireland (U.K.-R.O.I.) collection and the Finnish DKD Study (FinnDiane). None of the *FRMD3* polymorphisms were associated with DKD [rs1888747 (P = 0.77 and 0.25, U.K.-R.O.I. and FinnDiane respectively); rs1086805 (P = 0.52 for U.K.-R.O.I. and 0.25 for FinnDiane)] [29].

Park et al. [54] aimed to find candidate genetic determinants of renal function in 1007 individuals from 73 extended families of Mongolian origin. The strongest associations found were with rs17400257 ($P = 7.21 \times 10^{-9}$) and rs6559725 ($P = 9.12 \times 10^{-7}$). rs17400257 is located 45 kb downstream of *FRMD3*, and rs6559725 is located in the intronic region of *FRMD3* [54].

After initial findings in a T1DM population in 2009 [24], Pezzolesi et al. [49] proceeded to examine whether the SNPs at these susceptibility loci were associated with DKD in patients from the Joslin Study of Genetics of Nephropathy in a T2DM Family Collection. The 9q21.32 locus was significantly associated with high microalbuminuria, proteinuria, and ESRD [49]. Among diabetic family members, rs1888747 on chromosome 9q21.32 was associated with advanced nephropathy (P = 0.029). Furthermore, when the definition of DKD was expanded to include individuals with high microalbuminuria, the strength of the association improved significantly [49].

Martini et al. [48] proposed a transcriptional link that might explain how the rs1888747 polymorphism in *FRMD3* influences transcriptional regulation within the bone morphogenetic protein (BMP)-signaling pathway. *FRMD3* transcript levels decreased significantly with progression of DKD ($P < 0.02$). *FRMD3* gene expression was studied by comparing renal biopsies in a group of 22 Pima Indians with T2DM and normal GFR and a cohort of seven participants with T2DM and CKD stage 3 [48].

Hierarchical clustering using the *FRMD3* coexpressed transcripts expression detected two distinct clusters. In cluster 1, presenting higher ΔACR/year (Δ albumin-to-creatinine ratio per year), the gene expressions of seven out of the eight BMP pathway genes (BMPR2, CREB1, KRAS, MAP3K7, PRKAR2B, SMAD5, and XIAP) were lower than the expressions of these genes in cluster 2. These findings suggest a common molecular mechanism responsible for the coregulation of *FRMD3* and several BMP pathway members [48]. Additionally, *in silico* comparison of sequence variants of the risk allele identified a potential homeodomain factor (HOMF) transcription factor binding site (TFBS) covering the SNP position [48]. The rs1888747 polymorphism affects protein binding, suggesting the generation of a TFBS by that particular SNP [48]. However, the mechanism mediating the connection between *FRMD3* and BMP pathway members is still unknown, and no data are available at protein, RNA, or microRNA levels to elucidate this association [48]. It should be noted that Palmer and Freedman [52] suggest that the framework proposed by Martini et al. [48] is limited to be genes with known or predicted functional roles.

Recently, a GWAS was performed by Palmer et al. [55] with the objective of evaluating candidate DKD susceptibility genes in African Americans. The SNPs selected were the same previously studied in European ancestries by Pezzolesi et al. [24]. The results showed that SNPs in *FRMD3* trended toward association with T2DM-ESRD ($P < 0.05$).

CONCLUSION

Even though not much is known about the *FRMD3* gene, some studies provide insights into the relation between *FRMD3* variants and DKD. Major findings in the African American population show an association between DKD and many SNPs, especially rs10867977 (OR 1.31, 1.06-1.62). In the Japanese population, a significant

association was found only at rs1888747, whereas in Caucasians three SNPs have been found to be associated with DKD (rs1888746, rs1888747, rs10868025). Nevertheless, considering all SNPs and ethnic groups analyzed, the strongest association was found in Caucasians with polymorphisms rs1888747 and rs10868025. However, the data produced so far is not sufficient to ascertain a role of *FRMD3* in DKD pathogenesis. Also, because the relationship between the *FRMD3* gene and DKD was recently detected, very few studies focusing on its mRNA and protein expression are available.

In summary, *FRMD3* is a strong candidate gene for DKD. However, further studies are needed to explain the pathways through which *FRMD3* influences the onset of this diabetic complication.

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Figure Legends

Figure 1. Map of the *FRMD3* gene in chromosome 9q21-32. The 14 exons (boxes) are numbered from left to right according to the transcription region. Black boxes represent encoding regions, and the white box represents the 3'UTR region. Vertical arrows show the polymorphic sites associated with diabetic kidney disease. Figure adapted from http://www.ncbi.nlm.nih.gov/gene/257019.

Tables Legends

Table 1. *FRMD3* variants studied in the context of diabetic kidney disease

Figure

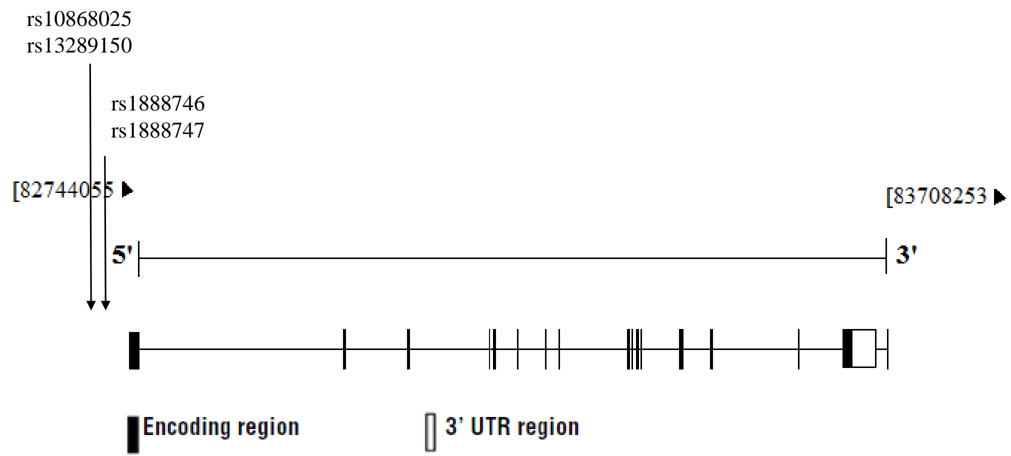

Figure 1

Table

Table 1

Variant	**Reference**	**Ethnicity**	**Outcome**	**Position on chromossome 9***	**Risk Allele**	**P value**	**Odds ratio (95% CI)**
rs942278	Freedman et al., 2011 [53]	**African American**	T2DM and DKD	85906066	T	0.0023	1.30 (1.11-1.53)
rs942280				85905861	G	0.00070	1.28 (1.09-1.51)
rs942283				85905666	C	0.0014	1.25 (1.07–1.47)
rs1535752				85905253	T	0.013	1.27 (1.03–1.57)
rs1535753				85905060	T	0.0024	1.24 (1.06–1.46)
rs2378658				85904778	C	0.0040	1.25 (1.06–1.46)
rs10867977				85907516	G	0.022	1.31 (1.06–1.62)
rs1888746	Hu et al., 2011 [51]	**Chinese**	T2DM and DKD	86155392	C	0.66	0.93 (0.60–1.38)
	Pezzolesi et al., 2009 [24]	**Caucasian**	T1DM and DKD (proteinuria and ESRD)	86155392	C	0.02	NA‡
rs1888747	Maeda et al., 2010 [25]	**Japanese**	T2DM and DKD	86155551	G	0.10	1.24 (0.96–1.61)
			T2DM, DKD and diabetic retinopathy			0.84	1.02 (0.83–1.26)
			T2DM and			0.004	0.28 (0.12–

			microalbuminuria				0.67)
			T2DM and DKD			0.43	0.87 (0.61–1.23)
	Pezzolesi et al., 2009 [24]	**Caucasian**				0.0000006 7	1.45 (1.25–1.67)
	Williams et al., 2012 [29]	**Caucasian**	T1DM and DKD			0.24	1.06 (0.96–1.17)
	Pezzolesi et al., 2011 [56]	**Caucasian**	T2DM and DKD			0.92	1.01 (0.86–1.19)
	Pezzolesi et al., 2013 [49]	**Caucasian**	T2DM and DKD			0.0014	NA§
	Moyaart et al., 2011 [28]	**Caucasians**	Meta-analysis T1DM and DKD			0.12	0.74 (0.65-0.83)
rs10868025	Pezzolesi et al., 2009 [24]	**Caucasian**		86164176	A	0.00005	1.45 (1.25–1.67)
	Williams et al., 2012 [29]	**Caucasian**				0.19	1.06 (0.97–1.17)
	Pezzolesi et al., 2013 [49]	**Caucasian**				0.0039	NA§§
	Pezzolesi et al., 2011 [56]	**Caucasian**				0.90	0.99 (0.84–1.16)
	Maeda et al., 2010 [25]	**Japanese**	T2DM and DKD			0.31	1.12 (0.89–1.42)
			T2DM, DKD and diabetic retinopathy			0.54	0.94 (0.78–1.14)
			T2DM and microalbuminuria			0.07	0.49 (0.22–1.06)
			T2DM and DKD			0.33	0.85 (0.62–1.18)
	Hu et al., 2011 [51]	**Chinese**				0.25	0.91 (0.76-1.07)
	Moyaart et al., 2011	**Caucasians**				0.60	0.72 (0.64-

	[28]					0.81)
rs13289150	Pezzolesi et al., 2009 [24]	**Caucasian**	83549513	A	0.05	NA‡‡

*Based on the hg19 genome assembly. ‡Hazard ratio 1.33. ‡‡Hazard ratio 1.23. §Z score 3.19. §§Z score 2.88. Abbreviation: NA, not applicable.

