Az öröklött és szerzett kataláz hiány klinikai és klinikai biokémiai vonatkozásai című OTKA pályázat eredményeinek ismertetése

I. A kataláz enzim és hidrogénperoxid metabolizmus

A szervezetben hidrogénperoxid képződik fiziológiás és patológiás folyamatok során. A nagy koncentrációjú hidrogénperoxid, de különösen a belőle képződő hidroxil gyök károsíthatja a különböző sejteket. A legújabb kutatások azonban a kis koncentrációjú hidrogénperoxid fizológiás szerepéről számolnak be. Ez főként a diabetesben, trombocita aktiválásban, gyulladásban és az immunválaszban lejátszódó jelátviteli folyamatokat érinti (hidrogénperoxid paradoxon). Az emberi szervezetben a hidrogénperoxid koncentráció 0,1 nmol/l és 100 μmol/l között változik.

A hidrogénperoxid koncentráció fő szabályozó enzime a kataláz, amely ezt a feladatot a speciális kinetikája révén biztosítja, gyorsan bontja a toxikus szubsztrát koncentrációt és szinte hatástalan a fiziológiás koncentrációra.

Az akatalazémia, a kataláz enzim veleszületett hiánya, amit korábban tünetmentesnek tartottak, az újabb eredmények ismeretében azonban inkább szindrómának tekinthető, mivel megváltozott lipid, szénhidrát (a diabetes mellitus fokozott előfordulása) és vörösvértest metabolizmus jár együtt. *(Góth L: A kataláz enzim klinikai vonatkozásai és mutációi Magyarországon. LAM 2005: 15: 274-278. Góth L: A hidrogénperoxid paradoxon. Orvosi Hetilap 2006: 147: 887-893).*

II. A vér kataláz aktivitás változás különböző megbetegedésekben, veleszületett kataláz hiányban és a felelős kataláz gén mutációk

Veleszületett kataláz hiányos családok kataláz hiányos tagjainál csökkent ($p<0,01$) fólsav (5,44 vs 7,56 ng/ml), vér hemoglobin (140 vs 153 g/l), emelkedett homocisztein (9,72 vs 7,36), MCV (96,5 vs 90,5 fl) értékeket mértünk, míg a szérum és vér hemolízis markerek nem változtak.

A veleszületett kataláz hiány az emelkedett homociszteinnel fokozottan járulhat hozzá a szabadgyök képződéshez, amely felelős lehet a mérsékelt anémiáért. *(Góth L, Vitai M. The effects of hydrogen peroxide promoted by homocysteine and inherited catalase deficiency on humán hypocatalasemic patients. Free Radical Biology and Medicine 2003: 35: 882-888.).*

Terhességben csökkent átlagos vér kataláz aktivitást mértünk (89 vs 109 MU/l), amely terhességi diabetesben még tovább csökkent (74 MU/l). A vér kataláz csökkenés nem mutatott nagyobb veszélyeztetettséget sem az anyára sem a magzatra. A kataláz aktivitás csökkenés nem volt magyarázható az ismert

kataléz gén mutációkkal. (*Góth L, Tóth Z, Tarnai I, Bérces M, Török P, Bigler WN. Blood catalase activity in gestational diabetes is decreased but not associated with pregnancy complications. Clinical Chemistry 2005: 51: 2401-2401).*

A diabetes mellitus 1-es típusában csökkent a vér kataláz aktivitás (71,2 vs 104, 7 MU/l, p<0,01), míg a 2-es típusnál ez a csökkenés (102,5 vs 104,7 MU/l) nem volt szignifikáns. A a kataláz gén 9. exon C111T polimorfizmusának vizsgálata azt mutatta, hogy a mutáns T allél kismértékű aktivitás csökkenéssel járt 1-es típusban és kimértékű aktivitás növekedéssel a 2-es típusnál. (Tarnai I, Csordas M, Sukei E, Shemirani A, Káplár M, Góth L. Effect of C111T polymorphism in exon 9 of the catalase gene on blood catalase activity in different types of diabetes mellitus. Free Radical Research submitted for publication 2006. November).

