Disease Markers 27 (2009) 29–35
DOI 10.3233/DMA-2009-0648
IOS Press

*Xba*I GLUT1 gene polymorphism and the risk of type 2 diabetes with nephropathy

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Abstract. Altered expression of the facilitated glucose transporter GLUT1 affects pathways implicated in the pathogenesis of diabetic nephropathy. There is indication that variation of *GLUT1* gene (*SLC2A1*) contributes to development of microangiopathy in diabetes mellitus type 2 (DM) patients. A genetic association study involving Caucasians was carried out to investigate the role of *Xba*I polymorphism in the *GLUT1* gene in diabetic nephropathy (DN). Study population ($n = 240$) consisted of 148 unrelated patients with DM (92 cases with diabetic nephropathy (DN)), and of 92 matched healthy control subjects. Diabetic nephropathy was defined as persistent albuminuria (> 300 mg/24 h) and/or renal failure, in the absence of non-diabetes induced renal disease. The analysis showed that the risk of developing DM and DN in *XbaI*$(-)$ carriers, when healthy individuals were considered as controls, was two-fold: odds ratio (OR) 2.08 [95% confidence interval (1.14–3.79)]. However, there was no evidence of association between *XbaI*$(-)$ and DN when patients with DM and without DN were considered as controls: OR $=$ 1.12 (0.55–2.26). Thus, the *GLUT1 XbaI*$(-)$ allele is associated with DM, and possibly with a more severe form of the disease that can lead to development of DN.

Keywords: Diabetes type 2, diabetic microangiopathy, diabetic nephropathy, GLUT1, polymorphism

1. Introduction

Diabetic microangiopathic complications, as diabetic nephropathy (DN) or diabetic retinopathy, are major causes of morbidity and mortality in patients with type 2 diabetes mellitus (DM). In industrialized countries, diabetic nephropathy has become the most frequent cause of end-stage renal disease [1] requiring chronic renal replacement therapy.

Since the publication of the Diabetes Control and Complications Trial [2] in the early 1990s, hyperglycemia remains incontrovertibly the most important factor for the development of diabetic microangiopathy. However, there is strong evidence for the involvement of genetic factors in its pathophysiology. Not all diabetic patients develop microangiopathy, indicating specific, genetically-defined predisposing factors [3]. Furthermore, significant familiar clustering of diabetic nephropathy [4,5] has been shown in type 2 DM and in different populations. It is therefore postulated that investigation of genetic susceptibility to diabetic nephropathy will elucidate the pathogenesis of renal and retinal involvement in diabetes.

In a survey of all published association studies on the relation between the *Xba*I *GLUT1* gene (*SLC2A1*) polymorphism and the risk of DN in type 1 and type 2 DM, a meta-analysis and subsequent sensitivity analyses supported an association between the allele*Xba*I (–) and DN [6]. However, individual studies reported

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ISSN 0278-0240/09/$17.00

Table 1
Profile of all participants grouped according to type 2 diabetes mellitus (DM) and nephropathy (DN) status. Values are mean ± SD

Study groups Variables	Type 2 DM	Healthy controls	*P	DM-DN	DM+DN	#P	¶P
N	148	92	n.a.	56	92	n.a.	n.a.
Sex (m/f)	81/67	57/35	0.	32/24	49/43	0.	0.734
Age (years)	69 ± 10	69 ± 11	0.785	70 ± 12	69 ± 10	0.855	0.278
BMI (kgr/m^2)	26.2 ± 4.2	26.0 ± 3.6	0.802	25.4 ± 3.7	26.8 ± 4.5	0.224	0.036
Hypertension (%)	57.4	0	n.a.	55.4	58.7	n.a.	0.733
DM duration (years)	19.8 ± 6.7	n.a.	n.a.	20.2 ± 5.8	19.5 ± 7.2	n.a.	0.079
HbA1c (%)	7.21 ± 1.03	n.a.	n.a.	6.81 ± 0.87	7.44 ± 1.04	n.a.	0.001
Albuminuria (mg/d)	497 ± 713	13.4 ± 6.3	0.001	14.9 ± 9.1	625 ± 768	0.001	0.001
Creatinine (mg/dl)	1.56 ± 1.20	0.77 ± 0.15	0.001	0.94 ± 0.38	1.98 ± 1.37	0.001	0.001
Urea (mg/dl)	65 ± 44	30 ± 8	0.001	39 ± 16	83 ± 48	0.001	0.001

n.a.: not applicable, DM = Diabetes mellitus,
DM + DN = Diabetes type 2 with diabetic nephropathy, DM–DN = Diabetes type 2 without diabetic nephropathy.
* means p values on comparison of the Type 2 DM group with healthy controls,
# means p values on comparison of the DM + DN group with healthy controls,
¶ means p values on comparison of the DM + DN group with the DM–DN group.

marginal or even controversial associations, particularly in the subgroup of studies concerning type 2 DM [7–9]. Therefore, we performed a new case-control analysis in order to replicate [10,11] the association described in the above meta-analysis.