CAPÍTULO II

Estudo de expressão

ESTUDO DA EXPRESSÃO GÊNICA E PROTEICA DO *FRMD3* EM CÉLULAS RENAIS HUMANAS

1. Objetivo

Analisar a relação entre o polimorfismo *rs1888747* no gene *FRMD3* com a expressão do gene *FRMD3* e a expressão da proteína *4.1* em células renais.

2. Materiais e métodos

2.1 População e amostra

No presente trabalho foi realizado um estudo envolvendo genotipagem do polimorfismo rs1888747 do gene *FRMD3*, expressão gênica e protéica. A amostra foi composta por 140 indivíduos submetidos à nefrectomia radical para tratamento de tumores renais malignos, urolitíase, entre outras causas, conforme protocolos já estabelecidos pelos Serviços de Urologia do Hospital de Clínicas de Porto Alegre (HCPA). A maior parte do rim retirado foi para avaliação histopatológica e confirmação do tipo de tumor no Serviço de Patologia do HCPA, quando indicado. Os procedimentos de retirada do rim e análise histopatológica foram realizados pelos médicos dos Serviços de Urologia e Patologia conforme sua rotina clínica. Logo após a retirada do rim e antes do envio da peça ao Serviço de Patologia, foram coletadas duas amostras pequenas de tecido renal não atingido pelo tumor para a realização das análises deste estudo. Um questionário padronizado foi aplicado aos participantes para coletar informações acerca da idade, gênero, peso, altura, presença de hipertensão arterial, diabetes e hábitos de fumar.

Os procedimentos realizados estão de acordo com a Resolução 196/96 do Conselho Nacional de Saúde, item IV3.c. A porção do tecido renal extraído para tratamento tumoral e que não é utilizada pelos técnicos do Serviço de Patologia para diagnóstico do tumor pode ser fornecida para pesquisa uma vez que seria descartada, conforme previsto no Artigo 3º da Resolução da Diretoria Colegiada da ANVISA – RDC Nº 67, de 30 de setembro de 2008.

Entrentanto, para que houvesse a concordância do doador para utilização deste tecido e coleta do sangue periférico, foi lido e assinado o termo de consentimento livre e esclarecido (TCLE) por estes doadores, quando de acordo.

Amostras de sangue periférico foram coletadas para extração de DNA e posterior genotipagem. Biópsias renais normais foram divididas em alíquotas para as análises de expressão gênica e protéica. A partir da extração de RNA, realizou-se a transcrição reversa em cDNA e posterior análise de expressão gênica por transcrição reversa de PCR em tempo real quantitativo (TR-PCRq). Na imunohistoquímica, a partir da confecção de blocos de parafina, as peças renais foram processadas e as lâminas resultantes analisadas por dois observadores, tendo como resultado a quantificação da expressão protéica além da localização no tecido. Já no western blot, foi possível quantificar a expressão protéica e observar três das cinco possíveis isoformas do gene *FRMD3*.

2.2 Análises laboratoriais

2.2.1 Coleta e preparação das amostras

Após a coleta do tecido renal não tumoral de doadores que se submeteram à nefrectomia radical foram realizados os seguintes procedimentos:

1) Amostra de tecido renal não-tumoral 1: para análises de expressão de RNAm. Aproximadamente 0,5g de tecido renal não atingido pelo tumor foram colocados em um tubo de 1,5 ml, o qual foi transferido imediatamente para uma recipiente contendo nitrogênio líquido e, após 30 min, foi colocado em freezer -80°C localizado no Laboratório de Biologia Molecular e Celular (LBMC) do Serviço de Endocrinologia do HCPA, até a realização das análises de expressão.

2) Amostra de tecido renal não-tumoral 2: para análise de imunohistoquímica. Aproximadamente 2-5g de tecido renal não atingido pelo tumor foi colocado em um tubo Falcon de 50 ml estéril contendo Formalina 10% e imediatamente levado ao LBMC do Serviço de Endocrinologia do HCPA. Nesse laboratório, a amostra foi mantida em formalina 10% por um período de 24 a 48h e, logo após, transferida para etanol 70% por 24h e finalmente mantida em etanol absoluto até a preparação dos blocos de parafina pelos técnicos do Serviço de Patologia do Centro de Pesquisa do HCPA.

3) Coleta de sangue total: para extração de DNA e genotipagem do polimorfismo estudado. De cada doador, algumas horas antes da cirurgia de nefrectomia, também foram coletadas 10 ml de sangue periférico, os quais também levados ao LBMC do Serviço de Endocrinologia.

2.2.2 Extração de DNA e Genotipagem

A extração do DNA foi realizada a partir de leucócitos periféricos (5 ml de sangue venoso), pelo método de Lahiri & Nurnberger, e posteriormente realizada a genotipagem por PCR em tempo real.

O polimorfismo *rs1888747* foi genotipado através de ensaios de discriminação alélica por PCR em tempo real utilizando-se primers e sondas contidas no *Human Custom TaqMan Genotyping Assay* (40x) (Assays-By-Design Service, Life Technologies, Foster City, CA; USA). Uma das sondas marcada com a fluorescência Vic e a outra sonda com a fluorescência Fam. A reação de PCR em tempo real foi conduzida em uma placa de 96 poços, em um volume total de 5µl contendo 2ng de DNA genômico, o tampão *TaqMan Genotyping Master Mix* (1x) (Life Technologies) e os primers e sondas contidos no *Custom TaqMan Genotyping Assay* (1x). As placas foram então colocadas em um termociclador para PCR em

tempo real (7500 Fast Real Time PCR System; Life Technologies) e aquecidas por 10min a 95°C, seguidos por 40 ciclos de 95°C por 15s e 63°C por 1min. Os resultados analisados no software *System Sequence Detection* v.1.4 (Life Technologies).

2.2.3 Extração de RNA e expressão gênica em rim humano

Inicialmente, as biópsias renais foram homogeinizadas em fenol/guanina isotiocianata (Invitrogen Life Technologies, Carlsbad, CA). O RNA foi extraído com clorofórmio e precipitado com isopropanol através de centrifugação (12,000 x g) à 4°C. O *pellet* de RNA foi lavado três vezes com etanol 75% e ressuspendido em 10-50 µl de dietilpirocarbonato tratado com água. Concentrações dos isolados de RNA foram quantificados utilizando o espectrofotômetro NANODROP 2000 (Thermo Scientific Inc., DE, USA). Somente amostras de RNA total com taxas adequadas de pureza (A260/A280 = 1.9-2.1) foram utilizadas para as subseqüentes análises [1]. Adicionalmente, integralidade e pureza foram conferidas em gel de agarose contendo *GelRed Nucleic Acid Gel Stain* (Biotium Inc, Hayward, CA). A concentração média (± DP) de RNA isolado foi de 2,68 ± 1,69 µg/250 mg de rim.

Posteriormente, a técnica de TR-PCRq foi realizada em duas reações separadas: primeiro, 5 µg de RNA total foi usado para transcrição reversa em cDNA utilizando-se o kit *SuperScript Vilo MasterMix* (Synthesis System for RTq-PCR; Invitrogen), de acordo com o protocolo fornecido pelo fabricante. Os experimentos de TR-PCR foram realizados no equipamento *7500 Real Time PCR System* (Life Technologies), através do monitoramento em tempo real da fluorescência do reagente SYBER Green (1x) [2]. Os primers foram construídos utilizando-se o programa Primer Express 3.0 (Life Technologies).

As reações de PCR foram realizadas utilizando 10 µl de SYBR Green (1x) (Life Technologies), 1 µl de primers específicos, *FRMD3* ou *cyclophilin* (Life Technologies), 7 µl de água e 1 µl de cDNA (0.2 µg/µl) em um volume total de 20 µl. Cada amostra foi testada em triplicada e um controle negativo foi incluído em cada experimento. As condições de termociclagem utilizadas para estes genes foram: um ciclo inicial a 95°C por 10 min, seguido por 50 ciclos a 95°C por 15 s e a 60°C por 1:30 min.

