SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Examination of association of catalase gene polymorphisms, DNA mutations and catalase activity

by Teréz Nagy Nyesténé

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The Examination takes place at the library of Department of Cardiology, Faculty of Medicine, University of Debrecen
at 11:00 a.m. on 26th Oktober,2017.

Head of the **Defense Committee**: Prof. Dr. Zoltán Papp MD, PhD, Dsc
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The PhD Defense takes place at the Lecture Hall of Auguszta Building, Faculty of Medicine, University of Debrecen
at 13:00 p.m. on 26th Oktober,2017.

1. INTRODUCTION

The enzyme catalase (EC1.11.1.6 hydrogen peroxide/ hydrogen peroxide oxidoreductase) was one of the earliest known and examined enzymes, and several of its unique characteristic features were described. A significant number of these features, however, could only be proved and explained later with the help of the most recent scientific methods.

The 90s' boom in molecular biology research followed by whole-genome sequencing first accomplished in 2000 gave a new impetus to research on catalase enzyme as well.
The determination of the full sequence of catalase gene was a great help to researchers, including our own research team. It helped us to map the coding and non-coding sequences in Hungarian populations, to detect mutations, polymorphisms and SNPs in catalase enzyme, and examine them in different diseases. My thesis advisor and his fellow researchers studied the reasons for decrease in catalase activity. Their research concentrated on the association of decrease in enzymatic activity detectable in different diseases and the catalase gene polymorphisms and mutations. I joined their research in 2008.
We measured the activity of enzyme catalase in patients with the diseases like type 1, type 2 and gestational diabetes, vitiligo, presbyacusis, microcytic anemia, and beta-thalassemia. The samples with decreased activity were further examined with molecular biology methods of PCR-SSCP, RFLP, heteroduplex analysis and sequence analysis in search for the genetic reasons of decrease. We gained several interesting and valuable results, which were first presented in student thesis papers, Students' Research Conference papers (TDK - Hungarian abbreviation), or at Hungarian and international conferences. Our novel results were published in international scientific journals.
I have been working on the detection of polymorphisms of the gene encoding the catalase enzyme and the reasons for decreased enzymatic activity ever since. My thesis is a summary of the results I have gained since 2008.

2. ENZYME CATALASE

Free radicals are being continuously produced as a result of oxidation-reduction biochemical processes in the human body. Oxidative stress occurs when the concentration of the reactive oxygen species (ROS) exceeds the antioxidant capacity of the body. The damaging effect of ROS is well-known in conditions like diabetes mellitus, atherosclerosis, chronic hepatic conditions, kidney failure, tumors, rheumatoid arthritis and neurodegenerative diseases.
Reactive oxygen species are capable of damaging proteins, lipids and the DNA, which carries the genetic information of cells. A possible result of this damaging process can be the failure in membrane function, the formation of oxidized lipids, as well as mutations in DNA. Prolonged oxidative damage may cause the aging of cells and could contribute to the development of chronic conditions and tumors.
The second line of defense against damaging agents like hydrogen peroxide is provided by antioxidant enzymes such as superoxide-dismutase, glutathione-peroxidase and catalase.. By now catalase enzyme seems to be of the greatest importance in the hydrogen peroxide removal.

Catalase (EC 1.11.1.6, hydrogen peroxide/ hydrogen peroxide oxidoreductase) forms a tetramer composed of four identical subunits with a molecular weight of 240 kDa. The specialty of the substrate decomposition process is that the enzyme does not function if the concentration of H_2O_2 is physiological.

The decomposition of hydrogen peroxide is a two-step process. In the first step enzyme substrate complex I (compound I) is formed in a reversible reaction.
In the second catalytic step complex I oxidizes a second hydrogen peroxide molecule forming oxygen and a molecule of water.

The blood cells contain the enzyme catalase in different concentrations with the highest in erythrocytes. Moe than 99 % of blood catalase derives from erythrocytes. The reference mean for blood catalase activity is 113.3 MU/L of blood. If we relate it to 1 L of erythrocytes, the activity is 251.7 MU/L of erythrocytes.
It could be assumed that the catalase found in high concentration in blood also has a general protective role against the hydrogen peroxide. Hydrogen peroxide could damage the tissues when the catalase concentration is low.
The decreased activity of the enzyme can lead to early manifestation of several diseases like tumors, anemias, vitiligo, diabetes mellitus.

