ORIGINAL PAPER

Association of +35A/C (intron3/exon3) polymorphism in SOD1-gene with diabetic nephropathy in type 1 diabetes

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Abstract

Diabetic nephropathy is a major complication of type 1 diabetes whose pathogenesis is insufficiently known, but oxidative stress and genetic susceptibility seem to be involved. The purpose of this study is to assess the possible association of +35A/C (rs2234694) polymorphism in SOD1-gene with advanced stages of diabetic nephropathy in patients with type 1 diabetes in Romania. There have been enrolled 238 unrelated patients, having type 1 diabetes, divided into group A (106 patients) with diabetic nephropathy – macroalbuminuria or ESRD (End Stage Renal Disease) and group B (132 patients) without diabetic nephropathy. The genomic DNA was extracted from the peripheral venous blood and the genotyping of +35A/C (rs2234694) polymorphism has been made using the PCR–RFLP technique. The statistical analysis has been made using De Finetti's program. There has not been a significant deviation from the Hardy–Weinberg equilibrium for any group (p=0.229 and p=0.894, respectively). The data analysis revealed that the presence of a C-allele confers a significant risk (p=0.008) for the advanced diabetes nephropathy (OR=4.940, 95% C.I.=1.341–18.198), and the CA-genotype (p=0.015) confers a little lower risk (OR=4.491, 95% C.I.=1.203–16.766). This study shows the association of a mutant C-allele of rs2234694 polymorphism in SOD1-gene with the advanced stages of diabetic nephropathy in patients with type 1 diabetes in Romania, suggesting the involvement of the defense against oxidative stress, as an important link in the pathogeny of diabetic nephropathy.

Keywords: SOD1, +35A/C polymorphism, diabetic nephropathy, type 1 diabetes, rs2234694.

Introduction

Diabetic nephropathy (DN) is developing in approximately 15–33% of type 1 diabetic patients. Oxidative stress is a key-component in developing this complication [1]. In diabetes mellitus, there are many ways to produce ROS (Reactive Oxygen Species) [2, 3]. At the same time, ROS may activate the formation of AGE [4], polyol pathway [5], Protein Kinase C (PKC), [6] NF-kB [7], p38MAPK and Jak/STAT, leading to endothelial dysfunction, trigger of cytokine and growth factors release [2], mesangial proliferation, glomerular basement membrane thickening [8], tubular cell apoptosis [9], podocyte apoptosis [10] and fibrosis. Moreover, the unified theory states that chronic hyperglycemia is responsible for producing mitochondrial dysfunction with ROS superproduction, which is the triggering factor of these processes [11]. A vicious circle is formed in which the antioxidant defense plays an important role, protecting against diabetes mellitus complications [12].

Probably the most important free radical scavenger enzyme is superoxide dismutase (SOD). Superoxide dismutase 1 (SOD1) is located at the cytosol level [13, 14] and represents between 50% and 80% of the total SOD activity [15, 16]. SOD1 is a key enzyme in diabetic nephropathy because its renal level is decreased in this disease [17], SOD1-deficient animal models develop accelerated kidney lesions [18] and the treatment with a SOD1-mimetics leads to the suppression of albuminuria [18] and improvement of nephropathic lesions [17].

Involvement of low levels of SOD1 in diabetic nephropathy, the existence of some polymorphisms which diminish SOD1-activity and the evidence of genetic susceptibility for diabetic kidney disease [19–22], render the study of SOD1-gene functional mutations as risk factors for diabetic nephropathy, an interesting one.

SOD1-gene has five exons and the +35A/C polymorphism (rs2234694) is adjacent to the splicing point (exon3/intron3) [23], being related to the SOD1-activity – AA-genotype having the higher SOD1-activity [25].

The purpose of this study is to assess the possible

association of +35A/C (rs2234694) polymorphism with the advanced stages of diabetic nephropathy in patients with type 1 diabetes in Romania.

Material and Methods

Two hundred and thirty-eight unrelated patients with type 1 diabetes were enrolled in the study. The enrollment in the study was made by obtaining the informed consent of patients, in compliance with the Declaration of Helsinki. The patients were divided in group A (106 patients) having diabetic nephropathy – macroalbuminuria or ESRD (End Stage Renal Disease), and group B (132 patients) which had diabetes with an evolution of over 20 years, without diabetic nephropathy. The diagnosis of type 1 diabetes was confirmed by determining the C-peptide (<0.3 nmol/L). In addition, for all the patients, the treatment with insulin has been initiated in the first 12 months from the diagnosis of diabetes and the diabetes onset was through ketoacidotic coma. The patients were included in the group with diabetic nephropathy if they showed a glomerular filtration rate of ≤59 mL/min./1.73 m^2 and albuminuria >300 mg/L in the first morning urine. Those from the control group showed a glomerular filtration rate of >90 mL/min./ 1.73 m^2 and less than 120 mL/min./1.73 m^2.

