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Simple PCR heteroduplex, SSCP mutation screening methods for the detection of novel catalase mutations in Hungarian patients with type 2 diabetes mellitus

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Abstract

Background: The enzyme catalase is the main regulator of hydrogen peroxide metabolism. Deficiency of catalase may cause high concentrations of hydrogen peroxide and increase the risk of the development of pathologies for which oxidative stress is a contributing factor, for example, type 2 diabetes mellitus. Catalase deficiency has been reported to be associated with increased frequency of diabetes mellitus in a cohort of patients in Hungary. In this cohort, the majority of mutations in the catalase gene occur in exon 2.

Methods: Type 2 diabetic patients (n = 308) were evaluated for mutations in intron 1 (81 bp), exon 2 (172 bp) and intron 2 (13 bp) of the catalase gene. Screening for mutations utilized PCR single-strand conformational polymorphism (SSCP) and PCR heteroduplex methods. Verification of detected mutations was by nucleotide sequence analysis.

Results: A total of 11 catalase gene mutations were detected in the 308 subjects (3.57%, $p < 0.001$). Five of the 11 were at two previously reported mutation sites: exon 2 (79) G insertion and (138) GA insertion. Six of the 11 were at five previously unreported catalase mutation sites: intron 1 (60) G→T; intron 2 (7) G→A and (5) G→C; exon 2 (96) T→A; and exon 2 (135) T→A. The novel missense mutations on exon 2 (96 and 135) are associated with 59% and 48% decreased catalase activity, respectively; the novel G→C mutation on intron 2 (5) is associated with a 62% decrease in catalase activity. Mutations detected on intron 1 (60) and intron 2 (7) showed no change in catalase activity. The G→C mutation on intron 2 (5) might be a splicing mutation. The two missense mutations on exon 2 (96) and (135) cause substitutions of amino acids 53 (Asp→Glu) and 66 (Glu→Cys) of the catalase protein. These are close to amino acids that are important for the binding of heme to catalase, 44 (Val) and 72–75 (Arg, Val, Val, His). Changes in heme binding may be responsible for the activity losses.

Conclusion: Mutations that cause decreased catalase activity may contribute to susceptibility to inherited type 2 diabetes mellitus. Exon 2 and neighboring introns of the catalase gene may be minor hot spots for type 2 diabetes mellitus susceptibility mutations.

Keywords: catalase gene; diabetes; nucleotide sequence; oxidative stress; PCR heteroduplex; PCR-SSCP

Introduction

Oxidative stress is associated with diabetes mellitus, especially type 2 diabetes mellitus, which reportedly involves elevated production of oxygen free radicals (1). Abnormally high glucose concentrations, both extra- and intra-cellular, increase oxidative stress by enhancing the transformation of pro-oxidants to oxidants. Reactive oxygen species, e.g., superoxide anions and hydroxyl radicals, formed in this process may contribute to both the development and later complications of type 2 diabetes (2, 3). In type 2 diabetes, there is evidence that the stress-activated signaling pathways lead to both insulin resistance and impaired insulin secretion (4).

Natural defense mechanisms against oxidative stress include the enzymes superoxide dismutase and catalase. Superoxide dismutase converts the highly reactive superoxide anion into less toxic hydrogen peroxide. If sufficient catalase activity is not available to metabolize the peroxide, high concentrations of the hydrogen peroxide may lead to cell death (5), change endothelial cell permeability (6), alter insulin secretion (7), and impair insulin signaling (8).

The enzyme catalase (EC1.11.1.6) is the major regulator of the hydrogen peroxide concentration (9, 10). A link between inherited catalase deficiency and susceptibility to diabetes mellitus was first detected in Hungary. In a cohort of 13 Hungarian families, 65 persons had catalase deficiency (two acatalasemic homozygotes; 63 hypocatalasemic heterozygotes) and 66 persons were normocatalasemic. Of those with inherited catalase deficiency, eight (12.3%) were diagnosed with diabetes (11). None of the 66 normocatalasemic family members had diabetes mellitus; in contrast, both of the acatalasemic and six of the hypocatalesmic subjects had type 2 diabetes mellitus.