Acatalasemia is a disorder marked by congenital absence of catalase, which is a genetically mutant homozygous condition. For these patients the substrate measuring methods can still detect an activity of 1-8 %. Hypocatalasemia is the heterozygous condition and these patients are with about 50 % of the blood catalase. The decrease in enzymatic activity can also be related to the polymorphisms of catalase gene which could cause a decrease in protein expression.
The first patient with acatalasemia was described by Professor Takahara in Japan in 1946. He observed almost total lack of hydrogen peroxide decomposition in an 11-year-old girl patient. To measure blood catalase activity, he applied the permanganometric titration of hydrogen peroxide and as a result, in 6 cases he could observe ”total” deficiency of catalase activity. In 5 cases the activity was <10 %, and in 2 cases the activity was 10 %. Following the discovery made by Professor Takahara, acatalasemia came to be detected in further 12 countries, with the highest numbers in Japan (91) and Switzerland (11).
The examination of a large Hungarian population (n=28,252) the acatalasemia has shown an incidence of 0.05/1000. Our research team detected the first acatalasemia cases in Hungary in 1989 (4.6 MU/L). On the other hand, the incidence of hypocatalasemia in the Hungarian population is 0.18/1000.

Type 2 diabetes mellitus can be considered a complex condition. Its development is influenced by environmental and genetic factors alike. Several research teams include in their scope the assessment of the association between catalase deficiency and diabetes. In Hungary we carried out detailed examination on this topic. The studies performed so far have shown that one or more catalase gene mutations may contribute to the early manifestation of type 2 diabetes mellitus. We found that in inherited catalase deficiency the diabetes is more frequent than in normocatalasemic subjects. We may suppose that the lack of catalase makes hydrogen peroxide concentration increase chronically, which will exert a damaging effect on the pancreatic beta-cells highly sensitive to oxidation.

In microcytic anemia the number and size of erythrocytes decreases. Red blood cells are smaller than physiological and their hemoglobin content is also lower, i.e. they are hypochromic.
Genetic disorders may also lead to the formation of small hypochromic red blood cells. It is also genetic disorder that causes thalassemia, which can be divided in two main groups. In α-thalassemia the hemoglobin α-chain fails to work sufficiently. In the more common β-thalassemia the synthesis of the β-chain is decreased or deficient. The decreased blood catalase activity in these erythrocytes may lead to an increase in oxidative processes as the damaging and active H_2O_2 is not eliminated. The examination of the association between decreased blood catalase activities and β-thalassemia may lead to a better understanding of the disease pathology.

Catalase protein is coded by a single gene, the catalase gene, which is located on the short arm of chromosome 11 at position 13. The gene spans 33,114 kb and has 13 exons and 12 introns. Its chromosome location is chr:11:34,417,054-34,450,176. On the basis of the information available in the NCBI database, by the year 2012 there were 245 nucleotide positions detected in the catalase gene that were different from the consensus. The majority of polymorphisms result in a benign condition with no effect on enzymatic activity. They do not affect catalase protein expression, and it either has no pathological effect or it has not yet been discovered.

Our research team has also been working for decades with the association between various diseases and the mutations, polymorphisms and SNP of the catalase gene. We could detect significant decrease in catalase activity in the presence of the T mutant allele and we found that the C111T missense mutation increases the susceptibility of vitiligo.

The mapping of the exonal and intronal regions of the gene encoding the enzyme is still undergoing. The increase of molecular biology techniques made it possible to detect in the Hungarian population acatalasemia mutations. We named the detected mutations as the A, B, C, D, E types of the Hungarian acatalasemia. Investigating the causes of acatalasemia and hypocatalasemia may help us get a better insight into the genetic and epigenetic factors influencing the activity of this enzyme. We may suppose the toxic effect of an increased hydrogen peroxide concentration in cells and tissues due to a decrease in catalase enzyme activity deriving from genetic abnormalities. The highly oxidative environment may favor for oxidative stress, which can further damage stress-sensitive organs and tissues. By examination of the catalase gene mutations we might get to a better understanding of pathology and possible treatment of several diseases.

3. AIMS

For my thesis entitled Examination of the association between catalase gene polymorphisms, mutations and catalase activity the aims are listed bellow**.**

1. To examine with a simple mutation/polymorphism screening method the effect of polymorphism rs769217 in exon 9 on the activity of the enzyme catalase in microcytic anemia and beta-thalassemia patients.