The study presented was designed to investigate the potential association of the *Xbα*I polymorphism of the GLUT1 gene with diabetic nephropathy in a group of patients with type 2 DM from Greece, which is a Mediterranean country with a racially homogeneous population of Caucasian origin.

2. Methods

2.1. Participants

The case-control study was conducted in the University Hospital of Larissa, Thessaly, Greece. The local ethical committee approved the study protocol and all participants signed an informed consent before enrolment. The study conformed with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

The study population ($n = 240$, 138 males, 102 females; mean age 69.4 ± 10.5) consisted of 148 unrelated patients with type 2 DM (81 males, 67 females; mean age 69.5 ± 10.4 years) and of 92 randomly selected apparently healthy control subjects with no family history of DM (57 males, 35 females; mean age 69.2 ± 10.8 years).

Both control and diabetic groups resided during the study in the same region in central Greece (Thessaly). They were recruited from patients attending the Clinics of Nephrology and Internal Medicine at the University Hospital of Larissa between January 2005 and June 2005. The characteristics of participants are described in Table 1. For the evaluation, type 2 DM patients with DN ($n = 92$) were matched according to age and body mass index (BMI) to patients without any microangiopathic complications (diseased controls; $n = 56$) and to healthy controls ($n = 92$) respectively.

Diagnosis of type 2 DM was confirmed according to the American Diabetes Association (ADA) criteria of 2003 [12]. Type 2 DM with DN (DM + DN) was diagnosed on the basis of a persistent albuminuria, urinary albumin excretion > 300 mg/24 h (> 200 μg/min; representing an overt glomerular proteinuria) with or without elevated serum creatinine levels and in the absence of clinical or radiological evidence of non-diabetic renal disease. Patients with microalbuminuria, i.e. urinary albumin excretion 30–300 mg/24 h (20–200 μg/min), were excluded. Although, microalbuminuria may be an early finding in diabetic nephropathy it is not invariably equivalent to it. Patients with type 2 DM were classified free of DN (DM–DN) if their albumin excretion rate were < 20 mg/24 h (<15 μg/min) and serum creatinine concentration within normal range in at least two examinations. In all patients of this group duration of DM was greater than 15 years.

Each subject had a standardized physical examination and provided a history especially regarding diagnosis, treatment, and complications of DM. The presence of hypertension was not an exclusion criterion. Variables known to be associated with raised urinary albumin concentration including hypertension, cardiovascular disease and glycosylated hemoglobin (HbA1c) were registered. A blood sample for biochemical mea-

surements and DNA extraction was taken from each individual.

2.2. Analytical procedures

DNA was extracted from peripheral blood monocytes by standard methods. Genomic DNA suspended in 10 mm Tris-HCl, 1 mm ethylenediaminetetraacetic acid, pH 8.0, and the concentration was measured by spectrophotometry.

On restriction enzyme analysis of the human *GLUT1* genomic clone (*SLC2A1*), the polymorphic *Xba*I site has been localized in the intron 2 of the gene (rs841853). It represents a transversion of guanine (G) to thymine (T) which abolishes the *Xba*I recognition site in the mutant form. For polymerase chain reaction (PCR) amplification a 5' primer (TGT GCA ACC CAT GAG CTA A) and a 3' primer (CCT GGT CTC ATC TGG ATT CT) were applied. A 1.1 kb DNA fragment of the intron 2 of GLUT1 including the polymorphic *Xba*I site was the PCR product [13]. After 30 cycles of amplification consisting of denaturation at 94 °C for one minute, annealing at 55 °C for one minute, extension at 72 °C for 1.5 minutes, the PCR products were digested with *Xba*I restriction enzyme and electrophoresed on a 1.2% agarose gel. Finally the *Xba*I Restriction Fragment Length Polymorphism (RFLP) was detected by ethidium bromide staining. A 1.1 kb band corresponded to the *XbaI*(−) allele and a set of 0.9 and 0.2 kb bands corresponded to the *XbaI*(+) allele.