A quantificação de cDNA do *FRMD3* foram realizadas utilizando o método ΔΔCq [1, 3] e os relativos expressos para o gene de referência (*cyclophilin*) em 91 amostras de tecido renal: 76 carreando o alelo G (G/G ou G/C) do SNP rs1888747, e 15 com genótipo C/C. O número de amostras foram suficientes para detectar uma diferença de 0,3-fold entre os genótipos. A validação dos ensaios foram feitos através da amplificação separada dos genes alvo (*FRMD3*) e de referência (*cyclophilin*) utilizando diluições seriadas das amostras de cDNA. Assim como este método requer, ambos os genes, alvo e de referência, apresentam eficientes amplificações (E = 95% - 105%) em todos os experimentos. O método ΔΔCq calcula mudanças na expressão gênica assim como a diferença relativa em número de vezes (*n-fold change*) entre uma amostra de calibrador experimental e uma externa [1]. A especificidade de RTq-PCR foi determinada através de análises de fusão das curvas e analisa todos as amplificações geradas pelos primers que produziram um único pico agudo durante as análises [4].

2.2.4 Expressão proteica do FRMD3

A expressão protéica do FRMD3 foi determinada em 48 amostras de rim humano. Destas, 39 eram carreadoras do alelo G do polimorfismo rs1888747 (GG = 23 e CG = 16). A localização da expressão protéica foi avaliada através da imunohistoquímica (IHC), enquanto

a quantificação da expressão proteica foi determinada por western blot (WB), considerado atualmente o teste padrão ouro para tal finalidade.

Para análises de IHC, foi utilizado o anticorpo policlonal de coelho anti-FRMD3 (Abcam, Cambridge, MA). As análises de IHC foram realizadas em 4 µm de tecido renal [5]. A rotina da técnica de IHC consiste em: desparafinização e rehidratação, recuperação antigênica, inativação da peroxidase endógena e bloqueio de reações não específicas. As frações seccionadas de tecido são incubadas com anticorpo primário e então incubadas novamente com anticorpo secundário biotinilado, *streptavidin horseradish peroxidase conjugate* (LSA; Dako Cytomation, Inc Carpinteria, CA), e tetrahidrocloridrato diaminobenzidina (Kit DAB Dako Cytomation, Inc). As imagens foram visualizadas através de um microscópio Zeiss (modelo AXIOSKOP-40; Carl Zeiss, Oberkochen, Germany) e capturadas utilizando câmera Cool Snap-Pro CS (Media Cybernetics).

Para as análises de WB, proteínas foram extraídas pelo tampão RIPA e quantificadas utilizando um kit de ensaio colorimétrico de proteína BCA™ (Thermo Scientific). As proteínas extraídas (20 µg) foram adicionadas em gel de poliacrilamida 10%, transferidas para membrana Immobilon®-P^{SQ} (Millipore, Billerica, MA), e incubadas com anticorpo monoclonal para *FRMD3* (Bioss, Woburn, MA) ou β-Actina (Millipore, California, CA) durante toda noite. Anticorpos secundários consistiram em *horsedish peroxidase conjugated goat anti-rabbit* (Millipore, Billerica, MA). A detecção foi realizada utilizando o substrato Immobilon Western Chemiluminescent HRP (Millipore, Billerica, MA), e as imagens foram adquiridas e quantificadas através do sistema de imagem digital ImageQuant LAS 500 (GE Healthcare, USA). Inicialmente, o processo foi aplicado para o gene *FRMD3*. Após a realização do procedimento de *stripping*, o processo foi aplicado então para o gene *β-Actina*. Dados foram expressos em relação a diferença relativa em número de vezes (*n-fold change*).

2.2.5 Análise estatística

As freqüências alélicas do SNP foram determinadas por contagem gênica, e o equilíbrio de Hardy-Weinberg foi verificado utilizando o teste de qui-quadrado. Dados contínuos com distribuição normal foram representados por média ± desvio padrão, enquanto variáveis contínuas com distribuição assimétrica foram logaritmizados antes das análises e apresentados como mediana (mínimo – máximo), com exceção da concentração protéica de FRMD3 (western blot). Neste último caso, a transformação logarítmica não normalizou a variável, então foi utilizado variável não logaritmizada e testes não paramétricos. Dados categóricos foram expressos em números de casos e percentual de indivíduos afetados. *One-way analysis of variance* (ANOVA), qui-quadrado ou teste t de Student foram utilizados para comparar grupos em termos de características clínicas e laboratoriais ou expressão gênica e protéica de FRMD3. O teste de correlação de Pearson foi utilizado para avaliar a correlação entre variáveis quantitativas. Análise de regressão linear múltipla foi aplicada com expressão do gene e da proteína como variáveis dependentes e idade e sexo como variáveis independentes. Os resultados com $p < 0,05$ foi considerado estatisticamente significativo. Essas análises foram realizadas através do pacote estatístico SPSS 18.0 (Chicago, IL, USA).

3. Resultados

3.1 Descrição da Amostra

Nas 140 amostras genotipadas, a freqüência genotípica foi de 62,1% (n=87) G/G, 25,0% (n=35) G/C e 12,9% (n=18) C/C. A frequência do alelo de risco (G) foi de 0,25 nessa população. A média de idade dos indivíduos foi 58,94 ± 14,52 anos. Destes 49,1% eram do sexo masculino. Hipertensão arterial estava presente em 54,7% dos pacientes e diabetes mellitus em 35%. Dezessete por cento eram tabagistas. As características clínicas de acordo

com os genótipos do polimorfismo rs1888747 estão descritas na Tabela 1. Os genótipos do polimorfismo rs1888747 desviaram do equilíbrio de Hardy-Weinberg ($p < 0,001$), porém, não foram detectados erros de genotipagem, logo, decidimos prosseguir com as análises.

3.2 Expressão gênica

A quantificação de mRNA (*n-fold change*) do *FRMD3* não diferiu entre os sexos [feminino: 0,31 (-1,92 – 2,05); masculino: 0,12 (-2,70 – 2,00); p = 0,200] ou presença de hipertensão arterial [hipertensos: 0,47 (-2,05 – 2,05); não hipertensos: -0,21 (-2,70 – 1,99); p = 0,077], e também não correlacionou com idade (r = 0,11; p = 0,305). Esta também foi similar em indivíduos com ou sem diabetes [0,37 (-2,70 – 1,98) *vs.* 0,16 (-2,22 – 2,05) *n fold change*; p = 0,779].

A expressão gênica em amostras renais categorizadas de acordo com o polimorfismo do rs1888747 (CC *vs*. GG+GC – modelo recessivo) está representada na Figura 1. Resultados mostram que a expressão foi similar em homozigotos para o alelo C [0,41 (-1,77 – 2,00)] e naqueles carreando o alelo G [0,21 (-2,70 – 2,05)] (P = 0,630).

3.3 Expressão Protéica

A expressão protéica (*n-fold change*) não diferiu entre os sexos [feminino: 0,73 (0,11 – 2,87); masculino: 0,79 (0,07 – 2,64); p = 0,959] ou presença de hipertensão arterial [hipertensos: 0,73 (0,07 – 2,31); não hipertensos: 1,05 (0,11 – 2,87); p = 0,212], e também não correlacionou com idade (r = -0,13; p = 0,364). A quantidade da proteína FRMD3 dos pacientes com diabetes foi maior que dos pacientes sem diabetes, entretanto, com valor de P limítrofe [0,66 (0,11 – 2,31) vs. 0,99 (0,07 – 2,87); p = 0,05].

A expressão protéica em amostras de rim humano categorizadas de acordo com o modelo recessivo (CC *vs*. GG+GC) para o polimorfismo rs1888747 está representada na

Figura 2. A expressão protéica foi similar em homozigotos para o alelo C e nos indivíduos carreando o alelo G [1,17 (0,07 – 2,27) vs. 0,74 (0,11 – 2,87); p = 0,190]. Além disso, os genótipos do polimorfismo rs1888747 não influenciaram os níveis de proteína após ajuste para idade, sexo e presença de diabetes (β = -0.019, p = 0,908). A Figura 2 retrata a representação dos géis de WB para a proteína de FRMD3 de acordo com os genótipos do rs1888747. A proteína FRMD3, demonstrada pela imunohistoquímica, está predominantemente expressa nos túbulos e podócitos renais (Figura 3).

