Polymorphism of Catalase Gene Promoter in Romanian Patients with Diabetic Kidney Disease and Type 1 Diabetes

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Hyperglycaemia leads to ROS (Reactive oxygen species) generation, affecting the cells that cannot decrease glucose uptake such as: glomerular epithelial cells, mesangial cells and proximal tubule cells. ROS excess seems to activate important pathogenic pathways of development of diabetic nephropathy. The decrease of CAT activity, one of the most important antioxidant enzymes, following to some genetic defects, may be a risk factor for diabetic nephropathy.

The purpose of this study is to investigate the association of 21A/T (rs7943316) polymorphism of CAT gene with advanced diabetic nephropathy in patients with type 1 diabetes in Romania.

There have been studied 238 patients with T1D (type 1 diabetes), divided into the group with diabetic nephropathy (DN) (106 patients) and the group without renal affection (132 patients). The genotyping has been made by using PCR-RFLP technique. The analysis of association has been made by using DeFinetti programme. The value considered significant has been p < 0.05.

There has been a deviation from Hardy-Weinberg equilibrium in the group with diabetic nephropathy (p=0.019), the equilibrium being preserved by the control group (p=0.771). T allele does not confer a risk for advanced diabetic nephropathy (OR_T = 0.757, 95%C.I. = 0.405–1.414; P=0.381), the result being statistically insignificant even taking into consideration the risk allele A (ORA=0.793, 95%C.I. = 0.465–1.350; P=0.392). The results remain concordant too after applying the Cochran – Armitage test.

Our data do not suggest an effect of 21A/T (rs7943316) polymorphism in the susceptibility for diabetic nephropathy in Romanian patients with type 1 diabetes. Further studies are necessary in order to demonstrate or exclude the role of CAT gene in diabetic nephropathy in patients with type 1 diabetes.

Diabetes mellitus leads to microvascular and macrovascular chronic complications. Diabetic nephropathy is an important complication of type 1 diabetes with a prevalence of approximately 15% in patients with type 1 diabetes in Europe [1]. Hyperglycaemia is the leading force of a pathologic process in diabetic nephropathy and despite of a prolonged effect of intensive treatment of hyperglycaemia, [2] the individual variability [3] for developing such complications is not only partly explained.

Hyperglycaemia affects mostly the cells which cannot decrease the glucoses capitation such as: glomerular epithelial cells, mesangial cells and proximal tubular cells [4]. The exposition of such cellular cells to hyperglycemia leads to ROS generation [5–7]. There are many ways of producing ROS in diabetes [8], but the most important seems to be that of mitochondrial electron transport chain [9]. At the same time, hyperglycemia may lead to ROS formation by non-enzymatic glycation of proteins [10][11] self-oxidative glycation [12] C protein activation [13] and the increase of flow by polyl pathway [14]. Irrespective of the mechanism of producing, the increase of secondary oxidative stress of hyperglycaemia leads to activation of important pathogenic pathways which support the oxidative stress and cause diabetic renal diseases, but also the overexpression of scavenger enzymes of free radicals.

ROS excess may seem to activate four important pathogenic pathways of diabetic complications development [15]: polyl pathway [16] formation of final products of glycosylation – AGE [17] and NF-kB [18] hexosamine pathway and C protein kinase (PKC) [19].

Oxidative stress leads also to overexpression and increase of antioxidant enzymes activity, especially CAT and GPX, trying to fight against ROS excess. CAT seems to be strongly activated.
by PKCζ subunit, PKCγ and 34cdc/cyclinaB [20]. However, catalase expression (CAT) in diabetes seems to be low [21]. Data about CAT activity in diabetes mellitus are contradicting, some laboratories have found its activity increased [22] [23] and others found it decreased [24] [25] in animal models. Under the conditions of hyperglycaemia CAT and GPX activity have been decreased in patients with diabetic nephropathy as compared to those with diabetes without nephropathy or with nephropathy without diabetes. In addition, ARNm expression of CAT, CuZnSOD and GPX is lower in PBMCs cells (peripheral blood mononuclear cells) of patients with microvascular complications as compared to those having type 1 diabetes for over 20 years without complications, which have normal antioxidant defence compared with that of non-diabetic patients [27]. CAT overexpression has an antiproliferative effect, delaying G0/G1 progression of cellular cycle [28] [29], NF-kB activation [30] and ROS normalization leads to an improvement of histological lesions in nephropathy. These data confirm the fact that individual susceptibility for diabetic nephropathy and microvascular complications seems to be given by the abnormal response of antioxidant genes to hyperglycaemia and especially of catalase and GPX.