Both acatalasemic and two hypocatalasemic subjects with diabetes had a GA insertion at position 138 of exon 2 in the catalase gene. Insertion of a G at

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position 79 of exon 2 was detected in one hypocatalasemic type 2 diabetes mellitus patient. These mutations in exon 2 (at 79 or 138) may be part of the descriptive link between catalase deficiency and diabetes for the majority (62.5%, i.e., 5 of 8) of the type 2 diabetic hypo/acatalasemic patients (12–15). Other mutations were detected in the 5′ promoter region of the catalase gene, but appeared to be benign polymorphisms with no association with diabetes mellitus (16).

Mean blood catalase activities were compared for two groups: 137 diabetics (type 1, 45; type 2, 92) and a non-diabetic population. The mean activities were 94.4±19.2 MU/L for the diabetic group and 113.3±16.5 MU/L for the non-diabetic subjects. The decrease was significant ($p < 0.005$). None of these subjects were known to be members of families with inherited hypocatalasemia (17).

Blood catalase activity provides a general defense against diffusible hydrogen peroxide, especially for tissues with low catalase activities, for example pancreas and brain (9, 18, 19). Inherited or acquired catalase deficiency may create chronic oxidative stress, because abnormally low catalase activity will result in chronic elevation of hydrogen peroxide concentrations; the tissues most likely to be damaged are those that normally have the least intracellular catalase activity, e.g., the pancreas. High concentrations of hydrogen peroxide may damage pancreatic β-cells (11), mitochondrial electron transport (20), and influence homocysteine metabolism (21).

The evidence cited above suggests that in some families there may be a link between type 2 diabetes mellitus and catalase deficiency. There has been no report on the frequency of catalase mutations in a large cohort of type 2 diabetes mellitus patients.

The current study examined a large Hungarian cohort of type 2 diabetes mellitus patients for catalase gene mutations. The goals were (a) to determine the frequency of the previously detected mutations associated with diabetes mellitus at exon 2 (79 and 138), which we call Hungarian type A and type B mutations; and (b) to detect new mutations in the catalase gene, which may also be associated with susceptibility to diabetes. The hope is that the frequency and pattern of mutations may suggest how catalase deficiencies are associated with increased risk of diabetes mellitus.

Patients and methods

Type 2 diabetic patients (n=308) from throughout Hungary were included in the study. Their ages ranged between 45 and 70 years, with a mean±SD of 58.4±6.5 years. The body mass index was 28.5±1.9 kg/m^2 for men and 27.3±2.1 kg/m^2 for women. The upper limit of the reference range for body mass index is 27 kg/m^2 for men and 25 kg/m^2 for women. The group included 128 males (41.6%) and 180 females (58.4%). The criteria for diagnosis of diabetes were as defined by the National Diabetes Data Group (22) (impaired glucose tolerance test, increased hemoglobin A_{1c}, glucosuria, anamnesis). Patients had stable diabetes as determined by hemoglobin A_{1c} tests. Exclusion criteria were stage III symptomatic diabetic polyneuropathy, uncontrolled hypertension, incipient renal insufficiency, history of cancer with significant invasion or recurrence within 3 years, endocrine disorders other than diabetes mellitus, peripheral neuropathy, poor CYP2D6 metabolism, known other causes of secondary diabetes (e.g., pregnancy, surgery) and the use of insulin therapy. There were no other selection criteria, e.g., severity of diabetes or time since diagnosis.

The age- and gender-matched control group included 120 healthy Hungarian subjects. Their ages ranged between 41 and 71 years (55.3±7.1 years). The exclusion criteria of this group were any form of diabetes mellitus, inherited catalase deficiency, anemia, and decreased (below the reference range) blood catalase activity.

Genomic DNA was obtained from lymphocytes by standard methods (23, 24). PCR amplifications were performed in a total volume of 10.5 μL containing 1 μL of genomic DNA (0.2 μg/μL), 1 μL of each primer (10 μM), and 5 μL of RED-Taq (Ready Mix) from Sigma-Aldrich (Budapest, Hungary). Amplification cycles (30 cycles at 94°C for 0.5 min, 62°C for 0.5 min and 72°C for 1 min) were carried out in a DNA Thermal Cycler (Perkin Elmer Cetus TC 1, Norwalk, CT, USA) as previously described (12, 14).