2. To examine the effects of polymorphisms of rs769217 in exon 9 and rs1001179 in the promoter region on blood catalase activity, and on lipid and carbohydrate biomarkers such as triglyceride, cholesterol, HDL, LDL, ApoA-I, ApoB, glucose, and HbA1c.

3. To be sorted out a cohort with blood catalase activity lower than 50%. We may suppose that cataalse gene polymorphisms presumably more frequent in this group than in normocatalasemics. For this purpose, to examine the 13 exonal and 12 intronal regions to detect further mutations, polymorphisms and SNPs. The newly detected DNA polymorphisms and mutations may have an effect on blood catalase activity and could contribute to the pathology of several diseases.

4. A further aim is to set up a control group with blood catalase activity within the reference range. The analysis of such samples would make it possible to perform comparative analysis of the catalase gene.

4. MATERIALS AND METHODS

4.1. Samples

The blood samples (EDTA-treated full blood samples) were used during my research work. They were collected from the clinics (Internal Medicine, Pediatrics, Otorhinolaryngology) of the Medical and Health Science Center of University of Debrecen. The samples had been referred for laboratory analysis to the Clinical Biochemistry and Molecular Pathology Department (today's Laboratory Medicine). The analysis only comprises the data of the patients whose blood hemoglobin concentration was in the reference range (above 120 g/L).

4.2. Patients and controls

During the examination of catalase gene exon 9: rs769217 polymorphism the samples of 98 patients (microcytic anemia and beta-thalassemia patients) and 50 control samples were examined.

The patient and control samples to be used for the analysis of catalase rs769217 and rs1001179 polymorphisms had been obtained from outpatients of the Diabetes Specialty Care of the Internal Medicine Department of the University, presented between 2007 and 2010.

We measured blood catalase activity to screen a big number of patients (617) and controls (295) samples for the examination of novel acatalasemia mutations.

The blood samples of controls were collected from staff members of the different clinics at the Clinical Center (former Medical and Health Science Center) of the University of Debrecen. These health care test samples were sent for analyses to the Department of the Laboratory Medicine. The control cohort included subjects with blood catalase activity (80.3-146.3 MU/L) and hemoglobin concentration (120-180 g/L) both within the reference range. The samples were assigned a code containing both numbers and letter to ensure anonymity. Our research had been approved by the Ethical Committee/Institutional Review Board of the University of Debrecen with the authorization number 2734-2008.

4.3. Measurement of catalase enzyme activity

In order to determine catalase activity, I used a spectrophotometric assay, which measures the concentration of hydrogen peroxide substrate.

Decomposition of hydrogen peroxide substrate was stopped after 60 seconds by adding ammonium molybdate. Ammonium molybdate forms a complex with the hydrogen peroxide stopping the enzymatic reaction. This complex of hydrogen peroxide and ammonium molybdate is yellow in color, and its absorbance is measured at 405 nm which is depends on the hydrogen peroxide concentration.

4.4. Determination of lipid parameters and glucose concentration

Lipid metabolism parameters (triglyceride, total cholesterol, HDL cholesterol, LDL cholesterol, ApoAI, ApoB) and glucose concentration were determined in serum with Roche Integra assays, Roche Integra 700, Modular P800 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

4.5. Determination of hematological parameters

Blood hemoglobin and mean cell volume (MCV) of red blood cells were measured with Sysmex XE 2011D hematological analyzer (Sysmex, Japan), while hemoglobin A2 and hemoglobin F were determined with a HPLC method, (Variant II instruments, Haemoglobin Testing System, BioRad, Hercules, CA, USA) from blood treated with anticoagulant EDTA.

The analyses were performed at the Division of Hematology and Separation Technique at the Department of Laboratory Medicine of the University of Debrecen.

4.6. Molecular biology techniques

DNA isolation

Genomic DNA was isolated from buffycoat (blood fraction rich in leucocytes) treated with anticoagulant EDTA using QIAmp Kit (DNA Blood Mini Kit, QIAGEN, Hilden, Germany).

PCR

For the PCR reaction the primers and steps recommended by Wen and Kishimoto were used in the amplification of exons.

Electrophoresis

The resulting PCR products were examined in 6 % polyacrylamide gel and visualized by silver staining. The lengths of DNA fragments were compared to molecular weight markers.

SSCP analysis

The dsDNA was single-stranded by denaturation and immediately cooled on ice and analyzed by electrophoresis.