The genomic DNA was extracted from the patients' peripheral venous blood, using Promega Wizard Isolation Kit. The genotyping of +35A/C (rs2234694) polymorphism was made using the PCR-RFLP technique. A fragment of 277 bp was amplified, using the following primers, previously used by Young RP *et al.* (Table 1) [26].

Table 1 – ***Components of PCR reactions for +35A/C polymorphism in SOD1-gene***

Polymorphism	Primers	Restriction conditions	Electrophoresis
+35A/C (exon3/intron3)	F 5-CTATCCAGAAAACACGGTGG GCC-3 R 5-TCTATAT TCAAT CAAATG CTACAAAACC-3	*Hhal* (37^{0}C)	8% Polyacrylamide

The mixture used for PCR-reaction contained: 1 µL genomic DNA, PCR Buffer (2X) 5 µl, dNTP 0.4 µL, primes F and R of 0.05 µL each, Taq polymerase 0.1 µL, water 3.6 µL, working in a final volume of 10 µL. PCR was made with the help of a Corbett Research CGI–96 appliance. PCR had a stage of initial denaturation of 2 minutes at 95^{0}C followed by 32 cycles – 40 seconds at 94^{0}C, 40 seconds at 55^{0}C, 40 seconds at 72^{0}C, and ended by a final elongation of 1 minute at 72^{0}C.

Amplicons of 277 bp in size were verified through electrophoresis in (2%) agarose gel, and then restricted using 5 U/reaction of *HhaI* enzyme (Fermentas). The enzymatic digestion lasted three hours at 37^{0}C. Restriction fragments were visualized under UV light, after the electrophoresis in 8% polyacrylamide gel and impregnation.

The first stage of the statistical analysis of genotype distribution in the two groups was the testing concerning the deviation from Hardy–Weinberg equilibrium using „Pearson's chi-square test", calculating also the "inbreeding coefficient" (F) for the population in the two groups. Then, odds ratio (OR) and 95% confidence intervals (95% C.I.) were calculated starting from the contingency tables, in order to assess the association between GPX-1 genotypes and diabetic nephropathy. At the end of the analysis, with the help of contingency tables, the Cochran–Armitage Test was performed [27, 28] to raise awareness of χ^2-test. The calculations for the case-control study were made using De Finetti's program. In all cases, the *p*-values considered to be statistically significant were <0.05.

Results

The clinical data of patients enrolled in the study are shown in Table 2. The average age of patients in the group of diabetic nephropathy cases was of 36.8 years (SD =±2.5), and that in the control group was of 38.3 years (SD=±-1.8), with a significant difference at *t*-test in favor of those in the control group, who were older, on the average, with 1.5 years. There has not been any significant difference between the two groups, with regard to gender or the smoker status.

Table 2 – ***Clinical data of patients enrolled in the study***

Parameter	Group A n=106 (%)	Group B n=132 (%)	*p*
Average age (SD)	36.8 (±2.5)	38.3 (±1.8)	<0.0001
Gender			
Males	46 (43.3)	54 (40.9)	0.6993
Females	60 (56.7)	78 (59.1)	
Status			
Smoker	36 (33.6)	35	0.2129
Non-smoker	70 (66.03)	97	

PCR–RFLP analysis revealed the existence of the three genotypes of +35A/C polymorphism. The presence of the 35C-mutant allele lead to the appearance of a situs of restriction for *HhaI* enzyme in the amplicon, this one being split into two fragments of 71 bp and 206 bp, respectively. The presence of 35A-allele (wt) does not lead to the appearance of the restriction situs, the amplicon remaining at a size of 277 bp (Figure 1).