Individual susceptibility in developing diabetic nephropathy caused by the inadequate response of antioxidant genes (especially CAT and GPX) to hyperglycaemia and oxidative stress, corroborated with the proofs concerning the existence of a genetic susceptibility for nephropathy in type 1 diabetes [31–34] as well as with the existence of some possible functional polymorphisms of CAT gene and the antiproliferative activity of this gene makes attractive the study of these polymorphisms as possible risk factors for diabetic nephropathy in type 1 diabetes.

In GENETRANS, CAT human gene (GeneID: 847 or HGNCid 1516) is located 11p13, and has 13 exons [35]. The polymorphisms are associated, in the last years, with the resistance to oxidant substances [36] amyotrophic lateral sclerosis, diabetic nephropathy [37] and vitiligo [38].

The 21A/T (rs7943316) polymorphism situated at the promoter region level, immediately proximal to start situs was less studied [39]. This polymorphism may influence, at least theoretically, the gene expression, whether by its position close to start codon or by its linkage to the interest areas of the promoter where transcription factors are tied up. This polymorphism has never been studied in diabetic nephropathy in patients with type 1 diabetes.

The purpose of this study is to assess the association of 21A/T (rs7943316) polymorphism of CAT gene promoter with diabetic nephropathy in patients with type 1 diabetes in Romania.

MATERIALS AND METHODS

In the study there have been enrolled 238 unrelated patients with type 1 diabetes, after obtaining their informed consent, in compliance with the declaration of Helsinki. They have been divided into group A (106 patients) with diabetic nephropathy – macroalbuinuria or ESRD (End Stage Renal Disease) and group B (132 patients) having diabetes with an evolution for over 20 years, without diabetic kidney disease. 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 since the diagnosis was made, or the onset of diabetes was through ketoacidotic coma. The patients have been included in the group with diabetic nephropathy if they showed a glomerular filtration rate of ≤ 59 ml/min/1.73 m² 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 m² and less than 120 ml/min/1.73 m².

The genomic DNA was extracted from the patients’ peripheral venous blood, using Promega Wizard Isolation Kit. The genotyping of (rs7943316) 21A/T polymorphism of CAT gene promoter has been made by PCR-RFLP technique. It has been amplified a fragment of 277 bp, using the following primers, previously used by Young RP[40]. (Table I).

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primers</th>
<th>Restriction conditions</th>
<th>Electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>21A/T promoter</td>
<td>F 5-AATCAGAAGGCAAGTCTCCC-3 &lt;br&gt; R 5-TGAGGGAGACACAGAGTGAC-3</td>
<td>Hinf I &lt;br&gt; (37°C)</td>
<td>Polyacrylamide 8%</td>
</tr>
</tbody>
</table>
The mixture used for PCR reaction contained: genomic DNA 1 µl, PCR Buffer (2X) 5 µl, dNTP 0.2 µl, primers F and R of 0.05 µl each, Taq polymerase 0.1 µl, water 3.6 µl, working in a final volume of 10 µ. PCR reaction has been made by using a Corbet Research CGI-96 instrument.

PCR program had an initial denaturation of 2 minutes at 95°C followed by 32 cycles –40sec at 94°C, 30sec at 54°C, 40sec at 72°C – and a final elongation of 1 minute at 72 degrees. The size of amplicons (249 bp) has been verified through electrophoresis in agarosis gel (2%) Then each amplicon has been restricted, using 5U/reaction of HinfI (Fermentas) enzyme. The enzymatic digestion lasted 3 hours at 37°C. Restriction fragments have been visualized after the electrophoresis in 8% polyacrylamide gel and argentie staining.

