The 262T>C promoter polymorphism of the catalase gene is associated with diabetic neuropathy in type 1 diabetic Russian patients

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SUMMARY
Objective: Oxidative stress plays an important role in the development of diabetic neuropathy (DN). Antioxidant enzymes reduce enhanced oxidative stress in the peripheral nerve. Genetic variations within the antioxidant genes therefore could be implicated in the pathogenesis of DN.

Methods: Using a PCR-RFLP assay, a total of 216 Russian type 1 diabetic (T1D) patients with DN and 250 T1D individuals without DN have been tested to verify whether the -262T > C and 1167C > T polymorphisms of the catalase (CAT), 197Pro > Leu amino acid substitution of the glutathione peroxidase 1 (GPX1) and +/null polymorphism of the glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) genes contribute to susceptibility to DN.

Results: Association between the -262T > C polymorphism of the CAT gene and DN was shown. The -262TT genotype of the CAT gene was significantly associated with higher erythrocyte catalase activity in blood of DN patients compared to the -262CC genotype (17.8 ± 2.7 x 10^4 IU/g Hb vs. 13.5 ± 3.2 x 10^4 IU/g Hb, P = 0.0022).

Conclusions: These data suggest a protective role of the -262T allele of the CAT gene against the rapid development of DN in T1D (Odds Ratio = 0.7 [95% confidence interval 0.54-0.9], P = 0.002).

Key-words: Diabetic neuropathy • Catalase promoter polymorphism • Oxidative stress • Type 1 diabetes.

RÉSUMÉ
Objectifs : Le stress oxydatif joue un rôle important dans le développement de la neuropathie diabétique. Les enzymes antioxydantes réduisent le stress oxydatif en cas d’augmentation de celui-ci dans le nerf périphérique. Les variations génétiques des gènes antioxydants pourraient donc être impliquées dans la pathogénie de la neuropathie diabétique.

Méthodes : 216 patients russes diabétiques de type 1 (DT1) atteints de neuropathie diabétique (DT1) et de 250 DT1 indemnes de neuropathie ont été inclus dans une étude qui avait pour but de préciser si les polymorphismes -262T > C et 1167C > T du gène de la catalase (CAT), la substitution de l’acide aminé 197Pro > Leu de la glutathione peroxydase 1 (GPX1) et le polymorphisme +/nul des gènes des glutathione S-transferases M1 (GSTM1) et T1 (GSTT1) contribuaient à la prédisposition vis-à-vis de la neuropathie diabétique.

Résultats : Une association entre le polymorphisme -262T > C du gène de la catalase et la prédisposition vis-à-vis de la neuropathie diabétique a été mise en évidence. Comparé au gène -262CC, le gène -262TT du gène de la catalase était significativement associé à une activité catalase érythrocytaire plus importante chez les patients atteints de neuropathie (17,8 ± 2,7 x 10^4 UI/g Hb vs 13,5 ± 3,2 x 10^4 UI/g Hb, P = 0,0022).

Conclusions: Ces données suggèrent le rôle protecteur de l’allèle -262T du gène CAT vis-à-vis du développement rapide de la neuropathie diabétique chez les patients atteints de DT1 (Odds Ratio = 0,7 [intervalle de confiance à 95% 0,54 – 0,9], P = 0,002).

Mots-clés : Neuropathie diabétique • Diabète de type 1 • Susceptibilité génétique • Polymorphisme du promoteur catalase • Stress oxydatif.
Introduction

Peripheral neuropathy is a common diabetic complication that contributes to mortality in most cases. A variety of genetic and non-genetic factors are considered to play a role in the pathogenesis of diabetic neuropathy (DN). Enhanced oxidative stress altering nerve blood supply, nerve structure and endoneural metabolism represents an important factor predisposing to peripheral nerve damage and dysfunction in diabetic conditions [1].

Oxidative stress occurs when the balance between the production of oxidation products and the ability of antioxidant mechanisms to neutralize these products is skewed in favor of the former. In the peripheral nerve, antioxidant mechanisms include a number of cytosolic and lypophilic low molecular-weight and enzymatic antioxidants. The key enzymatic scavengers are superoxide dismutase [EC 1.15.1.1], catalase [EC 1.11.1.6], glutathione peroxidase [EC1.6.4.2.], glutathione reductase [EC 1.11.1.9] and glutathione S-transferases [EC 2.5.1.18]. The antioxidant enzymes reduce oxidative stress through the inactivation of highly toxic free oxygen radicals and peroxides and, therefore, play an important protective role in the pathogenesis of DN. In animal model of diabetes, reduced glutathione (GSH) and GSH-containing enzymatic scavengers (glutathione peroxidase (GSH-Px) and reductase) were found to be decreased in the peripheral nervous system [2], while catalase levels have been elevated in the peripheral nerve [3]. Several studies have reported reduced activity of both GPH-Px and catalase in blood of type 1 diabetic patients with vascular complications [4, 5]. The decreased activity of enzymatic scavengers can result from the non-enzymatic glycation and modification with glycoxidation products followed by inactivation of the modified enzyme [6,7]. However, genetic variations within the antioxidant enzymes, particularly those which influence enzymatic activity and gene expression, could be also responsible for quantitative changes in the activity and expression profiles of antioxidant enzymatic scavengers in nerve tissue and hence contribute to genetic susceptibility for DN.