4. Tabelas e Figuras

Tabela 1. Características clínicas de acordo com o polimorfismo rs1888747 do gene *FRMD3*

	C/C (n=18)	C/G (n=35)	G/G (n=87)	p
Idade (anos)	48,50 ± 19	58,50 ± 20	60 ± 20	0,326
Gênero masculino n (%)	5 (27,8)	19 (54,3)	43 (49,4)	0,170
Hipertensão arterial n (%)	12 (66,7)	17 (48,6)	49 (56,3)	0,478
Índice de massa corporal (%)	27,9 ± 4,85	30,0±8,92	26.8 ± 5,65	0,389
Diabetes Mellitus n (%)	5 (27,8)	8 (22,9)	29 (33,3)	0,536

Dados expressos em média ou desvio padrão ou numero de casos (%)

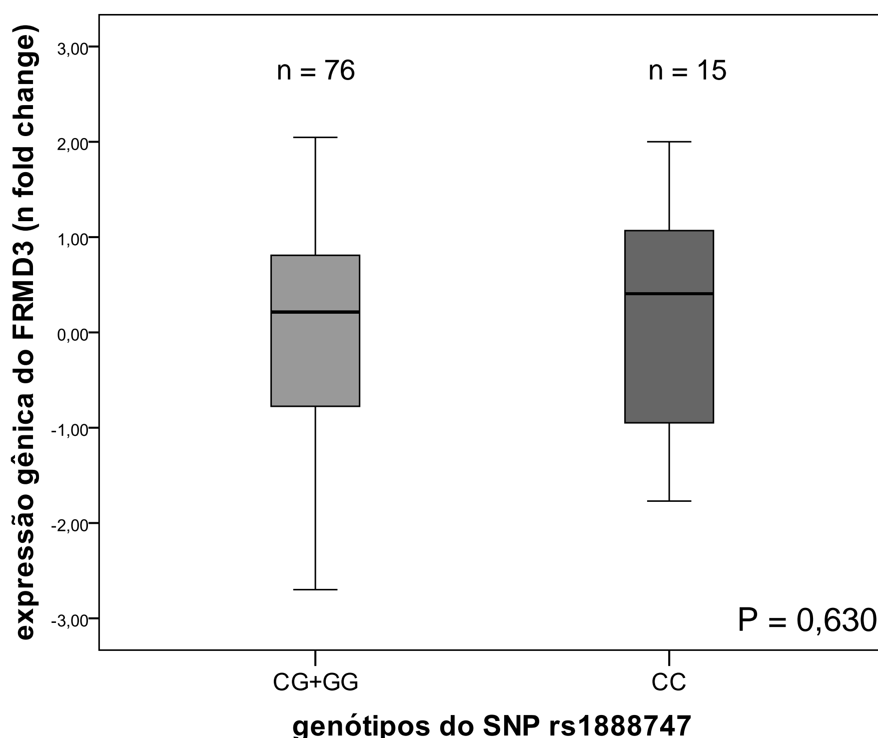

Figura 1. Expressão gênica do *FRMD3* de acordo com o polimorfismo rs1888747 do gene *FRMD3* (modelo recessivo). Dados expressos em mediana (mínimo – máximo).

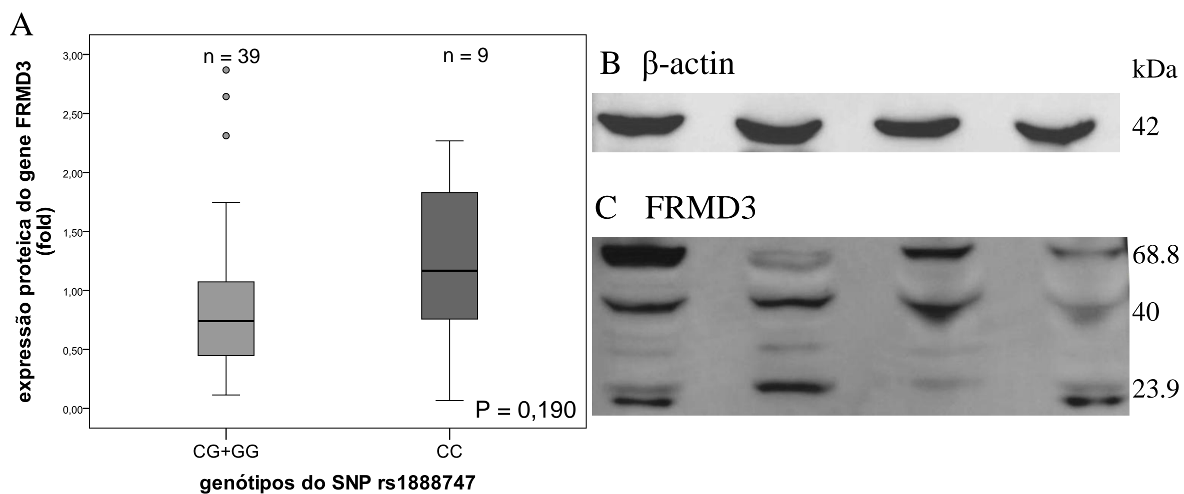

Figura 2. (A) Expressão protéica de FRMD3 de acordo com o polimorfismo rs1888747 do gene *FRMD3* (modelo recessivo). Representações de western blot para: (B) β-actina, e (C) três isoformas de FRMD3. Dados expressos em mediana (mínimo – máximo).

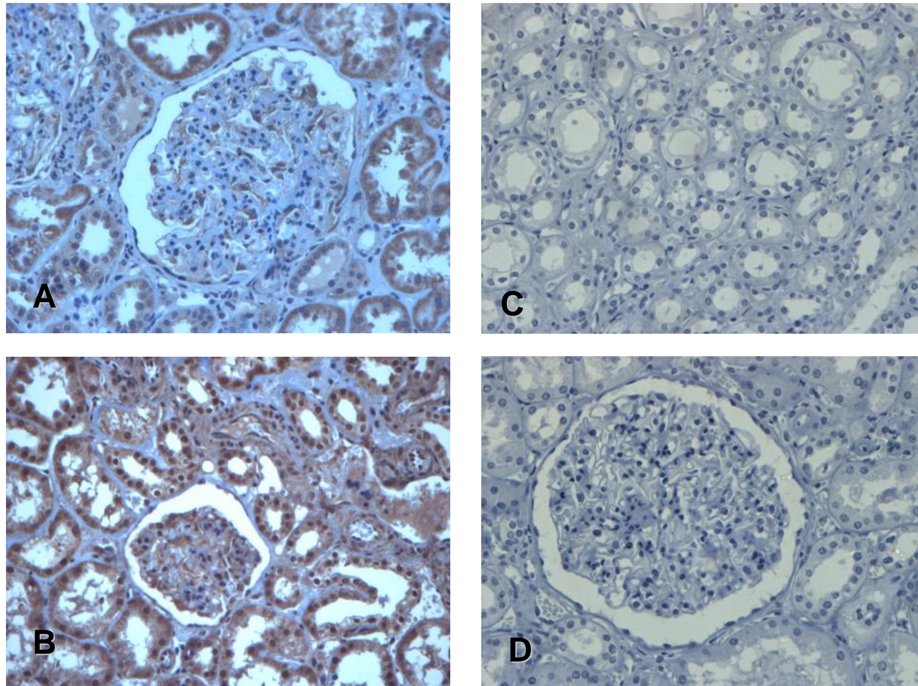

Figura 3. Representações fotomicrográficas de rim humano através de imunohistoquímica. Magnitude original de todas as imagens = ×400. (A, B) Expressão protéica de FRMD3 é predominantemente localizado nos túbulos. Nos glomérulos, a expressão é localizada exclusivamente nos podócitos. (C, D) Amostras de controles negativos.

5. Conclusões

Este estudo foi importante para ajudar a elucidar o papel do polimorfismo rs1888747, na expressão do gene até a sua tradução em proteína. Não foi possível afirmar que o polimorfismo esteja influenciando a expressão do FRMD3. O *FRMD3* é um gene relativamente novo e existem poucos estudos até o momento focando sua expressão, tanto gênica quanto protéica. Além disso, a confirmação da localização desta proteína nos podócitos renais corrobora o fato de que o produto resultante deste gene faz parte de uma família de proteínas envolvida na função de citoesqueleto, dando forma e integridade à célula.

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CAPÍTULO III

Artigo Original

"rs1888747 POLYMORPHISM IN THE *FRMD3* GENE, GENE AND PROTEIN EXPRESSION: ROLE IN DIABETIC KIDNEY DISEASE"

O POLIMORFISMO rs1888747 NO GENE *FRMD3*, EXPRESSÃO GÊNICA E PROTÉICA: PAPEL NA DOENÇA RENAL DO DIABETES

rs1888747 POLYMORPHISM IN THE *FRMD3* GENE, GENE AND PROTEIN EXPRESSION: ROLE IN DIABETIC KIDNEY DISEASE

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rs1888747 polymorphism in the *FRMD3* gene, gene and protein expression: role in diabetic kidney disease

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ABSTRACT

Aims/Hypothesis: Thirteen single nucleotide polymorphisms (SNPs) have been found to be associated with DKD in Caucasian patients with type 1 diabetes (T1DM). We carried out a case-control study to evaluate the possible association between seven of these SNPs and DKD in Brazilian patients with type 2 diabetes (T2DM). Additionally, we evaluated gene and protein expression of the genes which had associated polymorphisms with DKD in a group of non-DKD patients.

Methods: The association study included 1,098 T2DM white patients: 718 with DKD (cases) and 380 with normoalbuminuria (controls). The expression study included 140 patients undergoing nephrectomy without DKD. Out of the 13 polymorphisms associated with DKD in a previous study with T1DM, seven were chosen for evaluation in this study: rs1888747, rs9521445, rs39075, rs451041, rs1041466, rs1411766 and rs6492208. *FRMD3* gene expression was assessed by RT-qPCR. FRMD3 protein expressions in kidney tissue were quantified by western blot and it localization by immunohistochemistry.