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 for the population in the two groups. Then there were calculated Odds ratio (OR) and confidence intervals 95% starting from the contingency tables, in order to assess the association between CAT genotypes and diabetic nephropathy. At the end of the analysis, with the help of weighed contingency tables, has been made the Cochran – Armitage [41][42] as, because of the small number of patients, χ² test has to be made sensitive. The calculations for the study case-control have been made using De Finetti’s program. In all the cases the P values considered significant from the statistical point of view were < 0.05.

RESULTS

PCR-RFLP analysis showed the presence of all 3 genotypes of 21A/T polymorphism of CAT gene. The presence of the variant “wild type” A leads to the appearance of a restriction situs, the amplicon being digested in fragments of 175bp and 74 bp. The presence of mutant T allele leads to the disappearance of recognition sit for Hinf I, the amplicon remaining complete, with the size of 249 bp. Two supplementary fragments, of 145bp and 104bp, have been identified in polyacrylamide gel in all samples tested (Fig. 1). Following to blast analysis it has been found that primes amplified a duplicated area in a nearby region, which contains a restriction situs for Hinf I enzyme. This situs was monomorphic in our samples and thus it has not influenced our results (Table II).

![Fig. 1. – The result of the electrophoresis in polyacrylamide gel (8%) for 21A/T polymorphism in CAT gene promoter (lines1, 3 – TT; lines 2, 4 – AA; lines 5, 7, 8, 9 – AT; lines 6 – DNA Marker weight).](image)
Table 2
Distribution of genotypes of 21A/T polymorphism, allelic frequency, inbreeding coefficient and testing for the deviation from Hardy-Weinberg equilibrium

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of genotypes obtained in the study</th>
<th>Number of genotypes expected in the study (%)</th>
<th>Frequency of genotypes in the study (%)</th>
<th>Frequency of allele A (+/- SD)</th>
<th>Inbreeding coefficient</th>
<th>Statistical signification p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group – with type 1 diabetes, without diabetic nephropathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>25</td>
<td>24.18</td>
<td>18.93</td>
<td>0.43 (+/-0.031)</td>
<td>0.02526</td>
<td>0.771</td>
</tr>
<tr>
<td>AT</td>
<td>63</td>
<td>64.63</td>
<td>47.72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>44</td>
<td>43.18</td>
<td>33.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group with nephropathy – with type 1 diabetes and diabetic nephropathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>25</td>
<td>19.10</td>
<td>23.58</td>
<td>0.42 (+/-0.038)</td>
<td>0.22769</td>
<td>0.019</td>
</tr>
<tr>
<td>AT</td>
<td>40</td>
<td>51.79</td>
<td>37.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>41</td>
<td>35.10</td>
<td>38.67</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hardy-Weinberg equilibrium analysis stressed that there has not been any deviation of genotypes distribution in the control group (p=0.771), while in the group with patients having advanced diabetic nephropathy there existed a statistically significant deviation of genotypes distribution from the H-W equilibrium state (p=0.019), for which there is no immediate explanation.

The inbreeding coefficient in the control group has been very small (0.025), and in the group with advanced nephropathy has been 0.227. Genotypes distribution (Table III) has not been significantly different between the two groups, even if homozygotes AA and TT have been more frequent in a group with patients having advanced diabetic nephropathy (23.58 vs. 18.93 respectively 18.93 vs. 33.33). Contrarily, heterozygotes AT have been more frequently met in the control group without diabetic nephropathy (47.72 vs. 37.73). As it is shown by the difference among these allelic frequencies this polymorphism is not associated with advanced diabetic nephropathy (p=0.938).