PCR-RFLP analysis with enzyme BsTX1

Restriction fragment length polymorphism analysis (RFLP) was performed with BsTXI restriction enzyme (Amersham Pharmacia Biotech UK, Buckinghamshire, England). The enzyme recognizes the CCANNNNN*NT**GG sequence and cuts the 238 bp long PCR product into two fragments of 153 bp and 85 bp length.

DNA sequencing

Purified PCR products (QIAquick PCR Purification Kit QAGEN, Hilden, Germany) were sequenced with Taq Dye-DeoxyTermination Cycle Sequencing Kit of 50 ul final volume (BigDye Term v.1.1 Cycle Sequencing kit, Applied Biosystems, Foster City, CA USA) and separated with capillary electrophoresis (3100-Avant, Genetic Analyzer, ABIPRISM, Applied Biosystems, Foster City, CA, USA). The sequencing reactions were performed at the Division of Molecular Genetics at the Department of Laboratory Medicine of the University of Debrecen.

Heteroduplex analysis

This mutation screening method is used for examination of the heterozygous states. The amplified DNA products were analyzed either with SURVEYOR Mutation Detection Kit (Transgenomic Ltd., Elancourt, France) or with the conventional method.

The double-stranded DNA were heated and treated with surveyor nuclease. This enzyme recognizes and cleaves all types of mismatches arising from SNPs. DNA fragments were separated using 6 % polyacrylamide gel electrophoresis and visualized by silver staining.

The conventional method includes the high temperature for formation of single strands from the double strands of DNA. After this step, the slow cooling could help the formation of heteroduplexes which could be detected by electrophoresis and silver staining.

4.7. Statistical analysis

For the evaluation of data Student's *t*-test was used. For analysis of the genotype frequencies we used 3 x 2 contingency table, and for that of allele frequencies and odds ratio (OR) 2 x 2 contingency table was used. The Hardy-Weinberg (H-W) equilibrium was estimated according to the $\chi 2$ test. Values of p below 0.05 were regarded as significant.

5. RESULTS

5.1 Simple method for examination of catalase rs769217 polymorphism in exon 9

PCR products of the 2, 3, 7, and 9 exons of the catalase gene were examined by polyacrylamide gel electrophoresis and silver staining. The electrophoretic patterns of exons 2, 3, 7 showed the double-stranded PCR products in the 200 and 400 bp region. After denaturation the single-stranded DNA bands could be detected with a weaker intensity in the 400-600 bp regions.

For the exon 9 we got a different pattern when the step of denaturation was omitted. Double-stranded DNA 238 bp length bands have a lower intensity and 400- 600 bp regions showed a highly stained and well separated one or two bands. For the identification of this phenomenon we performed the following experiments.

1. Different DNA staining procedures of silver impregnation, SYBR Green II, and SYBR Green I were used. The bands appearing in the 400-600 bp region were only visualized by the single-stranded DNA (SYBR Green II) staining while the silver staining detected bands both regions.
2. DNA sequence analyses of the samples with the newly detected bands were performed. The DNA sequence analysis of the samples was consistent with the electrophoretic pattern. In the 400-600bp region, the fast migrating band is the homozygous TT mutant, the slowly migrating is the homozygous CC wild. When both bands appear it can be identified as the CT heterozygous genotype.
3. The simple method and the DNA sequencing yielded the same results for the genotypes in 74 samples.
4. Evaluation of samples of 72 subjects with the SSCP (denaturing with formamide / sodium hydroxide at 94^0C for 6 minutes) and the simple method without denaturation yielded the same genetic types for every sample. The denaturation free pattern does not contain any further conformers than denatured samples that make it.
5. During the treatment of PCR products with nuclear enzyme (SURVEYOR Mutation Detection Kit) the double stranded heteroduplex and single-stranded DNA are cut. After the cutting of the heterozygous mutant (CT genotype) and single stranded DNAs only the smaller (158bp) cutting product could be detected on the gel.
6. PCR-RFLP analysis was also used as a further comparative test. PCR products were digested with BstX1 (Amersham Pharmacia Biotech UK, Buckinghamsshire, England) restriction enzyme. The restriction enzyme could recognize the CCANNNNN * NT ** GG sequence which can be found in the exon 9 examined region. The two methods yielded the same genotypes.