A diabetes 2-es típusában szenvedő 308 betegnél a kataléz gén 2. exonjának analizíse 11 esetben mutatott mutációt. Ezekből 5 esetben a már korábban leírt Magyarország A és B típus volt kimutatható. A maradék 6 mutációból kettő bizonyult olyan új mutációnak, amely a kataláz hiányáért felelős. *(Vitai M, Fátrai Sz, Rass P, Csordás M, Tarnai I. Simple PCR-heteroduplex, SSCP mutation csreening methods for the detection of novel catalase mutations in Hungarian patients with type 2 diabetes mellitus. Clinical Chemistry and Laboratory Medicine 2005: 43: 1346-1350).*

Egy Magyarországi veleszületett kataláz hiányos családnál (D) meghatároztuk a kataláz hiányért felelős mutációt, amely a 9. exon 111-es poziciójában egy G→A szubsztitució révén kialakuló aminosavcsere (Arg354Cys) következtében alakult ki. A proband 2-es típusú diabetes mellitusban szenved. (Góth L, Vitai M, Rass P, Sükei E, Páy A. Detection of a novel catalase mutation (Hungarian type D) and the possible risk of inherited catalase deficiency for diabetes mellitus. Electrophoresis 2005: 26: 1646-1649).

Egy új, egyszerű gén mutáció kimutatására fejlesztettünk ki eljárást, amellyel veleszületett kataláz hiányban a Magyarországi E típust definiáltuk (9. exon C39T), amely az essenciális Arg254-et változtatja Cys-é. (Az eljárást szabadalmaztatás engedélyezésére a Debreceni Egyetemhez benyujtottuk, és addig ezt nem közöljük).

Egy Letter to the Editor-ban foglaltam össze a reaktív oxygen speciesek, a hidrogénperoxid, a kataláz enzim feltételezett szerepét a diabetes mellitus patomechanizmusában. Kiemelvtem, hogy nem a mutáció, hanem ennek hatására megnövekedett egész életen át ható hidrogénperoxid koncentráció lehet felelős a veleszületett kataláz hiányban detektált diabetes mellitus fokozott 12,1%-os előfordulásáért. *(Góth L. Reactive oxygen species, hydrogen peroxide, catalase and diabetes mellitus. Redox Report 2006: 11: 282-282).*

III. Összefoglaló közlemények

A katalázról és aveleszületett kataláz hiányról készített összefoglalomat az IFCC (International Federation of ClinicalChemistry) kongressusán Orlando, Miami, USA, 2005) Round Table presentációban ismertttem. A Research Sign Post kiadó felkért egy könyv szerkesztésére (ROS and Disease) amelyben két fejezetet írtam a katalázról.

Az irodalmi adatokat és saját ereményeinket felhasználva Review készítettünk az ismert kataláz gén polimorfizmusokról, az akatalazémiában detektált kataláz mutációkról és ezek kapcsolatáról a különböző betegségekkel. *(Góth L, Rass P, Páy A. Catalase enzyme mutations and their association with diseases. Molecular Diagnosis 2004: 8: 141-149).*

2006 februárban nyujtottam be MTA doktori pályázatomat, amelyben külön kiemeltem a jelenlegi és korábbi OTKA pályázatok pénzügyi támogatásának jelentőségét. Doktori értekezésem téziseit Gilyén Elemérné OTKA igazgatónőnek is eljutattam, és 2007. február 2.-án a sikeres védés megtörtént.
Az MTA doktori értekezés téziseinek az OTKA pályázat ideje alatt elért eredmények összefoglalása.

A SZÉRUM KATALÁZ ÉS A VÉR KATALÁZ CSÖKKENÉS VELESZÜLETETT ÉS SZERZETT FORMÁINAK PATOBIOKÉMIAI ÉS GENETIKAI VIZSGÁLATA MAGYARORSZÁGON

GÓTH LÁSZLÓ

6. 3. 2. A veleszületett kataláz hiány klinikai vonatkozásai

A kataláz hiányos egyéneknél fokozottan (12,7%) fordult elő a diabetes mellitus. A 63 kataláz hiányos egyén közül diabetes mellitusban szenvedett mindkét akatalazémiás és hat hypokatalazémiás egyén (1-es típus: 1 hypokatalazémiás, 2-es típus: 2 akatalazémiás és 5 hypokatalazémiás). Kontroll volt a hypo- és akatalazémiás családok 66 normokatalazémiás tagja, akik között nem fordult elő diabetes mellitus.

A jelenség magyarázata lehet, hogy az egész életen áttartó csökkent kataláz hiány révén megemelkedett steady state koncentrációjú, oxidatív hidrogénperoxid károsítja az oxidációra érzékeny pankreász β sejteket. Ezzel magyarázható a hypokatalazémiások csökkent inzulin és C-peptid koncentrációja is.