The primers for exon 2 (forward, 5′-tgcaaagctatgtaccccgtg-3′; reverse, 5′-acacagcacttacctgctcc-3′) were as reported by Kishimoto et al. (25) and purchased from Pharmacia Biotech (Uppsala, Sweden). The 265 bp of the PCR product contained 80 bp of intron 1, 172 bp of exon 2, and 13 bp of intron 2.

For single-strand conformational polymorphism (SSCP) analyses, 5 μL of PCR product, 5 μL of 99% formamide, 20 mM EDTA and 0.005% bromophenol blue were mixed and heated at 96°C for 6 min and rapidly cooled on ice. Electrophoresis was performed using a 6% polyacrylamide gel (150×150×1.5 mm) running at 300 V for 5–6 h. DNA bands were visualized by silver staining.

Our simple PCR heteroduplex screening method was used to detect GA (12) and G insertions (14) in exon 2. This method showed four bands (wild-wild homoduplex at 268 bp, mutant-mutant homoduplex at 270 bp, and two heteroduplex bands at 273 and 304 bp) for GA insertion and two bands (homoduplexes at 268–270 bp, heteroduplex at 275 bp) for G insertion. The PCR product (2 μL) was heated to 94°C, cooled slowly, and then loaded onto 6% polyacrylamide gel (150×150×1.5 mm) running at 300 V for 3–4 h. DNA bands were visualized by silver staining.

After screening-revealed mutations, nucleotide sequence analyses were carried. PCR products were purified by agarose gel electrophoresis. The separated DNA bands were visualized by ethidium bromide staining. The stained bands were cut out and their DNA was eluted in 100 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).The eluted DNA was ethanol-precipitated, washed with 70% ethanol and dissolved in 10 μL of distilled water. These purified PCR products were used for sequence analyses. The sequencing reactions were carried out using a Taq Dye-Deoxy Termination Cycle Sequencing Kit and a DNA Sequencer (Models 373 and 310, Applied Biosystem, Foster City, CA, USA).

Blood catalase activity was determined using a spectrophotometric method (26). Blood was taken with EDTA as anticoagulant. A 20-μL aliquot of blood was diluted with 4.0 mL of 60 mmol/L sodium-potassium buffer (pH 7.4) and the erythrocytes were lysed with 100 μL of a saponin solution (20 g/L). Then 30 μL of this hemolysate was immediately incubated at 37°C with 1.0 mL of substrate containing 65 μmol/mL hydrogen peroxide in the former buffer. After 60 s, the enzyme reaction was stopped by adding 1 mL of 32.4 mmol/L ammonium molybdate [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$]

Table 1 Catalase gene (intron 1, 80 bp; exon 2, 172 bp; intron 2, 13 bp) mutations and blood catalase activities in 11 of 308 diabetic patients with type 2 diabetes mellitus.

Localization, bp	Mutation	Type	Allelic frequency, %	Amino acid	Catalase, MU/L	%	Reference
Intron 1 (60)	G→T	Substitution	0.32	–	106.3	93.8	New
					90.3	79.7	
Intron 2 (7)	G→A	Substitution	0.16	–	80.5	71.0	New
Intron 2 (5)	G→C	Substitution	0.16	–	70.3	62.0	New
Exon 2 (96)	T→A	Missense	0.16	53 Asp→Glu	60.3	53.2	New
Exon 2 (135)	G→C	Missense	0.16	6 Glu→Cys	58.9	52.1	New
Exon 2 (138)	GA insertion	Frameshift	0.48	69 Ile→Glu	52.3	46.2	(12)
					60.3	63.2	
					61.2	54.0	
Exon 2 (79)	G insertion	Frameshift	0.32	48 Pro→Ala	58.4	51.5	(14)
					50.4	44.5	

and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm against blank. Enzyme activity was defined as follows: 1 U of catalase decomposes 1 μmol of hydrogen peroxide in 1 min under these conditions and is related to 1 L of blood. Due to the very high turnover of hydrogen peroxide by catalase and the high catalase content in blood, the blood catalase activity is reported as MU/L. The reference range for this method is 80.3–146.3 MU/L or mean (SD) = 113.3 (16.5) MU/L (26).

A χ^2 test was performed to assess the difference in allelic frequencies of mutation between diabetic patients and healthy controls.

The Student t-test was used to calculate the difference in blood catalase activity between the diabetic patients with mutations and healthy controls. The level for statistical significance was set at $p < 0.005$.