The C111T simple polymorphism detection method was investigated for the further known polymorphisms (C37T, G113A, G5) of exon 9 of the catalase gene. For the C37T, G113A and G5A polymorphisms, the method could not be a useful tool to identify the genotypes.

Summarizing the results of 1-6 experiments, the spontaneously formed single-stranded DNA pattern without denaturation can be used to examine C111T polymorphism (rs769217, +22348) in exon 9 of the catalase gene.

5.2 Examination of the rs769217 polymorphism of the catalase gene in Hungarian microcytic anemia, beta-thalassemia patients and control subjects

The method showed in the previous chapter was used to examine the C111T polymorphism of exon 9 in the catalase gene. In this study, genotypes of 55 microcytic anemia, 43 beta-thalassemia patients, and 50 controls were investigated and compared to European data. The C allele (71%) seems to be the same in the Hungarian control group and in European (80%)

individuals. There was no significant difference in the frequency of C alleles in microcytic anemia (67%, p=0.559) and beta-thalassemia (65%, p=0.265) when compared to the European control group.
The genotype distribution in the European control group showed significant differences compared to the Hungarian microcytic anemia (p=0.0059) and beta-thalassemia (p=0.049). The blood-catalytic activity of the microcytic anemic group is lower than that of the Hungarian control group ($p<0.025$), similarly to those of the beta-thalassemia group and control group ($p <0.016$). Comparison of the catalase activity of the microcytic anemic CC genotype (89 ± 25 MU/L) with the TT genotype (64 ± 31MU/L) yielded a significant (p=0.005) decrease for the TT genotype.
Compared with the TT genotype (75 ± 3 MU/L) of the CC genotype (92 ± 19 MU/L). a significant decrease (p=0.044) of blood catalase was detected in the beta-thalassemia patients.

5.3 The effect of blood catalase polymorphisms of rs769217 and rs1001179 on carbohydrate and lipid biomarkers in diabetes mellitus

5.3.1. rs769217 (C111T in exon 9, + 22348C>T) polymorphism

In the Hungarian diabetes and control group, the frequency of the C wild allele (68% and 72%) was higher than that of the T mutant allele (28% and 32%). The TT homozygous mutant genotype (12%) was higher in type 1 diabetes mellitus than in type 2 (9%, $p<0.02$) and in the control group (9%, $p<0.02$). However, the catalase enzyme activity did not show any significant decrease for these cases. We found that there is a significant association between the genotypes and the age (CC: 45 ± 13 years, CT: 39 ± 14 years, TT: 36 ± 10 years, $p <0.04$).
There were no significant differences in genotypes, lipid and carbohydrate parameters for type 1 diabetic patients ($p>0.08$).
In type 2 diabetes a significant decrease ($p<0.001$) was detected for the catalase activity in CC and CT genotype samples when they were compared either to those of the controls and or to type 1 diabetics. In type 2 diabetes, a significant increase of glucose concentration was detected when the patients with the TT genotypes were compared to those with the CT genotypes ($p<0.02$). Furthermore, hemoglobin A1c ($p<0.03$) and ApoB ($p<0.05$) concentrations were higher for CT genotypes than those of CC genotype.

5.3.2. rs1001179 (5'UTR region, – 262C/T) polymorphism

For his polymorphism we found significant decrease in blood catalase activity in type 2 (91 ± 20 MU/L, $p<0.001$) and in type 1 diabetes (95 ± 20 MU/L, $p<0.048$) compared to the control group (105 ± 17 MU/L). In type 1 diabetes, genotype analysis showed changes in blood catalase activity for CC (96 ± 26 MU/L) and CT (94 ± 30 MU/L) compared to control (CC: 109 ± 25 MU/L, CT: 102 ± 16 MU/L, $p <0.05$).
In type 2 diabetes the blood catalase was found significantly decreased ($p<0,05$) for all three genotypes (CC: 98 ± 30 MU/L, CT: 88 ± 34 MU/L, TT: 84 ± 23 MU/L) compared to the control group (105 ± 17 MU/L). The greatest difference was detected for the TT genotype group ($p <0.03$) compared to the control group.
There was a significant difference in genotypes of type 2 diabetes in carbohydrate biomarkers such as glucose (CC and TT, $p <0.02$), HbA1c (CC and TT, $p <0.05$) similarly to the lipid biomarkers of cholesterol (CC and TT, $p <0.01$), HDL (CC and TT, $p <0.05$), ApoB (CC and TT, $p <0.05$).
No significant difference ($p>0.1$) was found between type 1, type 2 diabetics and the controls either for the genotypes or for alleles.

