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A simple method for examination of polymorphisms of catalase exon 9: rs769217 in Hungarian microcytic anemia and beta-thalassemia patients

Teréz Nagy, Melinda Csordás, Zsuzsanna Kósa, László Góth *

Department of Biomedical Laboratory and Imaging Science, Medical and Health Science Center, University of Debrecen, P.O. Box 55, Debrecen H-4012, Hungary

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ABSTRACT

Catalase decreases the high, toxic concentrations of hydrogen peroxide but it lets the physiological, low concentrations in the cells mainly for signaling purposes. Its decreased activity may contribute to development of several pathological conditions. Catalase mutations occur frequently in exon 9, these were examined with different, complicated and costly methods.

The aim of the current study was to evaluate a method for screening of polymorphisms in catalase exon 9.

We used the slab gel electrophoresis of PCR amplicons without denaturation and silver staining for visualization of the DNA bands. We detected extra DNA bands in the 400–800 bp region of the catalase exon 9. Their single stranded nature was proved with nucleotide sequence analyses, comparison with the standard SSCP, staining with Sybr Green II and Sybr Green I, ethidium bromide, no digestion with RFLP (BstX I), and digestion with plant nuclease.

We used this method for examination of polymorphisms of catalase exon 9 in microcytic anemia and beta-thalassemia patients. The lowest blood catalase activities were detected in microcytic anemia and beta-thalassemia patients with the TT genotypes of the C111T polymorphism.

This method was sensitive for detection of G113A acatalasemia mutation, but poorly detected C37T and G5A acatalasemia mutations.

Introduction

There is an increasing need in the field of molecular diagnostics to develop new methods for detecting mutations associated with unique diseases, and today's laboratory professionals are inspired to develop both economical and practical laboratory developed tests to meet these demands. Often the off-the-shelf FDA cleared tests fall short of the laboratory's need for the above mentioned one-of-a kind assays. In some instances, the very nature of the disease makes the methodological choice obvious. But what if the method is not apparent or there is not an ideal methodology. One solution, that may be over looked, may be a stringent amplification paired with an electrophoretic technique. These two tried and true laboratory tools can be employed as simple and cost effective screening tools, before committing to more costly and time consuming laboratory techniques (such as gene sequencing). Furthermore, the re-tasking of this basic equipment mitigates any further capital expenditure. We have demonstrated this by developing screening assay using single-strand conformation polymorphism (SSCP) to detect known polymorphisms in exon 9 (http://peroxibase.toulouse.inra.fr/listing.php?action=view&id=5282) of the CAT (catalase)[1] gene (NM_001752.3).

Since its introduction by Orita [1], SSCP analysis has been widely used for mutation screening in both research and diagnostic laboratories [1]. A recent search in SCOPUS shows 1105 citations for SSCP in 2010 and 15,547 since 1989. Standard SSCP requires single strand formation (denaturation of double stranded DNA) of PCR amplicons. This can be achieved with heat or a denaturation buffer (such as formamide), or both. After denaturation, the sample mixture is "snap-cooled" causing sequence based single stranded conformers to form. Ideally, two conformers for homozygotes (wild type or mutant) and four conformers for heterozygotes are formed. These ssDNA fragments are then run under specific electrophoretic conditions and the conformational changes can be visualized as mobility shifts which allow for resolution of both wild type DNA and mutant DNA sequence [2]. Detection can be done with a number of products including homemade or precast gels or more technical capillary

* Corresponding author at: Department of Biomedical Laboratory and Imaging Science, Faculty of Medicine, Medical and Health Science Center, University of Debrecen, Nagyerdei krt 98, P.O. Box 55, Debrecen H-4012, Hungary. Fax: +36 52 255 170.

E-mail address: goth@dote.hu (L. Góth).

[1] *Abbreviations used:* BstXI, restriction enzyme; CAT, enzyme catalase (EC1.11.1.6); dsDNA, double stranded DNA; FDA, Food and Drug Administration (USA); H–W, Hardy–Weinberg equilibrium; MCV, mean cell volume; MFOLD, predicts secondary structures for RNA and DNA with method of Zuker; OR, odds ratio; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ssDNA, single strand DNA; SSCP, single strand conformational polymorphism; Sybr Green, DNA stain.

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electrophoresis technologies. The manual slab gel electrophoresis is slow and time consuming while capillary electrophoresis' more automated processing and high reproducibility comes at a higher cost. However, the performance of slab gel electrophoresis with denaturation can be competitive with the capillary electrophoresis technologies. The sensitivity of SSCP is influenced by several factors, including: amplicon size, denaturation conditions and efficacy, G + C composition and imbalance, and the conformation (stems, loops, and internal base-pairing) of single strands.

There are varying reports of its sensitivity (91–100%) and its specificity (88–100%), but there is an agreement that SSCP' sensitivity is optimal when DNA amplicon fragment sizes are less than 300 bp and proper optimization of SSCP is performed [3]. However, Glavac et al., Atha et al., and Liu et al., compared the single strand secondary structures predicted by the program MFOLD with the mobility shifts produced by DNA fragments and obtained acceptable correlations with amplicons between 52 and 131 bp [2,4,5].