Table III
The results of the association test of 21 A/T polymorphism of CAT gene promoter, with diabetic nephropathy in type 1 diabetes

<table>
<thead>
<tr>
<th>Difference of allelic frequency</th>
<th>Heterozygosis</th>
<th>Homozygosis</th>
<th>Allelic positivity</th>
<th>Odds Ratio corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>[A]&lt;-&gt;[T]</td>
<td>[AA]&lt;-&gt;[AT]</td>
<td>[AA+]&lt;-&gt;[TT]</td>
<td>[AA]&lt;-&gt;[AT+TT]</td>
<td>T risk allele</td>
</tr>
<tr>
<td>OR</td>
<td>1.014</td>
<td>0.635</td>
<td>0.932</td>
<td>0.757</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>0.704–1.462</td>
<td>0.321–1.255</td>
<td>0.463–1.874</td>
<td>0.405–1.414</td>
</tr>
<tr>
<td>Chi²</td>
<td>0.01</td>
<td>1.72</td>
<td>0.04</td>
<td>0.76</td>
</tr>
<tr>
<td>p</td>
<td>0.938</td>
<td>0.190</td>
<td>0.842</td>
<td>0.381</td>
</tr>
<tr>
<td>A risk allele</td>
<td></td>
<td></td>
<td></td>
<td>0.942</td>
</tr>
<tr>
<td>[T]&lt;-&gt;[A]</td>
<td>[TT]&lt;-&gt;[AT]</td>
<td>[TT]&lt;-&gt;[AA]</td>
<td>[AA+AT]&lt;-&gt;[TT]</td>
<td></td>
</tr>
<tr>
<td>OR</td>
<td>0.986</td>
<td>0.681</td>
<td>1.073</td>
<td>0.793</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>0.684–1.421</td>
<td>0.381–1.219</td>
<td>0.534–2.159</td>
<td>0.465–1.350</td>
</tr>
<tr>
<td>Chi²</td>
<td>0.01</td>
<td>1.68</td>
<td>0.04</td>
<td>0.73</td>
</tr>
<tr>
<td>p</td>
<td>0.938</td>
<td>0.195</td>
<td>0.842</td>
<td>0.392</td>
</tr>
</tbody>
</table>

Note: 21T risk allele–21A allele is considered reference (OR=1) and vice versa.
Odds Ratio corrected – after applying Cochran-Armitage test for setting up the trend.

When doing the test of allelic positivity, if we consider A allele as reference (OR=1), the presence of a T allele does not confer risk for developing advanced diabetic nephropathy (OR=0.757, 95% C.I. = 0.405–1.414), the result being statistically insignificant (p=0.381), even if we reverse the reference allele (OR=0.793, 95% C.I. = 0.465–1.350; p=0.392). When doing the heterozygosity test, considering A allele as reference, the presence of TA genotype does not confer significant risk for diabetic nephropathy (OR=0.635; 95% C.I. = 0.321–1.255; p=0.190). In the case of homozygote genotypes, the situation is similar, irrespectively of which allele is considered reference.
Due to the small number of patients, it has been made the correction by using Cochran – Armitage test, to raise the sensitivity of $\chi^2$ test. After this statistical test, the corrected values of OR$_{correctedA}$ and OR$_{correctedT}$ have also shown no association with advanced diabetic nephropathy in the population studied by us.

**DISCUSSION**

Our results show that there is no allele of rs7943316 polymorphism associated with advanced studies of diabetic nephropathy in patients with type 1 diabetes in Romania. The insignificant differences of allelic frequencies in Cochran-Armitage test suggest a raised possibility for these results to be reproducible in a larger study with the same population.

The group with nephropathy was not in Hardy-Weinberg equilibrium, and the breeding coefficient has been surprisingly raised. These observations may be the effect of a selection against rs7943316 genotype(s) (DN is a life threatening disease and the CAT gene may influence the patients life expectancy) or inclusion criteria (recruitment bias). However, the increase of inbreeding coefficient cannot be fully explained by a “selection bias” because an essential condition of subject recruitment was that they are not related.

There is only one study which investigates another polymorphism of CAT gene as possible risk factor for diabetic nephropathy in type 1 diabetes [43]. In the mentioned study it has not been found any association with diabetic nephropathy. This is the first study which investigates the association of 21A/T (rs7943316) polymorphism with diabetic nephropathy in type 1 diabetes. The functional role of this polymorphism is subject of speculations. In present the role of CAT gene in predisposition for diabetic nephropathy may not be denied, even the initial studies indicated that two of its polymorphisms are not associated with the disease. Investigation of other markers from this gene may bring new data regarding the role of CAT in DN.