5.4 Screening program for new acatalasemia mutations

Of 617 patients 51 subjects yielded blood catalase activity below 50% of the reference mean. These patients had diabetes mellitus (18, 35%), microcytic anemia (14, 27%), presbyacusis (10, 19.7%) and beta-thalassemia (4, 7.8%).

In patients with decreased blood catalase 14 patients had decreased MCV (71 ± 10 fL, reference 89 ± 9 fL, $p<0.0001$) and decreased blood hemoglobin concentrations (129 ± 14 g / L, 134 ± 20 g /L, $p>0.05$).

The mutation screening yielded 4 novel acatalasemia mutations in 7 patients. Based on these results, the novel acatalasemia mutations were named G1, H1, -2, and -3.

More than half of the new mutations (4, 57%) were found in diabetic patients. Of the 4 patients, three are type 2 and one with gestational diabetes.

5.4.1. The novel acatalasemia mutations

Four novel exon mutations were found in seven patients and the number of acatalasemia mutations increased from 11 to 15.

The novel G1 type is caused by a C insertion in exon 2 (c. 106_107insC). This insertion causes a frame shift change from amino acid 35Gly to a TGA stop codon at position 70 (p.G36Afs*5). This truncated catalase protein with its 70, instead of the 526, amino acids lack enzymatic function.

The Arg127Tyr substitution (Type H1) may decrease the activity of catalase by changing the shape of the main channel and decreasing access of the substrate to the active center. This missense c.379C>T mutation was detected in one female patient with microcytic anemia and in another female with gestational diabetes mellitus.

Type H2 is caused by a missense (c.390T>C) mutation with changing Arg129 to Leu. This mutation also could change the shape of the main channel of the catalase enzyme.

The H3 (c.431A>T) type of Hungarian acatalasemia was detected in a hypocatalasemic patient with type 2 diabetes mellitus.

6. DISCUSSION AND SUMMARY

Examination of PCR products of catalase exon 9 with polyacrylamide gel electrophoresis revealed extra band(s) in the 400-800 bp range. The use of PCR-SSCP denaturation and specific staining technique with SybrGreen II, and nucleotide sequencing confirmed that the bands in question may be single-stranded DNAs. These bands located in the 400-800 bp range can be used to examine exon 9 polymorphisms. The highest level of sensitivity was detectable for the C111T polymorphism of exon 9 (rs769217).

The method described by us is a simple, fast and cost-effective technique for genotype determination of C111T polymorphism in exon 9.

This method was used to examine the genotypes of C111T polymorphism in microcytic anemia and beta-thalassemia patients.

Decreased blood catalase activity was observed in the case of the TT genotype of C111T polymorphism.

The TT genotype of polymorphism C111T could be frequently detected (12%) in type 1 diabetes. However, no significant changes were detected in blood catalase activity, glucose, hemoglobin A1c, triglyceride, cholesterol, HDL, LDL, ApoA-I and APOB concentrations.

On the other hand, in type 2 diabetes significantly decreased blood catalase was measured in the samples of CC and CT genotypes, accompanied by increased glucose, hemoglobin A1c and ApoB concentrations.

We examined the nucleotide polymorphism -262C>T (rs1001179) in the 5' region which may play a role in regulatory processes. Decreased blood catalase activity could be detected in type 1 diabetics with CC and CT genotypes, as well as in type 2 diabetics with CC, CT, TT genotypes. The samples of type 2 diabetics with this polymorphism showed significant decreases when blood catalase activity, age and HDL concentration were measured. Furthermore, they showed an increase in glucose, hemoglobin A1c, cholesterol, and ApoB concentrations.

We set up a cohort comprising 51 patient samples for which patients' blood catalase activity was below 50% of the reference mean.

Four novel acatalasemia mutations were detected, which increased the number of so-far known types of Hungarian acatalasemia from 11 to 15. Seven out of the 51 samples revealed four novel mutations belonging to samples in the type 2 diabetes and microcytic anemia cohorts. These results provide a new evidence for acatalasemia being a potential risk factor in several age-related diseases.