Results: The presence of the C allele of the *FRMD3* rs1888747 SNP in homozygosis was associated with reduced risk of DKD (OR = 0.6, 95% CI 0.3-0.9; P = 0.022). None of the other polymorphisms were associated with DKD. FRMD3 gene and protein expressions were similar in subjects homozygous for the C allele and in those carrying the G allele (P = 0.630 and P = 0.190, respectively).

Conclusions/Interpretation: Replication of the association between SNP rs1888747 and DKD in a different population provides strong evidence that this link is not the result of chance. SNP rs1888747 is located at the *FRMD3* gene, which is expressed in human kidney, and a candidate gene for DKD. This study also suggests that, outside the

context of DKD, there is no genotype or specific allele influencing the gene expression and/or protein expression of the *FRMD3* gene.

Keywords: *FRMD3* gene expression, polymorphism, human kidney, diabetic kidney disease.

INTRODUCTION

Diabetic kidney disease (DKD) is currently the main cause of end-stage renal disease (ESRD) in developed countries [1-5], with an estimated incidence of 26% in patients starting dialysis [6]. In addition to ESRD, DKD also causes an important increase in cardiovascular-related morbidity and mortality [6, 7].

An increasing body of evidence supports a genetic basis for DKD [8, 9]. However, little is known about the mode of transmission, which is probably polygenic, or about the mechanisms of gene-environment interaction. Until this moment, genome wide-scan (GWS) and candidate gene studies have produced heterogeneous results [10-15].

A GWS study of 360,000 single nucleotide polymorphisms (SNPs) has identified an association of 13 SNPs in four genomic loci with DKD in two independent populations of patients with type 1 diabetes mellitus (T1DM) (Genetics of Kidneys in Diabetes Study - GoKinD) [17]. The strongest association was observed at the *FRMD3* (4.1 protein ezrin, radixin, moesin [FERM] domain containing 3) locus (OR = 1.45). Moreover, a strong association was also observed at the *CARS* (cysteinyl-tRNA synthetase) locus (OR = 1.36). Confirmation of implicated SNPs was obtained in 132 of 1,304 participants of the Diabetes Control and Complications Trial (DCCT) / Epidemiology of Diabetes Interventions and Complications (EDIC) study [17-19].

Because patients with T1DM and type 2 diabetes mellitus (T2DM) might share common genes related to DKD, the present study was designed to 1) investigate whether the SNPs found to be associated with DKD in T1DM are also associated with DKD in T2DM; 2) evaluate the association of these SNPs in early (microalbuminuria) and advanced stages of DKD (macroalbuminuria or ESRD) [in the same population];

and 3) evaluate *FRMD3* gene expression in kidney biopsies of individuals according to the SNP genotypes found to be associated with DKD.

SUBJECTS AND METHODS

Association study

A case-control study was conducted with 1,098 white T2DM patients selected from an ongoing cross-sectional study in the state of Rio Grande do Sul, Brazil. Patient recruitment began in 2002 [13]. T2DM was defined according to World Health Organization criteria [20]: 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 two categories: early DKD (albuminuria <30 mg/24h) or advanced DKD (albuminuria ≥300 mg/24h or ESRD). Stage of DKD was classified based on two out of three albuminuria measurements as normoalbuminuria (<30 mg/24h; n = 380), microalbuminuria (30-300 mg/24h; n = 323), macroalbuminuria (>300 mg/24h) or ESRD (need for dialysis) (n = 395). Six dialysis centers were involved: 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 and DKD 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 with renal diseases other than DKD were excluded. The protocol was approved by the ethics committees, and all patients gave their written informed consent.

All patients underwent an evaluation that included a standardized questionnaire and physical examination, as previously described [21]. Briefly, information was collected about age, age at T2DM 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.

Fasting blood samples were collected for laboratory and molecular analyses. 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 coefficient of variation (CV) of 2.4% and 0.5%, respectively (reference interval 4.1 to 6.0%) [22]. Serum creatinine was determined by the Jaffé reaction [23]. 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 CV of 4.5% and 11% respectively [24]. Use of angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists was interrupted at least one week before albuminuria measurement.

DNA isolation

DNA was extracted from peripheral blood leukocytes by a standardized salting-out procedure. Seven out of 13 SNPs initially associated with DKD in T1DM in the GoKinD Study [17] were chosen to be included in the present study (rs1888747, rs9521445, rs39075, rs451041, rs1411766, rs6492208, rs1041466). Not all 13 SNPs

were genotyped because some were in linkage disequilibrium (LD): rs1888747 and rs10868025 ($r^2 = 0.81$), both located at chromosome 9q; rs39059 and rs39075 (7p, $r^2 > 0.96$); rs451041 and rs739401 (11p, $r^2 > 0.87$), rs9521445 and 7989848 (13q, $r^2 > 0.87$), rs1411766 and 17412858 (13q, $r^2 = 1$), and rs6492208 and rs2391777 (13q, $r^2 = 1$) [17]. In LD pairs, the SNP having the strongest association with DKD was chosen for analysis. Polymorphism loci are shown as supplementary material (table S1).

Genotyping

All polymorphisms were genotyped using primers and probes contained in the Human Custom TaqMan Genotyping Assay (40x) (Assays-By-Design Service, Life Technologies, Foster City, CA; USA). The primer and probe sequences used for genotyping these SNPs are described in table S2. The reactions were conducted in 96-well plates, in 5 µl total reaction volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix (1x) (Life Technologies), and Custom TaqMan Genotyping Assay (1x). The plates were positioned in a real-time PCR thermal cycler (7500 Fast Real PCR System; Life Technologies) 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. Genotyping success was more than 95%, with a calculated error rate based on PCR duplicates of < 0.01%.

FRMD3 gene expression in kidney tissue samples

Kidney tissue samples were collected from an additional group including 140 consecutive patients undergoing therapeutic nephrectomy over a period of 30 months at Hospital de Clínicas de Porto Alegre. A standard questionnaire was used to collect information regarding age, gender, presence of arterial hypertension, diabetes mellitus, and smoking habit. A peripheral blood sample was collected from each subject for DNA extraction and genotyping. Excised normal kidney tissue was divided into aliquots for

mRNA expression analyses and evaluation of protein expression by western blot (WB) and immunohistochemistry (IHC).

The study protocol was approved by the Hospital's ethics committees, and all patients gave their written informed consent.

RNA isolation

Kidney biopsies were homogenized in phenol/guanidine isothiocyanate (Invitrogen Life Technologies, Carlsbad, CA). RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000 x g) at 4°C. RNA pellet was washed twice with 75% ethanol and resuspended in 10-50 µl of diethylpyrocarbonate treated water. Concentrations of isolated RNAs were assessed using NANODROP 2000 spectrophotometer (Thermo Scientific Inc., DE, USA). Only total RNA samples achieving adequate purity ratios (A260/A280 = 1.9-2.1) were used for subsequent analyses [25]. In addition, RNA integrity and purity were also checked on agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium Inc, Hayward, CA). The mean (± SD) concentration of isolated RNA was 2.68 ± 1.69 µg/250 mg kidney.

Quantification of FRMD3 gene expression by real-time reverse transcription PCR

Real-time reverse transcription-PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, and subsequently cDNA was amplified by quantitative real-time PCR (RT-qPCR). Reverse transcription of 5 µg of RNA into cDNA was carried out using the SuperScript Vilo MasterMix Kit (Synthesis System for RT-PCR; Invitrogen).

RT-qPCR experiments were performed in a 7500 Real Time PCR System (Life Technologies). Experiments were performed by real-time monitoring of the increase in

fluorescence of SYBER Green dye [26]. Primers were designed using Primer Express 3.0 Software (Life Technologies) and are depicted in Supplementary Table (S2).

PCR reactions were performed using 10 μl of SYBR Green (1x) (Life Technologies), 1 μl of specific primers, *FRMD3* or *cyclophilin* (Life Technologies), 7 μl of water and 1 μl of cDNA (0.2 μg/μl) in a total volume of 20 μl. Each sample was assayed in triplicate and a negative control was included in each experiment. The thermocycling conditions for these genes were as follows: an initial cycle of 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1:30 min.

Quantification of *FRMD3* cDNA was performed using the ΔΔCq method [25, 27] and expressed relative to the reference gene (*cyclophilin*) in 91 kidney tissue samples: 76 carrying the G allele (G/G or G/C) of the rs1888747 SNP, and 15 with the C/C genotype. The number of samples was sufficient to detect a 0.3-fold difference between genotypes. Validation assays were done by separate amplification of the target (*FRMD3*) and reference (*cyclophilin*) genes using serial dilutions of a cDNA sample. As a requirement of this method, both target and reference genes exhibited equal amplification efficiencies (E = 95% to 105%) in all experiments. The ΔΔCq method calculates changes in gene expression as relative fold difference (n-fold change) between an experimental and an external calibrator sample [25]. RT-qPCR specificity was determined using melting curve analyses and all primers generated amplicons that produced a single sharp peak during the analyses [28].