There are data which show that CAT expression and activity are poor in patients with type 1 diabetes and nephropathy, as compared to those with over 20 years with diabetes, without complications or the patients clinically healthy [26][27]. So, it is possible that this antioxidant enzyme plays an important role in the disease’s pathogeny. Most of the polymorphisms studied by researchers are functional exonic, while our polymorphism is not integrated in this category. The rs7943316 is situated near the start situs and the distance between the attachment elements of transcription factors is small, and it is likely to have a strong linkage with these segments. Also it is possible that this polymorphism has no functional effects, fact which is suggested by the lack of its association with BPCO in smokers of European origin, from the Great Britain [40]. Even if the polymorphism we chose is not the most, there is a study which demonstrates the lack of association of 262C/T polymorphism, also situated at the level of CAT gene promoter, with diabetic nephropathy in patients with type 1 diabetes [43]. This polymorphism is cited as being associated both with basal expression and with the level of enzyme at the level of erythrocytes [44]. The same polymorphism seems to contribute to renal manifestations in erythematous systemic lupus [45] and in type 1 diabetes it seems to have a protective role in nephropathy [37], without having any association with diabetic nephropathy. In addition, polymorphisms of CAT gene promoter seem to be associated with arterial hypertension [46]. So, it is very hard to demonstrate the implication of the polymorphisms of this gene in diabetic nephropathy, but we can speculate its role and the fact that it is possible that studied polymorphisms until present time may not be the most suggestive.

Another possible problem to be considered is the fact that the studies have been made on different populations, and the exclusion of the association of polymorphism with diabetic nephropathy in a population does not exclude its role to another subset of patients, the significant population variations being highly met.

**CONCLUSIONS**

Our data do not suggest an effect of 21A/T (rs7943316) polymorphism in the susceptibility for diabetic nephropathy in type 1 diabetes in the Romanian population. Further studies are necessary in order to demonstrate or exclude a role of CAT gene in diabetic nephropathy in patients with type 1 diabetes.

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Hiperglicemia conduce la generarea de ROS, cu afectarea celulelor care nu-și pot scădea captarea glucozei cum ar fi: celulele epiteliale glomerulare, celulele mezeangiale și celulele tubulare proximale. Excesul de ROS pare să activeze căi patogene importante ale dezvoltării nefropatiei diabetice. Scăderea activității CAT, una din enzimele antioxidante importante, ca urmare a unor defecte genetice, poate constitui un factor de risc pentru nefropatia diabetica.

Scopul acestui studiu este de a investiga asocierea polimorfismului 21A/T (rs7943316), al genei CAT cu nefropatia diabetica avansată, la pacienții cu diabet zaharat tip 1 din România.

Au fost studiați 238 pacienți cu T1DZ, împărțiți în lotul cu ND (106 pacienți) și lotul fără afecțiune renală (132 pacienți). Genotiparea s-a realizat prin tehnica PCR-RFLP. Analiza asocierii a fost realizată cu programul DeFinetti. Valoarea considerată semnificativă a fost p < 0.05.

A existat o deviere de la echilibrului Hardy-Weinberg în lotul de pacienți cu nefropatie diabetică (p=0,771), echilibrul fiind respectat pentru lotul control (p=0,019). Alela T nu conferă risc pentru nefropatia diabetica avansată (OR=0,757, 95%CI=0,405-1,414; P=0,381), rezultatul fiind nesemnificativ statistic chiar considerând factorul de risc alela A (OR=0,793, 95%CI=0,465-1,350; P=0,392).

Rezultatele se mențin concordante și după aplicarea testului Cochran – Armitage.

Datele noastre nu sugerează un efect al polimorfismului 21A/T (rs7943316) în susceptibilitatea pentru nefropatia diabetica, în diabetul zaharat tip 1, la populația din România. Studii viitoare sunt necesare pentru a demonstra sau a exclude un rol al genei CAT în nefropatia diabetica, la pacienții cu diabet tip 1.

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