A megnövekedett hidrogénperoxid koncentráció hatásának tulajdonítható a szintén oxidációra érzékeny fólsav koncentrációjának csökkenése, és az ennek következtében kialakult mérsékelt anemia.

Ez a két klinikai tanulmány arra hívja fel a figyelmet, hogy a korábban tünetmentesnek tartott kataláz hiány esetén az ebben szenvedő egyének fokozottabban érzékenyek a diabetes mellitusra és a fólsav hiányra/anémiára.

Ezt látszanak igazolni azok a klinikai tanulmányok, amelyekben a fokozottan képződő hidrogénperoxid (urikáz terápia, hidrogénperoxidos fertőtlenítés, terápiás koncentrációjú aszkorbinsav)a kataláz hiányos betegeknél hemolízist, methemoglobin képződést eredményezhet.

6. 3. 3 Biokémiai vizsgálatok kataláz hiányban

A kataláz hiányos egyéneknél a krónikus hemolízis nem detektálható, és az oxidatív stressz inkább a fólsavon keresztül gyakorol hatást a vörösvértest szám alakulására.

Az oxidatív stressz néhány megbetegedés, köztük az atherosclerosis, diabetes mellitus patomechanizmusában is szerepet játszik. És a nem konvencionális (Lp(a) és LDL oxidatív rezisztencia) kockázati tényezőkben jelentkező eltérések az akatalazémiás, hypokatalazémiás egyének fokozott arteriosclerosis veszélyeztetettségét jelenthetik. Ezt támasztja alá, hogy az elhalálozott 1 akatalazémiásnál és a négy hypokatalazémiás közül 3-nál a korához képest fokozott atherosclerosist mutatott a kórboncolás.

6. 3. 4. A kataláz hiány genetikai vizsgálata

A kataláz hiány genetikai okának meghatározásához protokollt dolgoztam ki és a tudomány mindenkori állásának megfelelő eljárásokat alkalmaztam.

Vizsgálataim során kimutattam, hogy a Japán A és B altípusok mutáció a magyarországi kataláz hiányos egyéneknél nem mutathatók ki.

A 14 ismert benignus kataláz poliformizmusok számát 8 újjal gyarapítottam.

6. 3. 5. A Magyarországi akatalazémia genetikai típusai

A Magyarországi A-típusnál a PCR-heteroduplex analizís jelezte a mutációt.

A 2. exonban a GA inszerció az akatalazémiás és 3 hypokatalazémiás családban 2 akatalazémiás és 29 hypokatalazémiás egyénnél volt detektálható. Az inszerció révén frameshift alakul ki, ami STOP kodont generálva trunkált proteint eredményez.

A Magyarországi B típusnál hasonló polimorfizmust jelzett a PCR-heteroduplex analizís. A 2. exonban jelentkező G inszerció eredménye a frameshift, amely STOP kodont generálva szintén trunkált proteint eredményezett.

A Magyarországi C típusnál a 7. intronban az PCR-SSCP mutáció szűrési eljárás mutatott polimorfizmust, ami egy splicing mutáció eredménye. A mutáció révén csökkent kataláz aktivitást Western blot vizsgálattal erősítettük meg.

A Magyarországi D típusnál a 9. exonban egy missense mutáció a 354 arginint változtatta hisztidinné. A 354 Arginin a katalitikus folyamatban játszik szerepet.

A Magyarországi E típusnál szintén a 9. exonban mutatható ki egy missense mutáció. Ez a mutáció a 364Arginint változtatja ciszteinné. A 364Arginin a hem vasának reakcióképességét határozza meg.

6. 3. 6. A Magyarországi örökletes kataláz hiány értékelése

A Magyarországon elsőként detektált örökletes kataláz hiány (1 akatalazémiás és 13 hypokatalazémiás családban), és ennek komplex (klinikai, klinikai biokémiai) jellemzése alapján állítható, hogy ez a régről ismert veleszületett kataláz hiány egy új formája, amelyet új genetikai mutációk is jellemeznek.

A klinikai elváltozások (diabetes, mérsékelt anemia) feltehetően a hidrogénperoxid révén inkább a fokozottabb oxidatív stressznek tulajdoníthatóak, mint a mutáció típusának, mivel ezek a különböző mutációk esetén tapasztalhatók.