Determination of* FRMD3 *protein distribution and concentration in kidney

FRMD3 protein distribution and concentrations were determined in 48 kidney sections. Thirty-nine of these sections belonged to subjects carrying the rs1888747 SNP G allele (GG = 23 and CG = 16). FRMD3 protein distribution was evaluated by IHC of

formalin-fixed, paraffin-embedded kidney sections, while FRMD3 protein quantities were determined using WB.

For IHC analyses, a goat rabbit anti-FRMD3 polyclonal antibody (Abcam, Cambridge, MA) was used to detect FRMD3 protein distribution in human kidney tissue. IHC analyses were performed on 4 μm-thick kidney sections [29]. The routine IHC technique comprised: deparaffinization and rehydration, antigenic recovery, inactivation of endogenous peroxidase and blocking of nonspecific reactions. Slides were incubated with primary antibody and then incubated again with a biotinylated secondary antibody, streptavidin horseradish peroxidase conjugate (LSA; Dako Cytomation, Inc Carpinteria, CA), and diaminobenzidine tetrahydrochloride (Kit DAB Dako Cytomation, Inc). Images were visualized through a Zeiss microscope (model AXIOSKOP-40; Carl Zeiss, Oberkochen, Germany) and captured using the Cool Snap-Pro CS (Media Cybernetics) camera.

For WB analyses, proteins from human kidneys were extracted by RIPA buffer and quantified using a colorimetric BCA™ Protein Assay Kit (Thermo Scientific). Protein extracts (20 µg) were resolved on 10% polyacrylamide gels, transferred to Immobilon®-P^{SQ} membranes (Millipore, Billerica, MA), and incubated with monoclonal antibodies to FRMD3 (Bioss, Woburn, MA) or β-Actin (Millipore, California, CA) overnight. Secondary antibodies consisted of horseradish peroxidase conjugated goat anti-rabbit antibodies (Millipore, Billerica, MA). Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA), and images were acquired and quantified in an ImageQuant LAS 500 (GE Healthcare, USA) digital imaging system. Initially, the process was applied for FRMD3 antibody. After stripping, the membrane was re-blotted with β-Actin antibody. Data are expressed as arbitrary units (AU).

Statistical analyses

In the association study, we compared controls *vs*. cases (defined as any degree of DKD) and controls *vs*. different DKD stages (microalbuminuria, macroalbuminuria, and ESRD). Data were analyzed assuming both dominant and recessive inheritance models to determine the best fit. Allele frequencies of all SNPs were determined by gene counting, and departures from Hardy-Weinberg equilibrium (HWE) were verified using the chi-square (χ^2) test. Allele and genotype frequencies were compared between groups using the χ^2 test. Continuous data with normal distribution are presented as means ± SD, while continuous variables with skewed distribution were log-transformed before analysis and are presented as medians (minimum – maximum values), with exception of FRMD3 protein concentration (WB) for which log-transformation did not normalized the variable, thus we used the non-log variable and non-parametric tests. Categorical data are expressed as number of cases and percent of individuals affected. One-way analysis of variance (ANOVA), χ^2 or Student's t test were used to compare the groups in terms of clinical and laboratory characteristics or FRMD3 gene/protein expression. The magnitude of the association was estimated using odds ratios (ORs) with 95% confidence interval (95%CI). Multiple logistic regression analysis was used to evaluate the independence of possible SNP associations with DKD, adjusting for covariables. Pearson's correlation test was used to evaluate correlation between quantitative variables. Multiple linear regression analyses were performed with FRMD3 gene and protein expression as dependent variables and age and sex as independent variables. Results for which $P < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS 18.0 (Chicago, IL, USA).

RESULTS

Association study

We evaluated 1,098 T2DM patients, of which 718 (65.4%) had DKD (323 microalbuminuric, 395 macroalbuminuric/ESRD). Patients with DKD had higher blood pressure, higher prevalence of diabetic retinopathy, and a worse lipid profile compared to the control group (Supplementary Table – S3). Both groups had similar HbA1c levels.

Genotypes of the seven SNPs analyzed in the present study were in HWE (P > 0.05). Genotype distribution are shown in Table 1. Of these seven SNPs, only rs1888747 was significantly associated with DKD (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; 95%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 was associated with DKD in this sample of T2DM patients (Table 1).

Table 2 shows the genotype distribution of rs1888747 SNP according to renal status (normo-, micro- or macro/ESRD). The same pattern described above was observed, with a decrease in the frequency of the homozygous genotype (C/C) for the minor allele of the rs1888747 SNP in subjects with microalbuminuria or macroalbuminuria/ESRD compared to controls. The frequency of the C allele was also lower in individuals with microalbuminuria or macroalbuminuria/ESRD compared to normoalbuminuric controls.

FMRD3 ***gene expression study – sample description***

Since only rs1888747 was associated with DKD in T2DM, FRMD3 gene and protein expressions in human kidney tissue were evaluated specifically according to the different genotypes of this SNP in the group of nephrectomized patients. The mean age of the 140 subjects genotyped for the rs1888747 SNP was 58.94 ± 14.52 years; 49.1% were males, 54.7% had arterial hypertension, 17.0% were smokers, and 35% had diabetes. *FRMD3* genotypes deviated from HWE ($P < 0.001$). Since no genotyping errors were detected, we decided to proceed with the analyses. Supplementary Table 4 presents the clinical data according to rs1888747 genotypes. Genotype frequencies were as follows: G/G, 62.14% (n = 87), G/C, 25% (n = 35), and C/C, 12.86% (n = 18). The minor allele (C) frequency was 25% in this sub-sample.

FMRD3 ***mRNA expression***

FRMD3 mRNA expression did not correlate with age ($r^2 = 0.11$; $P = 0.305$), and did not differ between females [0.31 (-1.92 – 2.05)] and males [0.12 (-2.70 – 2.00); P = 0.200], between patients with hypertension [0.47 (-2.05 – 2.05)] and without hypertension [-0.21 (-2.70 – 1.99); P = 0.077] and between patients with diabetes [0.37 (-2.70 – 1.98)] and without diabetes [0.16 (-2.22 – 2.05); P = 0.779]. *FRMD3* mRNA expression in kidney tissue samples categorized according to the minor allele recessive model (CC *vs*. GG+GC) for the rs1888747 polymorphism are represented in Figure 1. Gene expression was similar in subjects homozygous for the C allele [0.41 (-1.77 – 2.00)] and in those carrying the G allele 0.21 (-2.70 – 2.05)] (P = 0.630).

FRMD3 ***protein expression***

FRMD3 protein expression did not correlate with age ($r^2 = -0.13$; $P = 0.364$) and was similar in females [0.73 (0.11 – 2.87)] and males [0.79 (0.07 – 2.64); P = 0.959]

and in patients with [0.73 (0.07 – 2.31)] or without arterial hypertension [1.05 (0.11 – 2.87); P = 0.212]. FRMD3 protein expression differed in patients with [0.66 (0.11 – 2.31)] and without diabetes [0.99 (0.07 – 2.87); P = 0.05]. Protein concentrations were negatively correlated with *FRMD3* gene expression ($r^2 = -0.375$; P = 0.013). FRMD3 protein expression in kidney tissue samples categorized according to the recessive model (CC *vs*. GG+GC) of the rs1888747 polymorphism is shown in Figure 2. FRMD3 protein expression was similar in subjects homozygous for the C allele [1.17 (0.07 – 2.27)] and in those carrying the G allele [0.74 (0.11 – 2.87)] (P = 0.190). Moreover, *FRMD3* rs1888747 genotypes did not influence FRMD3 protein levels after adjustment for age, sex and presence of DM ($\beta = -0.019$, P = 0.908). It is worth noting that these other covariates also did not influence significantly FRMD3 protein levels. Figure 2 depicts representative WB gels for FRMD3 protein. Representative IHC photomicrographs of human kidney are represented in Figure 3. FRMD3 protein was predominantly observed in tubules and podocytes.

DISCUSSION

In the present study, we found that SNP rs1888747 is associated with DKD in T2DM, as previously described for T1DM patients [17]. The homozygous C allele conferred protection against the development of DKD. Additionally, a subgroup with microalbuminuria was evaluated. A similar group had not been evaluated in the original study of T1DM subjects [17]. So far, most association studies of genetic polymorphisms and DKD have not been replicated in additional populations [15, 30, 31]. DKD is probably a multifactorial disorder resulting from an interaction between environmental and genetic factors. GWS studies are capable of identifying unknown chromosomal regions that may be involved in the pathogenesis of DKD. However, replication of the

findings in distinct populations is essential to confirm the associations observed in such studies.