2.3. Statistical methods

For inter-group comparisons of continuous variables, i.e. between patients with type 2 DM and DN (DM + DN), patients with type 2 DM without DN (DM–DN), and healthy controls, the Mann-Whitney U test was applied. An initial exploratory analysis for differences in the genotype distributions among the three groups was carried out by comparing the genotype distributions with a chi-squared test. Findings from the initial analysis were followed by testing for the presence of association between the *XbaI*(−) alleles (number of alleles or *XbaI*(−) allele carriership) and diseased group with the use of the Fisher's exact test, and the association was expressed as an odds ratio (OR) with the corresponding 95% confidence interval (95% CI). Adjusted OR by sex, age and body mass index (BMI; kg/m^2) and additionally, if applicable, by diabetes duration was also considered using multiple logistic regression. A result with $p < 0.05$ was considered statistically significant. An exact test according to Weir was used in order to test whether the frequency distribution of genotypes in the control group was in Hardy-Weinberg equilibrium (HWE) (P$\geqslant$ 0.05) [14]. Analysis was performed using SPSS v13, and the HWE was tested using Compaq Visual Fortran90 [6,15].

3. Results

The *GLUT1 Xba*I genotype distribution of the three groups (DM + DN, DM–DN and healthy controls) is shown in Table 2. The genotype distribution of *Xba*I polymorphism was in HWE in the healthy controls group ($p = 0.231$). The initial exploratory analysis of genotype distributions comparisons showed that there was significant difference between the DM + DN group and healthy controls ($p = 0.046$) and between the group of diseased controls (DM–DN) and healthy controls ($p = 0.044$) whereas, this difference was not significant between the DM + DN and the DM–DN groups ($p =$ 0.433) (Table 2).

When the healthy controls were considered in investigating the association of the allele *XbaI*(−) and the risk of developing DM + DN, the analysis showed a non-significant result ($p = 0.083$) relative to the allele *XbaI*(+), and the OR was 1.49 [95% CI(0.97–2.28)]. However, there was evidence of association of *XbaI*(–) carriers with the risk of DM + DN relative to *XbaI*(–) non-carriers ($p = 0.024$), and the OR was 2.08 [95% CI(1.14–3.79)]. The OR adjusted by sex, age and body mass index (BMI; kg/m^2) was significant ($p = 0.039$) and similar to the unadjusted OR: ORadjusted = 1.92 [95% CI(1.03–3.56)] (Table 2). In addition, an increased risk of DM + DN was found for heterozygous *XbaI*(–) carriers relative to *XbaI*(–) non-carriers ($p =$ 0.017): OR = 2.22 [95% CI(1.17–4.19)], ORadjusted = 2.20 [95% CI(1.12–4.32)].

When the diseased controls (DM–DN) were considered, then, there was no significant ($p = 0.463$) association between alleles and the risk of developing DN: OR = 1.22 [95% CI(0.75–1.98)]. In addition, there was lack ($p = 0.857$) of association between *XbaI*(–) carriers and DN: OR = 1.12 [95% CI(0.55–2.26)]. The OR adjusted by sex, age, body mass index (BMI; kg/m^2) and type 2 DM duration was similar to the unadjusted: ORadjusted = 1.09 [95% CI(0.53–2.23)] (Table 2).

The comparison between the DM–DN group and healthy controls yielded non-significant results, nullifying the positive finding from the exploratory analysis. There was lack of association between *XbaI*(–) alleles

Table 2
The distribution of the GLUT1 XbαI genotypes (A) and the XbαI alleles (B) for the three groups of patients, namely patients with type 2 diabetes and diabetic nephropathy (DM + DN), patients with type 2 diabetes without nephropathy (DM–DN) and healthy controls are shown. P-values and Odds Ratios (ORs) with 95% Confidence Intervals (CIs) are provided, when applicable