We have already reported finding an association of the -9Ala > Val molecular variant of mitochondrial Mn2+-dependent superoxide dismutase (Mn-SOD), encoded by the SOD2 gene, and 213Arg > Gly polymorphism of the SOD3 gene, encoding extracellular superoxide dismutase (EC-SOD), with diabetic neuropathy in Russian type I diabetic patients [8]. In this study, we evaluate whether variations within other genes encoding important antioxidant enzymes, such as catalase (CAT), glutathione peroxidase 1 (GPX1) and glutathione S-transferase M1 (GSTM1) and T1 (GSTT1), could contribute to genetic susceptibility to DN in type I diabetes.

Methods

Patients

Patients were recruited in the Department of Neurology of the I.M. Sechenov Medical Academy (Moscow). They represented unrelated Russian individuals who lived in Moscow and the Moscow region. Informed consent was obtained from all subjects prior to participation in the study. The research protocol was carried out according to principles of the Helsinki Declaration and approved by the Ethics Committee of the Medical Academy.

This study enrolled a total of 466 patients with diabetes of short duration (less than 3 years). Type 1 diabetes (T1D) was diagnosed in the clinical setting according to the American Diabetes Association diagnostic criteria [9]. DN patients were selected according to recommendations of the San Antonio conference on diabetic neuropathy [10] by presence of one or more typical syndromes (pain, numbness, cramps), diminished heart rate variation with deep breathing, abnormal quantitative tests for tactile, vibration, thermal warming and cooling thresholds and reduced nerve conduction velocity. The case group (DN+) included 216 patients with clinical DN, whereas the control group (DN-) included the remaining 250 individuals with no neuropathy. None of the patients had been treated with antioxidants. Patients with causes of neuropathy others than diabetes (i.e., chronic alcohol abuse), truncal neuropathy, neurodegenerative disorders such as Parkinson’s disease and others, and ischemic-related diabetic foot disease were excluded from this study. Clinical and metabolic characteristics of the patients selected are shown in table I.

Clinical measurements

The deep breathing test assessed parasympathetic nerve function. In the deep breathing test, six maximal expirations and inspirations are performed continuously during 1 min in the supine position during the recording of a continuous electrocardiogram. Sensory nerve conduction velocity (SCV) and sensory nerve action potential amplitude (SNAP) in the sural nerve and motor nerve conduction velocity (MCV) and compound muscle action potential amplitude (CMAP) in the peroneal nerve were measured using surface electrodes and the MS92a EMG machine (Medelec Limited, Old Woking, Surrey, U.K.) for surface stimulation and recording. Quantitative sensory testing (QST) included assessment of vibration and thermal perception. A vibration perception threshold (VPT) test was applied using a Biothesiometer (Bio-Medical Instrument Co., Newbury, Ohio, USA). Temperature perception threshold was determined using a thermo-esthesiometer (Hokushin Seiki Kogyo, Japan) on the top of the middle finger of both hands. All the QST procedures were done...
Polymorphisms of antioxidant enzymes in diabetic neuropathy

Antioxidant determination assays

Reduced gluthatione (GSSG) in erythrocyte hemolysates was quantified as described by Bentler et al. [11]. Antioxidant enzyme activities (CAT, GSH-Px and glutathione S-transferase) in erythrocyte hemolysates have been measured according to Bentler [12].

DNA analysis

Genomic DNA was isolated from whole-blood samples pre-treated with proteinase K (Fermentas, Vilnius, Lithuania) using extraction with phenol and chloroform.