A veleszületett kataláz hiányt korábban tünetmentes, egyszerű enzimhiánynak tartották. A Magyarországi akatalazémia klinikai, klinikai biokémiai és genetikai vizsgálatai alapján talán inkább syndromanak tekinthető.

A kataláz hiányos egyének fokozottan érzékenyek a nagy hidrogénperoxid koncentrációt előidéző patológiás, terápiás hatásokra.

Az 1 akatalazémiás és 13 hypokatalazémiás családból az akatalazémiás és 8 hypokatalazémiás család genetikai jellemzése már megtörtént, míg a további családok és a kataláz mutációk, valamint és a betegségek kapcsolatának vizsgálata a jövő feladata.

IV. Jelen beszámolómhoz csatolom a két nyomtatásban még nem jelent kézirat másolatát.

Effect of C111T polymorphism in exon 9 of the catalase gene on blood catalase activity in different types of diabetes mellitus

Tarnai I[1], Csordas M[1], Sukei E[1], Shemirani A[2], Káplár M[3], Góth L[1,2].*

[1]Department of Clinical Analytical Chemistry,

[2]Department of Clinical Biochemistry and Molecular Pathology,

[3]Department of Internal Medicine

Medical and Health Science Center,

University of Debrecen, Debrecen H-4032, Hungary

*Corresponding author

Running title: catalase C111T polymorphiosm in diabetes

Key words: diabetes mellitus: type 1, type 2, gestational, blood catalase, C111T polymorphism in exon 9, mutation determination.

ABSTRACT

Hydrogen peroxide plays a major role in the pathomechanism of diabetes mellitus. Catalase is the main regulator its metabolism.

The blood catalase and the C111T polymorphism in exon 9 was examined in type 1, type 2 and gestational diabetes mellitus.

Compared to the control group (104.7±18.5 MU/L) significantly decreased ($p<0.001$) blood catalase activities were detected in type 2 (71.2±14.6 MU/L), gestational (68.5±12.2 MU/L) diabetes mellitus and without change in type 1 (102.5±26.9 MU/L). The blood catalase did not change ($p>0.063$) with age for type 1, gestational diabetic patients and controls. Type 2 diabetic patients yielded an adverse, significant (p:0.043) effect. Blood catalase showed a weak association with hemoglobin A1c for type 1 diabetic patients (r=0.181, increasing).

The mutant T allele was increased in type 1 and gestational diabetes mellitus, and CT + TT genotypes showed decreased blood catalase activity for type 1 and increased activities for type 2 diabetic patients.

The C111T polymorphism may implicate a very weak effect on blood catalase activity in different types of diabetes mellitus.

INTRODUCTION

Increasing evidence in both experimental and clinical studies suggest that oxidative stress plays a major role in the pathogenesis and in the long term complications of diabetes mellitus. Free radicals are formed in diabetes by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins [1]. Increased flux of glucose may cause generation of excess of reactive oxygen species. These species and the

impaired antioxidant status are involved in the oxidative stress associated with diabetes mellitus. The pancreatic beta-cells are sensitive to oxidative stress due to their very low activities of the main antioxidant enzymes which are superoxide dismutase, glutathione peroxidase and catalase. The high glucose flux may change the expression of these enzymes that protect against oxidant damage and may accentuate the oxidative injury [2]. Superoxide dismutase converts the superoxide anion into less toxic hydrogen peroxide but its high concentration also causes damage of cells, proteins and DNA. This effect is more serious when the very aggressive hydroxyl radicals are formed from hydrogen peroxide in the Fenton reaction. On the other hand, the low concentration of reactive oxygen species, including hydrogen peroxide which is generated in response to insulin stimulation, may serve as a second messenger in the insulin action cascade [3]. It can mimic insulin action by oxidation of the cystein rich part of protein-tyrosine kinases and phosphatases such as insulin receptor and insulin substrates [4].

The enzyme catalase is the main regulator of the hydrogen peroxide metabolism especially in the erythrocytes [5.6]. Due to the small, chargeless, and diffusible hydrogen peroxide molecule the high concentration of erythrocyte catalase can control the hydrogen peroxide formed inside and outside the erythrocytes [7]. High concentration of hydrogen peroxide is decomposed by catalase in a very effective and fast reaction [8] but due to the special Ser 198 and Asp 144 gating mechanism, catalase reveals a very weak affinity to the physiological concentration of hydrogen peroxide [8,9].