	Distribution of *GLUT1 Xba* I genotypes					
	*Xba*I (+/+)	*Xba*I (+/–)	*Xba*I (–/–)	*Comparison* (*p- value; OR*(*95% CI*))		
	N(%)			DM + DN vs Healthy controls	DM + DN vs DM−DN	DM − DN vs Healthy controls
DM + DN	29 (31.5)	50 (54.3)	13 (14.1)			
DM –DN	19 (33.9)	33 (58.9)	4 (7.1)			
Healthy controls	45 (49.0)	35 (38.0)	12 (13.0)	0.046 (NA)[a]	0.433 (NA)[a]	0.044 (NA)[a]
	non *XbaI* (–) carriers		*Xba* I (–) carriers			
	N(%)					
DM + DN	29 (31.5)		63 (68.5)			
DM –DN	19 (33.9)		37 (66.1)			
Healthy controls	45 (48.9)		47 (51.1)	0.024; 2.08 (1.14–3.79)[b]	0.857; 1.12 (0.55–2.26)[b]	0.090; 1.86 (0.94–3.71)[b]
	Distribution of *GLUT1 Xba* I alleles					
	Allele *Xba* I (+)		Allele *Xba* I (–)			
	N(%)					
DM + DN	108 (58.7)		76 (41.3)			
DM –DN	71 (63.4)		41 (36.6)			
Healthy controls	125 (67.9)		59 (32.1)	0.083; 1.49 (0.97–2.28)[b]	0.463; 1.22 (0.75–1.98)[b]	0.449; 1.22(0.75–2.00)[b]

NA: non-applicable.
[a]p-values for comparison of genotype distributions.
[b]p-values and corresponding ORs with 95%CIs.

or *XbaI*(–) allele carriership and the risk of developing DM: OR = 1.86 [95% CI(0.94–3.71)] and OR = 1.22 [95% CI(0.75–2.00)], respectively. Additionally, the heterozygous XbaI(−) carriers were not at an increased risk of developing DM compared to *XbaI*(–) non-carriers ($p = 0.102$): OR = 2.83 [95% CI(0.82–9.65)].

A secondary, probably data-driven result of our study was a statistically significant association between the *XbaI*(–) carriage and the presence of arterial hypertension in type 2 DM patients: OR 2.01 [95% CI(1.01–4.04)], $p = 0.053$. In investigating the risk of developing DN (DM +DN vs DM–DN), we used a subset analysis for patients with and without hypertension. Carriers of the *XbaI*(−) allele were not at increased risk of developing DN, independent of presence of hypertension: OR = 1.19 [95% CI(0.47–3.04)] and OR = 1.22 [95% CI(0.40–3.72)], for hypertensive and normotensive patients, respectively.

4. Discussion

The glucose transporter GLUT1 is the most important representative of the family of facilitative glucose transporters in glomerular mesangial cells. Its expression on the cell surface is probably pivotal in raising intracellular glucose levels in DM [16,17]. In mesangial cells elevated intracellular glucose as a result of DM is thought to affect a number of cellular pathways known to be involved in cellular growth and in the accumulation of the extracellular matrix [18–20]. Exactly these pathological changes are central factors in the pathogenesis of diabetic nephropathy. From this perspective it becomes clear that the activity of glucose transporter GLUT1 on the cell surface of the mesangial cells may be rate limiting for the development of the pathological changes and in the pathogenesis of DN [20–22]. Glucose transport across the blood retina barrier (endothelial cells or pigment epithelial cells) is also mediated by GLUT1 [23].

Taking these experimental findings into account it is plausible to choose *GLUT1* as a candidate gene to study the risk of diabetic microangiopathy or more specific of DN. Three previous case-control studies on the relationship of the *Xba*I *GLUT1* gene polymorphism with the risk of DN in type 2 DM rendered contradictory results [7–9]: the allele *Xb*αI(-) was shown either to be a risk [7], or neutral [8], or even protective for the development of the disease [9].

In our study, when the healthy controls were considered, there was a significant association of *XbaI*(−) carriers with the risk of DM + DN ($p = 0.024$) relative to *XbaI*(−) non-carriers, and the OR was 2.08 [95% CI(1.14–3.79)]. When diseased controls were considered as a comparison group, no significant association between carriership of the *XbaI*(−) allele and the risk of developing DN was detected. Additionally, the com-

parison of *XbaI*(−) alleles and *XbaI*(−) allele carriership between the DM–DN group and healthy controls did not show any significant association. Taken these results together, the *XbaI*(−) allele is associated with the development of DM leading to DN (DM + DN vs healthy controls); however, no association is evident with the development of DN (DM + DN vs DM–DN) or susceptibility to DM alone (DM–DN vs healthy controls).