For each gene studied, polymorphic regions were amplified using a polymerase chain reaction (PCR). The PCR cocktail contained 67 mM Tris-HCl, pH 8.8, 16.7 mM ammonium sulfate, 1.0 mM magnesium chloride, 0.1% Tween-20, 10% dimethyl sulfoxide, 0.2 mM each dNTP, 5 pmol of each primer, 100 ng of genomic DNA and 1.0 unit of Taq DNA polymerase (Fermentas) in a total volume of 20 µl. PCR was run on a GeneAmp® PCR System 9600 (Applied Biosystems, Foster City, California, USA) at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C to detect the 1167C > T dimorphism of the CAT gene and polymorphic markers within the GSTT1 and GSTM1 genes, or 65°C to determine the -262T > C substitution of the CAT gene and 197Pro > Lys polymorphism of the GPX1 gene, for 30 s and extension at 72°C for 1 min, with final extension at 72°C for 7 min. To identify the 1167C > T polymorphism of the CAT gene and 197Pro > Leu dimorphism of the GPX1 gene, a PCR product was further digested with Bst XI and BstDEI restriction enzyme, respectively [13]. The -262T > C polymorphism of the CAT gene was characterized using digestion with Smal as described by Folsberg et al. [14]. Polymorphic markers within the GSTT1 and GSTM1 genes have been simultaneously detected using a multiplex PCR [15]. PCR products and digestion fragments were analyzed electrophoretically in 2% agarose gel with ethidium bromide.

Statistical analysis

For statistical analysis, the Statgraphics Plus for Windows version 3.1 software (Statistical Graphic Corp., Rockwille, Madison, USA) was used. The ANOVA test was applied for comparisons between groups. The χ² test was used to compare allele and genotype frequencies in control subjects and diabetic patients complicated with DN. Odds ratios (OR) and 95% confidence interval (95% CI) were calculated to assess the strength of relationship between the polymorphic marker and diabetic neuropathy. A P value of < 0.05 was considered statistically significant.

Results

Characteristics of patients

When the groups of patients were compared, significant differences were observed between all characteristics used to evaluate nerve function except for CMAP (table I). Therefore, these parameters could represent strong and helpful criteria for the selection of diabetic patients with neuropathy.
Levels of reduced glutathione were significantly decreased in blood of DN patients compared to non-complicated individuals (P < 0.001). This could result from enhanced peroxidation indicating development of accelerated oxidation stress in DN patients. Significant changes in activity of antioxidant enzymes were found only for catalase, whose activity was increased in complicated subjects (P < 0.05) (table I).

Polymorphisms of antioxidant enzymes and oxidative stress

Among the polymorphisms tested, significant differences were shown only for the -262T > C nucleotide substitution of the CAT gene (table II). The -262C allele was associated with higher risk of DN in the Russian population sample (OR = 1.98, P corrected = 0.002). Carriage of the -262T > C molecular variant of the CAT gene was found to be correlated with catalase activity in erythrocytes. Higher enzyme activity was detected in blood of DN patients carrying the -262TT genotype compared to those who had the -262CC genotype (17,800 IU/g Hb vs 13,500 IU/g Hb, P < 0.002) (table III). Additionally, diabetic individuals without complications carrying the -262TT and -262CT genotypes had significantly higher levels of the reduced glutathione than T1D patients homozygous for -262CC (table III). These observations, therefore, suggest a protective role of the -262T CAT allele against rapid development of the oxidative stress in T1D.

For the 1167C > T polymorphism of the CAT gene, no significant correlation with enzyme activity and blood levels of reduced glutathione was found (table III). Proline-to-leucine amino acid change in codon 197 of the GPX1 showed no significant relationship to total glutathione peroxidase activity and GSH levels in blood of both complicated and non-complicated patients (table III). Similar results have been obtained for the +/null polymorphism of the GSTT1 gene encoding glutathione S-transferase θ1, which showed no significant association with total glutathione S-transferase activity nor with reduced concentration of glutathione in blood of the patients (table III). However, the “+” allele of the GSTM1 encoding glutathione S-transferase μ1 was shown to be significantly associated with