Results

PCR products from 92.5% (285 of 308) of the samples from diabetic subjects revealed no mutations either by PCR-SSCP or PCR heteroduplex analyses.

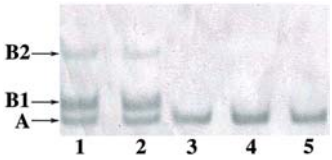

Figure 1 PCR heteroduplex mutation screening of catalase exon 2 in diabetes mellitus. A, wild-wild homoduplex; B1, B2, wild-mutant heteroduplexes. Lane 1, heteroduplex control; lanes 2–5, diabetic patients with heteroduplex formation (2) and without heteroduplexes (3–5).

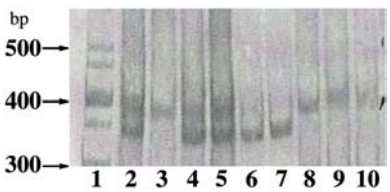

Figure 2 PCR-SSCP mutation screening of catalase exon 2 in diabetes mellitus. Lane 1, molecular mass marker; lanes 2–10, diabetic patients with SSCP polymorphism (2, 4–7) and without SSCP polymorphism (3, 8–10).

SSCP analyses revealed polymorphisms in samples from 18 (5.8%) of the patients. Sequence analyses of the PCR products identified two mutations (2 patients) in intron 2, one mutation in intron 1 (2 patients), two missense mutations (2 patients) in exon 2, and no mutation sites in 12 of the 18 subjects.

Screening by the PCR heteroduplex method was positive for samples from five patients (1.6% of 308). Sequence analyses revealed a GA insertion in exon 2 (Hungarian type A) in three patients (~1%) and a G insertion (Hungarian type B) in two patients (~0.6%), as shown in Table 1.

The mutation screening tests (Figures 1 and 2) were positive for 23 (7.5%) samples of 308 type 2 diabetes mellitus patients. Nucleotide sequence analyses verified seven mutations in 11 patients. All of these 11 patients were heterozygotes for catalase deficiency.

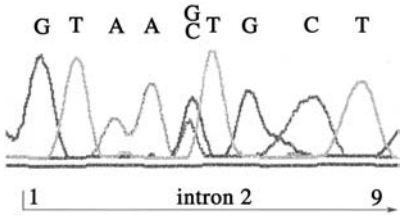

Figure 3 Nucleotide sequence analysis of catalase intron 2 in a diabetic patient. Upper line shows the nucleotides, lower line, the nucleotide positions. The new mutation is a G→C substitution at position 5 of intron 2.

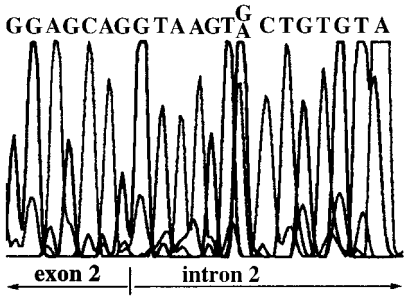

Figure 4 Nucleotide sequence analysis of catalase exon 2 in a diabetic patient. The upper line shows the nucleotides and the lower line their positions. The new mutation at position 7 of intron 2 is a G→A substitution.

These mutations (Figures 3 and 4) were associated with decreased (44.5–93.8%) blood catalase activity. These data indicate that in 308 patients with type 2 diabetes mellitus, catalase gene mutations on exon 2, intron 1 and intron 2 were detected in 3.57% (n = 11) of the patients. The allelic frequency of these mutations was 1.78%.

Catalase activities for the 11 heterozygotes are shown in Table 1. Mutations on intron 1 (60) caused the least significant decrease in catalase activity (93.8% and 79.7%) among these 11 heterozygotes, with values of 106 and 90 MU/L compared to the population mean of 113.3 ± 16.5 MU/L.

There was a significant difference ($p < 0.001$) in allelic frequencies of mutations between diabetic patients (11 of 616) and healthy controls (0 of 240).

The mean and SD of blood catalase activity of diabetic patients with mutations (68.1 ± 17.4 MU/L, n = 11) was significantly ($p < 0.001$) lower compared to that for all diabetic patients (90.5 ± 16.9 MU/L, n = 308). A similar decrease ($p < 0.001$) was found when diabetic patients (90.5 ± 16.9 MU/L, n = 308) were compared to the control subjects (103.8 ± 17.5 MU/L, n = 120).