We have reported on the high incidence (11.7 % vs 1.7 %) of diabetes mellitus in inherited catalase deficiency [10], and decreased blood catalase activities in different types (1, 2, and gestational) of diabetes mellitus [11,12]. The chronic exposure to relatively high levels of hydrogen peroxide have also been associated with functional beta-cell impairment and the chronic

complications of diabetes [3,13,14]. The experiments with cell lines showed that the overexpression of catalase protected the insulin producing pancreatic beta cells against hydrogen peroxide toxicity [15].

There are only few papers on the association of diabetes and catalase mutations [16]. Chystakov et al. found a weak association of C1167T polymorphism in the flanking region of the catalase gene and diabetes mellitus [17].

The C111T polymorphism of exon 9 was first detected in Japan [18]. It is a by-product which was detected during the molecular analysis of human acatalasemia. In 2002, Casp et al. found a possible genetic association of this polymorphism and vitiligo susceptibility [19] supposing the different gene expression of the wild and mutant alleles. The different expression of the wild and mutant alleles have been discussed widely but these catalase alleles have not been reported to support this phenomena [20]. Moreover, Gavalas et al. in a recent paper [21] could not confirm this association in vitiligo.

The decreased blood catalase activity together with the increased production of hydrogen peroxide in diabetes mellitus [22] leads to the increase of hydrogen peroxide concentration and its toxic effects. The low catalase activity is likely to result from allelic variants in the catalase gene which may cause different expression or different regulation. There are no data concerning the effect of C111T single nucleotide polymorphism on the catalase activity in diabetes mellitus.

The aim of this study was to measure the blood catalase activities, to examine its relation to the diabetic therapeutic marker of haemoglobin A1c, to determine C111T polymorphism in exon 9, and to examine the possible effect of this single nucleotide polymorphism on blood catalase activity in different types (type 1, type 2, gestational) of diabetes mellitus.

MATERIALS AND METHODS

33 pregnant women with gestational diabetes but without family history of diabetes mellitus were included in this study, who underwent treatment at the Department of Obstetrics and Gynecology of Medical Health Science Center. All received insulin therapy and blood samples were taken in the second trimester for women between the ages of 23-41 years (mean±sd= 30.8±4.6 years).

106 patients with type 1 (insulin dependent) diabetes mellitus were from the Diabetes Clinics of Internal Medicine Department of Medical and Health Science Center. They were treated with insulin and their therapy was followed by measurement of blood hemoglobin A1c. This group had 46 males (43.4 %, 43.3±21.8 years) and 60 females (56.6 %, 48.7±20.5 years).

100 patients with type 2 (non-insulin dependent) diabetes mellitus were treated at same department. This group included 33 males (33 %, 56.6±17.1 years) and 67 females (67 %, 58.9±15.9 years).

The control subjects were 60 hospital employees of the Medical Health Science Center of Debrecen University from the Eastern region of Hungary. Subjects were excluded if they had medical history of diabetes or if they were gravid.

The patients and controls were randomly selected during a period from 2003 to 2005.

Blood samples were stored at -20°C for two days for blood catalase determination and not more than 7 days for extraction of genomic DNA.

Blood catalase activity was measured by a spectrophotometric assay with the reference range of 113.3±16.5 MU/l (n=1756). The within run and day to day precision (coefficient of variation) of blood catalase determination is 3.1 % and 5.1 % [23,24].

Blood hemoglobin A1c (HbA1c) was measured with a HPLC system (Diamat, Bio-Rad, Hercules, CA, USA) with a reference range of 4.2 % to 6.1 %.

Genomic DNA was extracted from leukocytes using a QIAmp Blood Kit from Qiagen (Hilden, Germany). PCR and primers (forward primer:

tgttactgccctagtcagt, reverse primer: agagggcacgtggagcagat) were the same as described by Kishimoto et al. [25] and the region amplified included 139 nucleotides in exon 9, plus 56 nucleotides of intron 8 and 43 nucleotides of intron 9. Reagents (ReadyMix REDTaq with $MgCl_2$) and primers were purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri, USA). Amplifications were performed in total volumes of 12.0 μL. The mixture of 5.0 μL H_2O, 1 μL of each primer (10 μmol/L), and 2.0 μL of genomic DNA (0.2 μg/μL) was incubated at 94°C for 5 min. After that 5.0 μL ReadyMix RED Taq (20 mmol/L Tris-HCl pH:8,3, 100 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.002 % gelatine, 0.4 mmol/L dNTP mix, 60 U TaqDNA) was added. Thirty amplification cycles (94ºC, 55ºC, and 72ºC for 0.5 min, 0.5 min, and 1.0 min, respectively) were performed in a DNA thermal cycler (TC1, Perkin Elmer-Cetus, Norwalk, CT, USA).