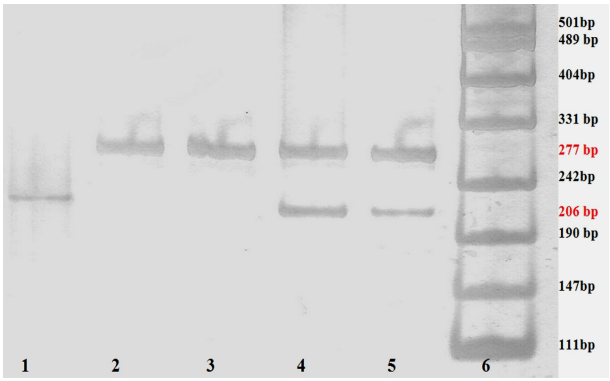

Figure 1 – ***The result of the electrophoresis in 8% polyacrylamide gel and argental coloring for +35A/C polymorphism (line 1 – CC-genotype; lines 2 and 3 – AA-genotype; lines 4 and 5 – AC-genotype; line 6 – Weight DNA Marker).***

When analyzing the Hardy–Weinberg equilibrium we noticed that there is no significant deviation (p=0.894) from the equilibrium, in both group B – patients with type 1 diabetes without diabetic nephropathy, and group A – patients with type 1 diabetes and advanced diabetic nephropathy (p=03229) (Table 3).

Table 3 – ***Distribution of +35A/C genotypes, allelic frequency, inbreeding coefficient and testing for deviation from Hardy–Weinberg equilibrium***

Data Genotype	Number of genotypes obtained in the study	Number of genotypes expected in the study	Genotype frequency in the study (%)	Frequency of C-allele (±SD)	Inbreeding coefficient	*p*
Control group – type 1 diabetes, without diabetic nephropathy						
CC	0	0.02	0.0	0.01 (±0.007)	0.011	0.894
AC	3	2.97	2.27			
AA	128	128.02	96.96			
Group with nephropathy – type 1 diabetes and diabetic nephropathy						
CC	1	0.34	0.94	0.06 (±0.017)	0.116	0.229
AC	10	11.32	9.43			
AA	95	94.34	89.62			

The inbreeding coefficients (0.011 and 0.116) were low within the two groups, an additional argument to validate our groups. The genotype distribution (Table 4) shows that AA-genotype is the most frequent in the studied population, but the frequency is higher in group B – control without diabetic nephropathy (96.96% *vs.* 89.62%). AC genotype is more frequent in group A – patients with diabetic nephropathy (9.43%) in comparison with the control group (2.27%). As for AA-genotype, it was found only in a patient from the group with diabetic nephropathy. The frequency of C-mutant allele was different in the two groups, being higher in the group with diabetic nephropathy (0.06 *vs.* 0.01). As the difference between the allelic frequencies, at the association test, also shows, C-allele seems to confer risk in developing advanced diabetic nephropathy (OR=5.180, 95% C.I.=1.442–18.603), and A-allele seems to be protective (OR=0.193, 95% C.I.=0.054–0.693) (Table 4).

Table 4 – ***The results of the association test of +35A/C polymorphism in SOD1 gene with diabetic nephropathy in type 1 diabetes***

	Difference of allelic frequency	Heterozygosity	Homozygosity	Allelic positivity	Odds Ratio corrected
			A-risk allele		
	[C]<->[A]	*[CC]<->[CA]*	*[CC+]<->[AA]*	*[CC]<->[CA+AA]*	
OR	0.193	1.000	0.248	0.267	0.335
95% C.I.	0.054–0.693	0.033–30.618	0.010–6.148	0.011–6.632	
χ^2	7.80	0.29	1.34	1.24	7.06
p	0.00524	0.58786	0.24716	0.26526	0.00790
			C-risk allele		
	[A]<->[C]	*[AA]<->[CA]*	*[AA]<->[CC]*	*[CC+CA]<->[AA]*	
OR	5.180	4.491	4.037	4.940	6.561
95% C.I.	1.442–18.603	1.203–16.766	0.163–100.179	1.341–18.198	7.06
χ^2	7.80	5.86	1.34	6.89	
p	0.00524	0.01549	0.24716	0.00865	0.00790

Note: 35A risk allele–35C allele is considered reference (OR=1) and vice versa. Odds Ratio corrected – after the application of Cochran–Armitage test to establish the trend.

Because of the small number of homozygotes, a correction was made the through Cochran–Armitage test, in order to increase the test sensitivity of χ^2. After this statistical test, the corrected value of $OR_{correctedA}$ is 0.335 (p=0.007), and of $OR_{correctedC}$ is 6.561 (p=0.007), which emphasizes the association of C-mutant allele, +35A/C (rs2234694) polymorphism in SOD1-gene with the advanced stages of diabetic nephropathy.