Discussion

PCR-SSCP and PCR heteroduplex mutation screening was useful for the detection of catalase gene mutations in hereditary catalase deficiencies in Hungarian patients (12–14, 16).

The PCR-SSCP method indicated mutations in 23 patients. Nucleotide sequence analyses revealed two previously unreported mutations on intron 2 near the exon/intron boundary (G → A at position 7 and G → C at position 5). The subject with the nucleotide substitution at position 5 with decreased catalase activity (70.3 vs. 113.3 MU/L) may be associated with a defect in exon splicing, as found for exons 4 (27) and 7 (13), which were also associated with a decrease in catalase activity.

The other patient with an intron 2 mutation (G → A at position 7) had a blood catalase activity (81.5 MU/L) at the low end of the reference range (80.3–146.3 MU/L).

The new G → C substitution at position 60 of intron 1 was detected in two unrelated patients. This mutation did not cause a significant decrease in catalase activity (100.3 and 90.9 MU/L). Due to the position of this mutation and to the lack of a decrease in catalase activity, we may suppose that this mutation might be a benign polymorphism of the catalase gene.

The novel exon 2 mutations T → G (position 96) and G → C (position 135) are missense mutations; the corresponding amino acid changes are asparagine → glutamine (amino acid position 53) and glutamine → cysteine (position 66). The amino-terminal domain of catalase has 75 residues, which form an arm extending from a globular region of the enzyme. The arm consists of two helices, both of which are involved in intersubunit contacts. The second helix is essential for binding of heme. Several amino acid residues (Val 44, Arg 72, Val 73 and 74, His 75) are most important for heme-protein binding.

The novel missense mutations (amino acids 53 and 66) are in the vicinity of these important amino acid residues (28–30). Therefore, amino acid substitutions in this region (exon 2 T → G at 96 and G → C at 135) may decrease the catalase enzyme activity (60.3 and 58.9 MU/L, respectively vs. the reference range of 113.3 ± 16.5 MU/L). Such a chronic decrease in blood catalase activity may yield a lifelong increase in hydrogen peroxide concentrations, which may contribute to the development of type 2 diabetes mellitus (11, 17, 20).

The PCR heteroduplex analyses detected heteroduplex formation characteristic of the Hungarian type A (12) and B (14) catalase mutations. These are frameshift mutations with early stop codons that truncate catalase proteins at 133 and 57 amino acids instead of 517 amino acids. These results for diabetes mellitus patients not known to be members of families with inherited catalase deficiency are consistent with earlier reports. These two mutations were detected in six of eight hypocatalasemic patients with type 2 diabetes mellitus. The frequency of diabetes mellitus in patients with inherited catalase deficiency was significantly higher (12.7%) compared to the frequency of 1.8% in the general Hungarian population (11).

In the current study, catalase mutations on or near exon 2 were detected in 3.57% of type 2 diabetes mellitus patients. The mutations detected included two new benign polymorphisms with no significant change in catalase activity (three patients), and mutations with decreased catalase activity, including one novel splicing mutation (one patient), two new missense mutations (two patients) and two known mutations (five patients).

None of these new mutations were detected in the 120 non-diabetic control subjects.

Decreased catalase activity due to the mutations may result in high steady-state concentrations of hydrogen peroxide and oxidative stress. Peroxide may damage pancreatic β-cells (11), mitochondrial electron transport (20), homocysteine metabolism (21), and insulin secretion and signaling (7, 8). These factors may lead to insulin resistance and impaired insulin secretion (4), although the mechanism responsible for the development of this disease is not yet known.

Our observations of catalase mutations on or near exon 2 are consistent with a hypothesis that catalase deficiency might be a minor factor that can contribute to inherited susceptibility to type 2 diabetes mellitus. This region of the catalase gene might be a hot spot for mutations that contribute to the pathogenesis of type 2 diabetes mellitus.

In conclusion, the new mutations on exon 2 (missense mutations at positions 96 and 135) and intron 2 (substitution at position 5), together with the previously identified exon 2 mutations (frameshift at positions 135 and 79) might be involved in the pathomechanism of diabetes mellitus. These catalase mutations might be regarded as risk factors for type 2 diabetes mellitus.

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