For the Single Strand Conformational Polymorphism (SSCP) method 10 μL of PCR product was mixed with 4 μL of loading dyes (0.05 % bromphenol blue and 0.05 % cylene cyanol in glycerol) and loaded directly into the gel.

Electrophoresis was performed in 6 % polyacrylamide (Acrylamide for molecular biology from Sigma-Aldrich and 5xTAE puffer) gel (175x160x1.5 mm) at 170 V and room temperature for three hours. DNA bands were visualized by silver staining.

For DNA sequence analyses, the PCR products were purified by agarose gel electrophoresis.

Sequencing reactions were carried out using Taq Dye-Deoxy Termination Cycle Sequencing Kits and DNA fragments were separated and detected by capillary electrophoresis (3100-Avant, Genetic Analyzer, ABI PRISM, Applied Biosystems, Foster City, CA, USA).

The Student t-test was used to evaluate the statistical significance of differences in blood catalase activities. We used the Microsoft Excel program to perform the multivariate analyses and trend test. For case/control association

studies, the significance of observed differences in allelic or genotypic frequencies between the diabetic patients and controls was determined using standard $\chi 2$-tests. Values were considered significant at $p<0.05$.

RESULTS

The type 2 and gestational diabetes mellitus showed significant ($p<0.001$) decrease in blood catalase activities. The blood catalase was higher for males than females inpatients and in controls. This increase was significant (p:0.033) only for type 2 diabetic males.

The blood catalase showed a non significant increase ($p>0.063$) with age for type 1, gestational diabetic patients and controls. The blood catalase of type 2 diabetic patients yielded an adverse, significant (p:0.043) effect of age. These patients had the highest blood catalase (78.9±10.8 MU/L) at their lowest ages (between 20-40 years) and the lowest blood catalase (68.0±14.4 MU/L) at the their highest ages (between 61-80 years) (Table 1).

The change in blood catalase with blood hemoglobin A1c yielded an increase (m=2.412 r=0.1819) in type 1 diabetes and decreases with very weak association (m=-0.309, r= 0.036 and m=-1.537, r=0.067) in type 2 and in gestational diabetes (Fig. 1-3).

The C111T polymorphism of exon 9 of catalase gene with PCR-SSCP and nucleotide sequencing analysis is shown in Fig 4.

The mutant genotypes (CT+TT) yielded a significant ($p<0.02$) decrease in blood catalase activity for type 1 and an increase for type 2 diabetic patients (Table 2).

The frequency of mutant T allele was significantly increased in type 1 and gestational diabetes when it was compared to that of the controls (Table 3).

DISCUSSION

Our previous report [11] showed significantly ($p<0.001$) decreased blood catalase activity of 94.4±19.2 MU/L in 137 diabetic patients. In this study, we analyzed the type 1 and type 2 of diabetes mellitus separately. For comparison we used the values of a control group of 60 hospital employees whose blood catalase activity was lower (104.7±18.5 MU/L, $p<0.001$) than that of the reference subjects (113.3±16.5 MU/L n:1756). This difference may be attributed to the more serious criteria of exclusion which we used for definition of a reference subject [23].

Type 2 diabetic group revealed significantly ($p<0.001$) decreased blood catalase activities. The mean of blood catalase activity was 67.9 % when it was compared to the reference mean. The blood catalase of these patients decreased ($p:0.043$) with age, also males had higher ($p<0.001$) catalase activities than females.

For 33 patients with gestational diabetes a decreased (68.5±12.2 MU/L) blood catalase activity was received, which was similar to that (74.0±14.0 MU/L, n: 60) we reported previously [12].

These data suggest that due their decreased blood catalase activities the type 2 and gestational diabetic patients had a lower antioxidant capacity against the hydrogen peroxide, the formation of which is increased in diabetes mellitus [22].