<table>
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<tr>
<th>Marker</th>
<th>Allele/genotype</th>
<th>DN+ (n = 216)</th>
<th>DN- (n = 250)</th>
<th>χ²</th>
<th>P</th>
<th>OR [95% CI]</th>
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<tr>
<td>1167C &gt; T CAT</td>
<td>Allele C (%)</td>
<td>347 (80.3)</td>
<td>390 (78.0)</td>
<td>0.76</td>
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<td>Allele T (%)</td>
<td>85 (19.7)</td>
<td>110 (22.0)</td>
<td>1.98</td>
<td>[1.53-2.58]</td>
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<td>Genotype CC (%)</td>
<td>141 (65.3)</td>
<td>146 (58.4)</td>
<td>5.32</td>
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<td>Genotype CT (%)</td>
<td>65 (30.1)</td>
<td>98 (39.2)</td>
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<td></td>
<td>Genotype TT (%)</td>
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<td>6 (2.4)</td>
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<td>-262T &gt; C CAT</td>
<td>Allele T (%)</td>
<td>186 (43.1)</td>
<td>266 (53.2)</td>
<td>9.55</td>
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<td>0.70 [0.54-0.90]</td>
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<td>Allele C (%)</td>
<td>246 (56.9)</td>
<td>234 (46.8)</td>
<td>1.98</td>
<td>[1.53-2.58]</td>
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<td>Genotype TT (%)</td>
<td>53 (24.6)</td>
<td>96 (38.4)</td>
<td>0.52</td>
<td>[0.35-0.78]</td>
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<td>74 (29.6)</td>
<td>10.27</td>
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<td>197Pro &gt; Leu GPX1</td>
<td>Allele Pro (%)</td>
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<td>Allele Leu (%)</td>
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<td>+/null GSTT1</td>
<td>Allele “+” (%)</td>
<td>272 (63.0)</td>
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<td>Allele “null” (%)</td>
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<td>170 (34.0)</td>
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<tr>
<td>+/null GSTM1</td>
<td>Allele “+”</td>
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<tr>
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<td>Allele “null”</td>
<td>278 (64.4)</td>
<td>344 (68.8)</td>
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</table>

1 χ² test: df = 1 for allele frequency comparisons; df = 2 for genotype frequency comparisons
increased total GST activity as well as with elevated GSH levels in both complicated and non-complicated diabetic subjects (table III).

**Discussion**

Decreased levels of glutathione in blood of DN patients (table I) could result from enhanced oxidative stress in blood vessels. These observations are consistent with results of other studies reporting significant reduction of GSH in the peripheral nerve and blood of rats with experimental diabetic neuropathy [3] and patients with diabetic microangiopathy [16]. Peripheral nerve, which requires exogenous glutathione due to the deficiency of its own γ-glutamylcysteine synthetase, an enzyme involved in GSH biosynthesis, is particularly sensitive to oxidative stress [17]. Thus, depletion of reduced glutathione could represent an important characteristic for the estimation of oxidative stress in diabetes and late diabetic complications because of the unique role of the altered ratio between reduced and oxidized forms of glutathione in molecular mechanisms of DN.

Significantly higher activity of catalase in blood of DN patients compared to individuals without complications (table I) is likely to reflect a primary response of the organism to high glucose-induced oxidative stress in short-term diabetes. This is supported both by the observations showing an increase in serum catalase activity in diabetic patients recently affected with microvascular complications [18] and also the elevated levels of catalase mRNA in the peripheral nerve of rats with experimentally induced diabetic neuropathy [3].

Folsberg et al. [14] reported a positive association between the -262T molecular variant of catalase and higher transcriptional activity of the CAT gene promoter that could explain significantly increased levels of erythrocyte catalase activity as well as with elevated GSH levels in both complicated and non-complicated diabetic subjects (table III).
in Swedish individuals with the -262TT and -262CT genotypes compared to subjects homozygous for -262CC. We also observed a significant association between the -262TT genotype of CAT and higher activity of the enzyme in DN patients (table III). This is likely to result from the increased transcriptional activity of the -262T CAT promoter variant providing higher levels of catalase mRNA compared to DN patients, who are homozygous for -262CC. This suggests a protective role of the -262T molecular variant of catalase against increased oxidative stress in type I diabetes and rapid development of diabetic neuropathy.

For the GSTM1 gene, a significant relationship between the “+” allele and high total activity of glutathione S-transferase was found, while the null allele was associated with decreased GST activity (table III). A null GSTM genotype results from gene deletion [19]. Approximately 50% of the Caucasian population are deficient for GSTM1. The +/null polymorphism of GSTM1 is usually associated with different intracellular concentrations of the enzyme, which are extremely low in individuals with the null GSTM1 genotype [19]. However, some investigators reported earlier on a relationship between +/null GSTM polymorphism and the enzyme activity, with high activity for the “+” allele and low activity for the null allele [20]. This correlation could result from different levels of the enzyme observed depending on genotype carriage.

We showed earlier on an association of certain molecular variants of Mn-SOD and EC-SOD with DN in Russian type I diabetic patients [8]. Here we report finding an association between -262T > C polymorphism of the CAT gene and DN. Both superoxide dismutase and catalase represent phase I antioxidant enzymes metabolizing toxic chemicals (superoxide and peroxides) to less toxic products further detoxified by phase II enzymes, such as glutathione peroxidase, glutathione S-transferase, and glutathione S-transferase, into metabolites which can be easily excreted from the body. In our studies, we found evidence for relationships between phase I enzymes and DN that could underline a unique role of phase I enzymatic scavengers in DN pathogenesis.

References