In our assay, we interrogate a number of polymorphisms in exon 9 of CAT which codes for the enzyme catalase (EC 1.11.1.6). Catalase is the main regulator of hydrogen peroxide metabolism and it eliminates toxic concentrations of hydrogen peroxide [6,7]. It has been reported that catalase deficiency may contribute to the development of diabetes mellitus, vitiligo, dyslipidemia, abnormal erythrocyte metabolism, Parkinson disease, hearing loss and bone mineral density [8–18]. Exon 9 is a hot spot for polymorphisms in the CAT with two acatalasemia mutations (G113A [12] and G5A [14] also known as Hungarian type D and one silent substitution (Asp389Asp, +22348C → T, rs:769217 [9]).

Materials and methods

Patients

Peripheral blood was collected from 98 patients and 50 staff members of the Medical Center from the University of Debrecen. Their demographic and hematological values are shown in Table 1.

Their genomic DNA was extracted with QIAmp Kit (DNA Blood Mini Kit, QIAGEN, Hilden, Germany). Included in this cohort were two patients from catalase deficient families (Hungarian type D (G5A, exon 9) and Hungarian type E (C37T, exon 9)).

Institutional Review Board approval and written informed consent were obtained.

Blood catalase activity determination

Blood catalase activity was measured with a spectrophotometric method. The yellow complex of hydrogen peroxide and ammonium molybdate was measured at 405 nm. One unit of catalase decomposes 1 μmol of hydrogen peroxide in 1 min and it is related to 1 L of blood. Due to the high activity of catalase in blood we used mega (10^6) units (MU) [19].

Hematological analyses

The hematological parameters were measured with SYSMEX XE 2011D hematological analyzer (Sysmex, Japan).

PCR analyses

PCR was performed as described by Wen and Kishimoto for exons 2, 3, 7, and 9 [20,21] for screening of C111T polymorphism. Briefly, a 7-μL denaturation of 400 ng of genomic DNA and 10 μmol/L of each primer (Sigma–Aldrich, St. Louis, MO, USA) was performed at 95° for 5 min. Then, 0.4 mmol/L dNTP mix, ReadyMix REDTaq with $MgCl_2$, (20 mmol/L Tris–HCl pH 8.3, 100 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.002% gelatine, 60 U TaqDNA) (preceding two reagents: Sigma–Aldrich, St. Louis, MO, USA) was added for a 14-μL final reaction volume. Primers and polymorphism in these exons are described in Table 2. Thermal cycling conditions in a TC1 DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, CT, USA) were thirty amplification cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, and final elongation of 72 °C for 5 min [14].

Standard and simple SSCP

For standard SSCP, 5-μL of PCR products were denatured by adding 5-μL of 99% formamide, 20 mM/L EDTA), 0.05% and 0.05% cylene cyanol and heated at 96 °C for 6 min and immediately cooled on ice. Alternately, the simple SSCP method was without the denaturation step. For this 5-μL of PCR product was added to 4-μL of loading dye mix (0.05% bromphenol blue and 0.05% cylene cyanol in 20% glycerol). Then, they were mixed and loaded directly into the gel. Electrophoresis was performed in 6% polyacrylamide gel ($100 \times 110 \times 1.5$ mm) at 90 V and room temperature for 90 min. DNA bands were visualized by silver stain and the bands were quantified with Gel Doc 1000 from Bio-Rad (Hercules, CA, USA). These reagents for molecular biology were purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA). Different dyes were used for detection of single stranded and double stranded PCR bands. For this, the polyacrylamide gel was cut horizontally into three parts: each part had the same loading pattern (lane1: molecular mass marker, lane 2: CC111 genotype, lane 3: TT111 genotype). These parts were stained with ethidium bromide, SYBR Green I and SYBR Green II (Sigma–Aldrich, St. Louis, MO, USA) overnight and pictured. After that the gel pieces was washed and silver stained.

PCR–RFLP analyses

Restriction fragment length polymorphism analyses (RFLP) was performed on PCR products with BstXI (Amersham Pharmacia Biotech UK, Buckinghamshire, England) overnight at 45 °C. This enzyme recognition sequence is CCANNNNN*NT**GG and the enzyme cuts it at nucleotide 153* of the 238 bp long PCR product forming two (153 bp and 85 bp) fragments from the mutant strand. The wild strand with C** in the recognition site will be unchanged (238 bp long). The restricted fragments were separated on 6% polyacrylamide gel and visualized with silver staining [22].

Bi-directional DNA sequencing

DNA sequencing reactions were carried out from purified PCR products (QIAquick PCR Purification Kit from QIAGEN, Hilden, Germany) using Taq Dye-Deoxy Termination Cycle Sequencing Kits (50 μL final volume with BigDye Term v1.1 Cycle Sequencing Kit

Table 1
The values of age (mean (range)), gender, blood hemoglobin, blood catalase, mean cell volume (MCV), hemoglobin A2 of patients and controls.

Patients	Age (year)	MCV (fL)	Hemoglobin (g/L)	Male/female	Blood catalase (MU/L)	Hemoglobin A2 (%)
Microcytic anemia	40 (20–57.5)	71.9 ± 9.7	104.9 ± 10.9	24/31	87 ± 29	2.6 ± 1.0
Beta-thalassemia	43.5 (18–45)	70.6 ± 8.7	102.9 ± 8.9	18/25	85 ± 23	5.56 ± 0.84
Controls	40 (18–62)	89 ± 9	134 ± 20	23/27	102 ± 20	2.5 ± 1.1