The rs1888747 SNP (C/G) is an intergenic polymorphism, located near the promoter region of the *FRMD3* gene [32]. This gene encodes a structural/cytoskeletal protein involved in maintaining cellular shape [33, 34], but whose function remains otherwise unknown. Studies focusing on the relation between rs1888747 and DKD have been developed in T1DM and T2DM individuals of various ethnic backgrounds, such as Caucasians, Japanese, African Americans, and Chinese, with conflicting results [17, 35-39].

A second SNP found in the GoKinD sample collection has been replicated in DCCT/EDIC subjects – rs451041, located in chromosome 11p. The nearest gene to rs451041 is *CARS*, which is expressed in mesangial and proximal tubule cells. Mutations in this gene have been associated with neurodegenerative diseases and cystinosis [40, 41]. However, neither this nor the other SNPs originally associated with DKD in T1DM patients were associated with DKD in the present study with patients with T2DM. Although this finding requires further validation, if confirmed it might suggest that different genes are involved in the development of DKD in T1DM and T2DM.

The evidence to infer susceptibility genes contributing to the pathogenesis of DKD remains elusive. Advances in genotyping technologies have allowed the identification of several chromosomal regions potentially associated with DKD, but the studies carried out so far lack statistical power; important differences in terms of the populations studied and the phenotypes analyzed make it difficult to compare findings and build up on results.

In the present study, we aimed to evaluate if the rs1888747 risk allele was influencing gene and/or protein expressions, but did not observe an association of these variables with the different genotypes. The *FRMD3* gene (ID: 257019) is located on chromosome 9q21.32, size of 2.282 pb; 14 coding exons; 81.4Mb position [42]. Members of the protein 4.1 family have well characterized functions in a variety of cell types, including rat nephron [33, 34]. *FRMD3* is present in adult ovaries as well as fetal skeletal muscle, brain, and thymus [43]. Our findings confirm that FRMD3 is expressed in proximal renal tubular cells and human podocytes, as previously shown [16, 37], suggesting that this protein is involved in maintaining the function and integrity of the slit diaphragm.

Recently, changes in FRMD3 expression have been linked to progression of DKD [44]. The same authors propose an influence of the rs1888747 polymorphism in the *FRMD3* promoter on transcriptional regulation within the bone morphogenetic protein (BMP)-signaling pathway [44], suggesting that the transcriptional coregulation of BMP pathway members and *FRMD3* might be mediated by the four transcription factor binding site (TFBS) promoter modules in the functional context of DKD [44]. The mechanism mediating the connection between *FRMD3* and BMP pathway members remains unknown. No evidence at the protein, RNA, or microRNA levels was found [44].

The present findings, derived from genotyping, gene and protein expressions studies, are important to elucidate the role of the rs1888747 polymorphism. Nevertheless, further research is necessary to confirm our observations and to unveil the mechanisms underlying the association of the rs1888747 polymorphism with DKD.

A limitation of case-control studies of DKD such as the present one is survival bias. However, we do not believe that this had an impact on the results we report,

because 1) minor allele frequency was similar in mild and severe stages of DKD; and 2) data from the prospective DCCT/EDIC cohort [18] suggest that the rs1888747 C allele had a faster progression to severe DKD. Another possible limitation of our study is the fact that the samples for the expression studies were not obtained from patients with DKD, but instead from patients undergoing therapeutic nephrectomy. Thus, the present gene and protein expression findings cannot be translated to patients with DKD. Further studies are necessary to determine if the rs1888747 polymorphism is or not related to changes in gene and protein expression in DKD.

In conclusion, the present study replicates the protective effect of SNP rs1888747 against established DKD previously described for T1DM. It also shows that this effect is already present in less advanced stages of DKD, such as microalbuminuria. Outside the context of DKD, this study was not able to show an influence of SNP rs1888747 on FRMD3 gene and/or protein expression.

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DUALITY OF INTEREST

All the authors declared no competing interests.

CONTRIBUTION STATEMENT

M.P.B conceived and designed the study, collected and analyzed data, and wrote the manuscript; D.C. conceived and designed the study, analyzed data, and wrote the manuscript; A.S. collected and analyzed data and contributed to the discussion; R.C analyzed data, contributed to the discussion, and reviewed the manuscript; M.P.C collected data and contributed to the discussion; M.I.E analyzed data and contributed to the discussion; M.B. analyzed data and contributed to the discussion; D.A.S. collected and analyzed data and contributed to the discussion; L.H.C. conceived and designed the study, analyzed data, and wrote the manuscript.

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Tables Legends

Table 1. Genotype distribution of polymorphisms in cases and controls

Table 2. Genotype distribution of SNP rs1888747 according to renal status

Figure legends

Figure 1. *FRMD3* gene expression according to the *FRMD3* rs1888747 polymorphism (recessive model). Data expressed as medians (minimum – maximum values).

Figure 2. (A) FRMD3 protein expression according to the *FRMD3* rs1888747 polymorphism (recessive model). Representative western blots for: (B) β-actin, and (C) three isoforms of FRMD3. Data expressed as medians (minimum – maximum values).

Figure 3. Representative photomicrographs of human kidney by immunohistochemistry. Original magnification of all images = ×400. (A, B) FRMD3 protein expression is predominantly localized in tubules. In glomerus, the expression is localized exclusively in podocytes. (C, D) Negative control samples.

Supplementary material legends

Table S1. Localization and allele of polymorphisms analyzed.

Table S2. Primer sequences used to genotype polymorphisms and to analyze gene expression.

Table S3. Clinical characteristics of association study.

Table S4. Clinical characteristics according to *FRMD3* rs1888747 polymorphism in the expression study.

Tables

Table 1

		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
rs451041	AA/AG/GG	23.4 / 48.2 / 28.4	21.3 / 47.1 / 31.6	0.318
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 2