Discussion

Our results show the association of C-allele of rs2234694 polymorphism with advanced stages of diabetic nephropathy in patients with type 1 diabetes in Romania. The observation of Hardy–Weinberg equilibrium, for both groups and the small enough inbreeding coefficient constitute an additional reason to validate our groups. The significant difference of allelic frequencies at Cochran–Armitage test reveals that these results are probably reproducible in a large size study within the same population.

This is not the first study to investigate the association of +35A/C (rs2234694) polymorphism in SOD1-gene with diabetic nephropathy in type 1 diabetes. A previous similar study assessed rs17880753 polymorphism, which considered it identical with rs2234694 polymorphism [24] studied by us, and has not found its association with diabetic nephropathy – microalbuminuric stage (OR=1.23, 95% C.I.=0.79–1.91; p=0.37) or with the severe nephropathy stage (OR=0.75, 95% C.I.=0.33–1.68; p=0.49), in patients within the DCCT/EDIC-study. This polymorphism was studied in diabetes mellitus, irrespective of the type of diabetes, and no association with microvascular complications was found, but the polymorphism was associated with macrovascular complications in the Czech population [25].

Our results revealed an association of this polymorphism with advanced stages of diabetic nephropathy ($OR_{correctedC}$=6.561; p=0.007), in patients with type 1 diabetes in Romania. Our study does not contradict the results of Al-Kateb H *et al.* [24] or Flekac M *et al.* [25], but some differences of susceptibility may exist depending on the studied population, fact that is encountered in other studies. Furthermore, as we have shown in the introduction, Flekac M *et al.* [25] found a decrease in SOD1-activity in patients with CC-genotype, which may be a possible argument in favor of the functional intervention of this polymorphism in diabetic complications, fact that is confirmed by the same author for the macrovascular complications.

There are many possible mechanisms through which this polymorphism, with its action over the SOD1-activity, may exercise its functional effects for the appearance of diabetic nephropathy in patients with type 1 diabetes: the impossibility of increasing SOD1-activity and expression as a response to NO, the defense against endothelial dysfunction, intervention on apoptosis, anti-hypertensive and anti-fibrotic protection, insulin resistance, diminished protection due to the low birth weight. The decoupling of eNOS and iNOS activation and the production of large quantities of NO lead to Cu/Zn SOD-activation in mesangial cells in order to compensate an endothelial dysfunction [31]. This fact is also confirmed by the increase of SOD1-levels in patients with ESRD where the endothelial dysfunction is marked [32]. In addition, the circulant levels of Cu/Zn SOD in adolescents with type 1 diabetes seem to be protective against endothelial dysfunction, the low SOD1-levels being a susceptibility marker for diabetic vascular complications [33]. Tubular cell and podocyte apoptosis is an early event in diabetic nephropathy, and the simultaneous release of SOD1 and cytochrome C regulates the mitochondrial apoptosis [34]. Hypertension and the rennin–angiotensin system are key factors in diabetic nephropathy, closely related to SOD1-levels [35]. The fibrosis mediated by TGF-β is the corner-stone of glomerulosclerosis in diabetic nephropathy, and SOD1 is a strong antifibrotic agent, lowering the TGF-β_1-expression [36]. The insulin resistance is increased in patients with nephropathy, [37, 38] in inverse ratio to GFR-decrease (glomerular filtration rate) [39] and seems to be the most important predictor for development of diabetic nephropathy [40–42], as early as the diagnosis of patients with type 1 diabetes. Insulin resistance is also related to SOD1, this preventing up to 50% of the reduction of intra cell picking-up of glucose stimulated by insulin [43]. Low birth weight is a risk factor largely discussed in diabetic nephropathy within type 1 diabetes [44]. A possible mechanism through which low birth weight exercises its effects is the low expression of Cu/Zn SOD in these children, which make them susceptible to oxidative stress [45]. It is hard to guess the mechanism through which the decreased SOD1-expression and activity leads to the development of nephropathy, but our results suggest that this polymorphism with functional role in antioxidant defense is associated with diabetic nephropathy.

The previous studies did not show any effect of gender or age on SOD1-activity [25], but there is a modifying factor of SOD1-gene expression – light exercise, which increases the SOD1-expression on animal models [29, 30]. This is also a limitation of the study, which has no data about physical exercise of enrolled patients.

Conclusions

This study shows the association of C-mutant allele of rs2234694 polymorphism in SOD1-gene with advanced stages of diabetic nephropathy in patients with type 1 diabetes in Romania, suggesting the involvement of defense against oxidative stress as an important link in the pathogeny of diabetic nephropathy.

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