Previous reports had supported a role of the *GLUT1 XbaI* polymorphism in predisposition to risk of type 2 DM [24,25]. Additionally, the meta-analysis previously conducted by our group, comparing DM + DN and DM–DN groups in order to explore the association between *GLUT1 XbaI* polymorphism and the risk of DN, supported an association [6]. The negative results of this study could be attributed to a limited statistical power to detect modest genetic effects, given the available sample size. Nevertheless, the positive association with the development of DM + DN raises the hypothesis the *XbaI*(−) might be associated with a more severe form of DM that ultimately leads to the development of DN. The validity of this hypothesis could be further enlightened by the construction of a diseased controls group (DM–DN) with the patients who had long history of poorly controlled DM. Since well-controlled DM is rarely associated with diabetic microvascular complications [26], the removal of these patients would leave only the most informative diseased controls in the study, i.e. patients at risk of DN who do not develop it. Given the retrospective nature of our case-control comparison, longitudinal data on the level of glycemic control were not available. Nevertheless, previous observations report suboptimal management of DM in the Greek population [27].

The*XbaI* polymorphism is located on the second intron of the *GLUT1* gene and it cannot possibly cause changes in the protein sequence. Therefore, it may be readily assumed that the *Xba*I polymorphism is in linkage disequilibrium with another locus which does have functional implications at the protein level and might play an etiopathogenic role in disease. In this case, the difference in haplotype structure, based on the linkage disequilibrium around *Xba*I, may explain the different results in the studies of different populations.

The involvement of GLUT1 transporter in the pathogenesis of DN may be independent from hyperglycemia. Over-expression of GLUT1 in mesangial cells can lead in matrix accumulation even in the absence of enhanced glucose levels in the medium [17]. Previous reports describe some cases of diabetic glomerulosclerosis without DM, also called "idiopathic nodular glomerulosclerosis" [28–31]. In fact idiopathic nodular glomerulosclerosis has been closely associated with hypertension and cigarette smoking and sometimes diabetic retinopathy was also present [30]. Although idiopathic nodular glomerulosclerosis is a very rare condition (biopsy incidence 0.45%) [28], its existence shows that albuminuria and/or nodular glomerulosclerosis can be developed without the presence of DM. These data provide justification for using both diseased [32,33] and healthy control subjects in genetic association studies for diabetic complications [34].

Another explanation could be the confounding existence of non-diabetic renal disease among cases considered to suffer from DN according to clinical criteria, i.e. patients with DM and proteinuria or renal failure. In particular, hypertensive nephroangiosclerosis can be difficult to differentiate from nodular glomerulosclerosis in patients with type 2 DM who very frequently suffer from longstanding hypertension. Hypertension is highly prevalent in DM and was present in >50% of our cases with type 2 DM (Table 1). In fact, considering the presence of arterial hypertension in our study, an interesting secondary finding resulted. Among patients with type 2 DM, *XbaI*(−) carriers had a marginally significant increased risk for hypertension relative to XbaI(−) non-carriers: OR 2.01 [95% CI(1.01–4.04)], $p = 0.053$. Genetic factors have been long postulated to explain the mechanisms for development of hypertension in type 2 DM [35]. Hypertension is significantly more prevalent in patients with type 2 DM than in the general population [35] and blood pressure shows a linear upward trend from normal glucose metabolism to DM [36]. Furthermore, DN is closely associated with hypertension in type 1 and 2 DM. Therefore, factors (e.g. genes) predisposing to hypertension may be also involved in the pathogenesis of DN and vice versa.

Nevertheless, the association between genetic markers and hypertension in type 2 DM has only scarcely been investigated, most probably because the majority of studies have focused on DN. Only in one previous study, a weak association between the homozygosity for the *XbaI*(−) allele of the *GLUT1* gene and the presence of high diastolic blood pressure in type 2 DM patients was reported [37]. Although our data show a marginal association of the *XbaI*(−) allele with hypertension, this finding is only hypothesis-generating and warrants further investigation in future studies.

In conclusion, this study in a Mediterranean Caucasian population provides some evidence that the

GLUT1 *XbaI*(−) polymorphism is associated with type 2 DM and possibly with a severe form that leads to the development of DN. In addition, there is indication that the *XbaI*(−) carriage may be related to hypertension in type 2 DM. The above findings reinforce the need of additional association studies in order to clarify the role of the *GLUT1* gene in susceptibility to DN.

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