7. NEW RESULTS

1. We examined the catalase gene exons for possible polymorphisms. The polyacrylamide gel electrophoresis of PCR product of catalase exon 9 yielded a new pattern. By using mutation screening methods (PCR-SSCP, single-strand and double-strand DNA staining, nuclease digestion, PCR-RFLP) we could clearly demonstrate that conformational changes may result in single-stranded DNAs in the 400-800bp region.
2. The bands seen in the electrophoretic pattern can be used for the detection of polymorphisms of catalase gene in exon 9. With the help of this simple, fast and cost-effective technique polymorphisms can be determined without sequence analysis.
3. The method developed by us could be used to detect C111T (rs769217) polymorphism and determine genotypes with very high efficiency.
4. Further exon 9 polymorphisms (C37T, G113A, G5A) can also be detected using this method with a lower efficiency. For these nucleotide changes the genotypes could not be determined clearly.
5. Our research group was the first to use the method we had validated to examine Hungarian microcytic anemia and beta-thalassemia patients as well as the Hungarian control group. Significantly lower blood catalase activity was detected in both microcytic anemia and beta-thalassemia samples of TT homozygous mutant genotype.
6. The results of the two cohorts were compared to Hungarian controls, the European population and to data from the NCBI international database.
   Both cohorts (Hungarian microcytic anemic and beta-thalassemia patients) showed decreased blood catalase activity compared to the control group.
7. We were the first to examine the association between polymorphism C111T and decreased blood catalase.
8. Furthermore, it was our group to first perform a detailed analysis of the two known catalase gene polymorphisms (rs769217 and rs1001179) in type 1 and type 2 diabetes mellitus cohorts.
9. Detailed comparative analysis was done on the effect of polymorphisms rs769217 and rs1001179 in the Hungarian control group and patients with type 1 and type 2 diabetes mellitus.
10. We followed up on the change in 10 important diagnostic parameters (age, blood catalase, glucose, HgbA1c, triglyceride, cholesterol, HDL, LDL, ApoA-I, ApoB) in cohorts with type 1 and type 2 diabetes and the control group.
    The samples of the mutant (rs1001179 TT genotype) patients revealed decreased blood catalase activity accompanied by increased glucose and HbA1c concentrations. The same cohort showed increased cholesterol, HDL and ApoB concentrations. Associations were detected between type 2 diabetes and the TT mutant genotype of polymorphism rs1001179.
11. We set up a cohort comprising 51 patients with their blood catalase below 50% of the reference mean.
12. For these patients we examined all exons of the catalase gene.
13. For seven patients four novel acatalasemia mutations were detected.
14. These mutations are listed below.
    G1 type is caused by a C insertion in exon 2 (c. 106_107insC).
    The H1 type is caused by a missense c.379C>T mutation with Arg127Tyr substitution.
    Type H2 is caused by a missense (c.390T>C) mutation with changing Arg129 to Leu.

The H3 type has a nucleotide (c.431A>T) substitution which yields an Asp143Val amino acid change.

15. The number of Hungarian acatalasemia mutations increased from 11 to 15.
16. The novel acatalasemia mutations were detected in three microcytic anemia patients and four diabetics.
    These results suggest that the decreased blood catalase especially the increased hydrogen peroxide concentration due to the catalase deficiency may play a role in the pathology of these diseases.

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List of publications related to the dissertation

1. **Nagy, T.**, Pászti, E., Káplár, M., Bhattoa, H. P., Góth, L.: Further acatalasemia mutations in human patients from Hungary with diabetes and microcytic anemia.
Mutat. Res. Fundam. Mol. Mech. Mutagen. 772, 10-14, 2015.
DOI: http://dx.doi.org/10.1016/j.mrfmmm.2014.12.008
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2. **Nagy, T.**, Csordás, M., Kósa, Z., Góth, L.: A simple method for examination of polymorphisms of catalase exon 9: rs769217 in Hungarian microcytic anemia and beta-thalassemia patients.
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3. Góth, L., **Nagy, T.**, Kósa, Z., Fejes, Z., Bhattoa, H. P., Paragh, G., Káplár, M.: Effects of rs769217 and rs1001179 polymorphisms of catalase gene on blood catalase, carbohydrate and lipid biomarkers in diabetes mellitus.
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List of other publications

4. Góth, L., **Nagy, T.**, Paragh, G., Káplár, M.: Blood Catalase Activities, Catalase Gene Polymorphisms and Acatalasemia Mutations in Hungarian Patients with Diabetes Mellitus. *Glob. J. Obes. Diabetes. Metab. Syndr. 3* (1), 001-005, 2016.

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