		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
	G (allele)	0.69	0.71	0.74	

Figure

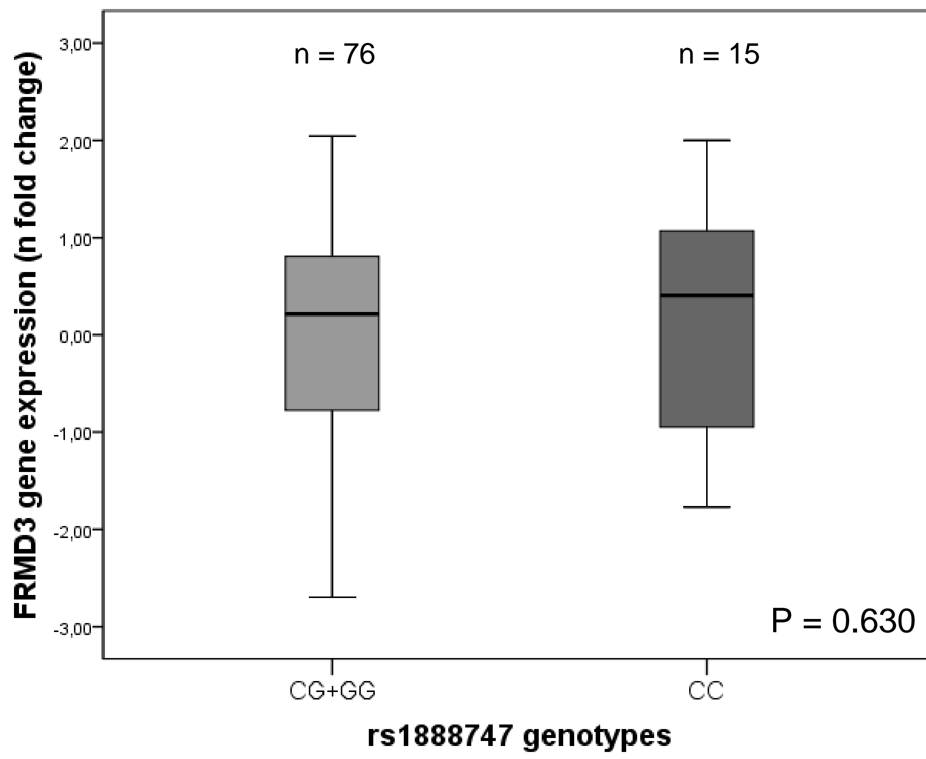

Figure 1

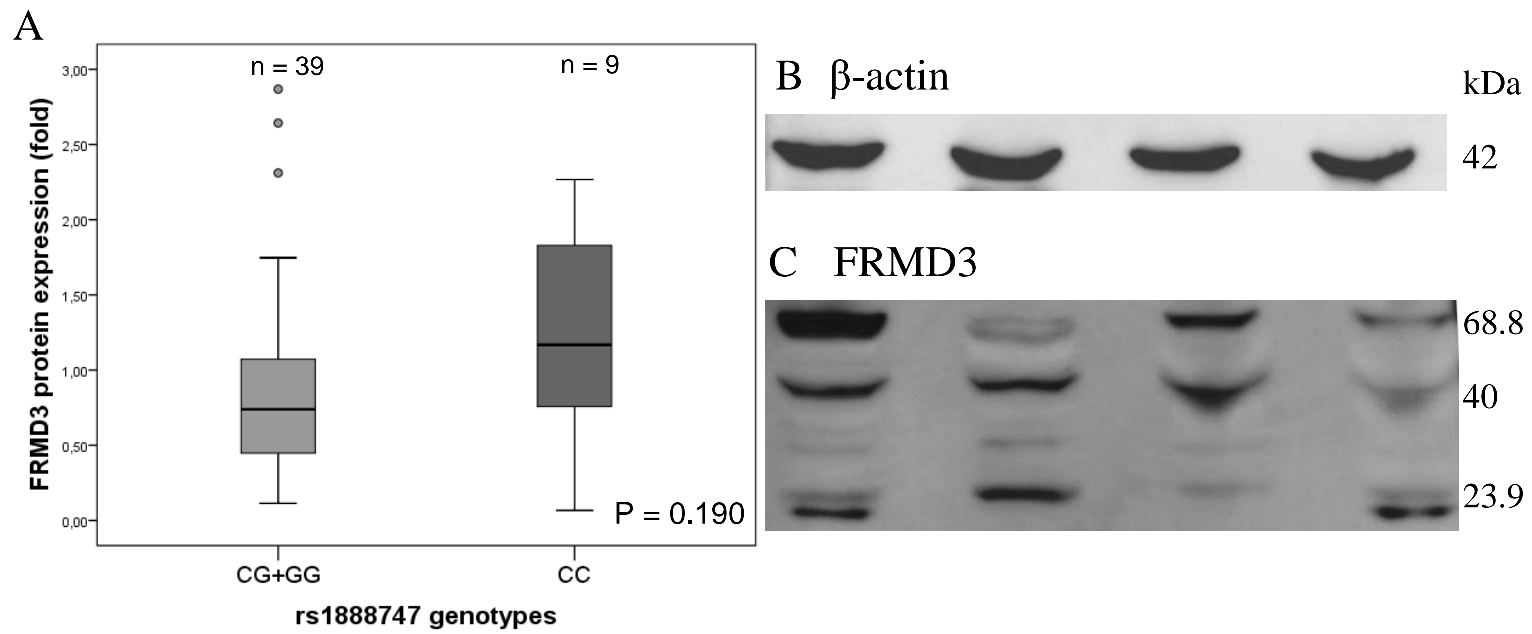

Figure 2

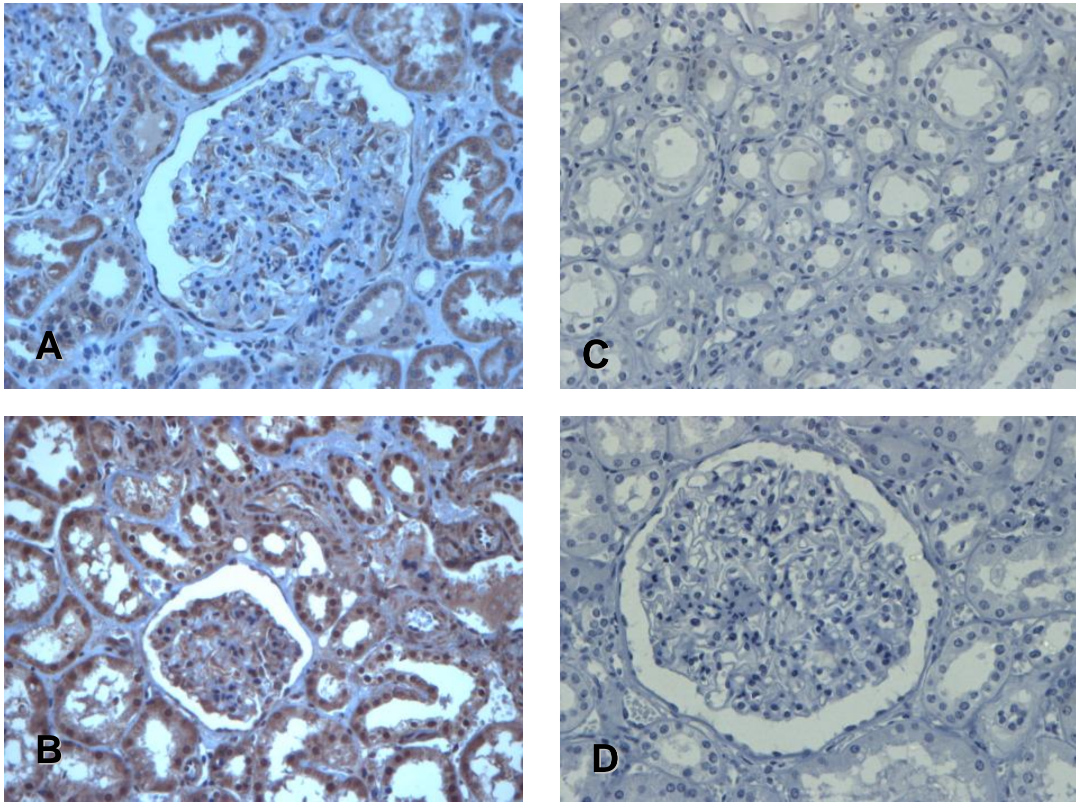

Figure 3

Supplementary material

Table S1

Polymorphism	**Alelle**	**Chromossome**	**Near gene**
rs39075	A/G	7p	CHN2
rs1888747	C/G	9q	FRMD3
rs451041	G/A	11q	CARS
rs9521445	C/A	13q	__
rs1041466	A/G	13q	__
rs1411766	G/A	13q	__
rs6492208	C/T	13q	__

Table S2

rs39075	F 5' - CCCAAGGTGCCAAGGATGA - 3' R 5' - GCTGTTAGCAAAGAAAGCACTTCA - 3' VIC 5' - CAGTACCGAAACAGC - 3' FAM 5' - CAGTACCAAAACAGC - 3'
rs451041	F 5' - CTCCCACCTGCCAAGTAGTG - 3' R 5' - GGCTCATTTCATTAAGCGTCAGAA - 3' VIC 5' - AAGGCCTAACATGATC - 3' FAM 5' - AAGGCCTAACGTGATC - 3'
rs9521445	F 5' - CCTTACTCTCTAGCCCCAAGTTGA - 3' R 5' - GACTATTCAGACGAATCTGGACACA - 3' VIC 5' - TGGAAGTGCAAGGTTA - 3' FAM 5' - CTTGGAAGTTCAAGGTTA - 3'
rs1041466	F 5' - GCATTTTTATTGACTGGGACAGCTT - 3' R 5' - CTCAGTGACTACAGAAATTACCAGGAA - 3' VIC 5' - AAGCACTGTCATTACC - 3' FAM 5' - AAGCACTGTCGTTACC - 3'
rs1411766	F 5' - CACTTCTCTTCCTCTGTCTCCAAAA - 3' R 5' – CCTAGGAAAAGGCGGCTAGTTG - 3' VIC 5' - CATTCGTGCGATTCAG - 3' FAM 5' - TCATTCGTGTGATTCAG - 3'
rs6492208	F 5' - CCACACCATGCCAACTACTTCAT - 3' R 5' - CCGACTAAATCAGTGTTCATGACAAC - 3' VIC 5' - TGTTGATAATGCACTTGACAAT - 3' FAM 5' - TTGATAATGCACCTGACAAT - 3'
rs1888747	F 5' - TCACCTGGATTGAACTACCCAATG - 3' R 5' - GAGTTCATTCTTTGAGTTCTTCCTAATCTTCATA - 3' VIC 5' - CAATGGCCAGTTGGTTT - 3' FAM 5' - AATGGCCACTTGGTTT - 3'
FRMD3 gene*	F 5' - TTTTCCCCAAGCAGTCACA- 3' R 5' - TGCCCCCTGAGTTCATTTT- 3'
Cyclophilin gene*	F 5' - GCCGATGACGACCCTTG- 3' R 5' - TGCCGCCAGTGCCATTATG- 3'

F, forward primer; R, reverse primer.

* Primers were designed using published human gene sequences and Primer Express 3.0 software (Life Technologies - Applied Biosystems) and projected to target two consecutive exons, so as to prevent the amplification of any contaminating genomic DNA.

Table S3

	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 S4

	C/C n=18	C/G n=35	G/G n=87	P
Age (years)	48.50±19	58.50±20	60±20	0.326
Male Gender (%)	5 (27.8)	19 (54.3)	43 (49.4)	0.170
Hypertension (%)	12 (66.7)	17 (48.6)	49 (56.3)	0.478
Body Mass Index (%)	27.91±4.85	30.0±8.92	26.84±5.65	0.389
Diabetes (%)	5 (27.8)	8 (22.9)	29 (33.3)	0.536

Data expressed as mean ± SD or number of cases (%).

Perspectivas

Estudo de outros fatores que possam influenciar a expressão do *FRMD3* e de outros genes já descritos como relacionados à doença renal do diabetes. Tais fatores englobam os epigenéticos, que referem-se à padrões hereditários de expressão gênica que não dependem da informação na sequência de DNA, tais como mudanças na metilação de DNA, que possam modificar a regulação gênica.