The blood haemoglobin A1c is a widely used indicator of the carbohydrate metabolism and the control of therapy, as it reflects the mean glucose concentration of the previous 6-8 weeks. The blood catalase reflects the mean of the catalase synthesis for the same period. The relationship between blood haemoglobin A1c and blood catalase showed an increasing slope of +2.412 with a weak correlation ($r=0.182$) for type 1 diabetes mellitus. For type 2 and gestational diabetes mellitus this slope had adverse values of -0,309 and - 1,537 with a poor correlation ($r=0.036$ and 0.067).

To explain the decrease of blood catalase we could not find human examinations but some animal experiments are available in the literature. It was reported for rat mitochondrial catalase that high insulin diminishes rates of catalase synthesis [26]. Type 1 diabetes may be associated with higher as well as lower insulin concentrations due to difficulties in this therapy. The high concentration of insulin may decrease the formation of hemoglobin A1c and the synthesis of catalase which may yield low hemoglobin A1c concentration and low catalase activity. Furthermore, the low concentration of insulin favours the formation of hemoglobin A1c and increases catalase synthesis. These two antagonistic effects may cause the unchanged mean value (102.5±26.9MU/L, n:106 vs 104.7±18.5 MU/L, n:60) of blood catalase due to the wide range (4.3-17%) of hemoglobin A1c in insulin dependent diabetes mellitus.

In type 2 and gestational diabetes, due to their other pathomechanism and therapy, a limited importance could be attributed to the former effect. In other animal experiments the appropriate diabetes therapy was able to normalize the activity and protein expression of antioxidant enzymes [27] and the decrease in reactive oxygen species prevented the development of insulin resistance [28]. The elder, type 2 diabetic patients had lower catalase which may mean a decreased defense against hydrogen peroxide.

In vitiligo Casp [16] supposed that the transcription from the T allele is slower than from the C allele.

In type 1 diabetes mellitus our genetic results are in agreement with this hypothesis. The T allele frequency increased (28.7% vs 19.2%) and catalase activity of mutant genotypes decreased (0.86 vs 0.96). Contrary to this, the mean value of blood catalase did not change (102.5±26.9 MU/L vs 104.7± 18.5 MU/L).

In type 2 diabetes mellitus the T allele frequency did not change (20.0% vs 19.2%) but activity of mutant genotypes increased (1.24 vs 0.96) and mean of blood catalase significantly decreased (71.2±14.6 MU/L vs 104.7±18.5 MU/L).

In gestational diabetes the T allele frequency increased (27.2% vs 19.7%), the activity of mutant genotypes did not change (0.93 vs 0.96) but the mean catalase activity significantly decreased (68.5±12.2 MU/L vs 104.7±18.5 MU/L).

In diabetes mellitus the examination of C111T polymorphism with catalase activity determination did not support Casp's findings which were detected in vitiligo.

The allele frequencies were similar in Hungarian and USA control patients (C: 80.8% vs 82.4) but they were different from the Japanese (C: 80.8% vs 52.5%) patients [18,19].

In conclusion, the decreased blood catalase activity in noninsulin dependent and gestational diabetes means a lower capacity against hydrogen peroxide which is increased in these diseases. The C111T polymorphism seems to have a very weak effect on blood catalase activity in diabetes mellitus. The pathomechanism of these types of diabetes is different and the ways of the production of hydrogen peroxide and its regulation by catalase also seem to be different. The strong decrease in blood catalase in type 2 and gestational diabetes may be attributed to other new mutations or regulatory mechanisms which require further examinations.

Acknowledgement

This work was supported by grant from Hungarian Scientific Research Fund (OTKA TO42685).

References

1. Martim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress and antioxidants. J Biochem Mol Toxicol 2003: 17: 24-38 (review).

2. Hodgkinson AD, Bartlett T, Oates PJ, Millward BA, Demaine AG. The response of antioxidant genes to hyperglycemia is abnormal in patients with type 1 diabetes and diabetic nephropathy. Diabetes 2003: 52: 846-851.

3. Goldstein BJ, Kalayankar M, Wu XD. Redox paradox-Insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signalling targets. Diabetes 2004: 54: 311-321 (review).
4. De Meyts P. Insulin and its receptor: structure, function and evolution. BioEssays 2004: 26: 1351-1362.
5. Gaetani GF, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HN. Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. Blood 1996: 87: 1595-1599.
6. Mueller S, Reidel HD, Stremmel W. Direct evidence for catalase as the predominant hydrogen peroxide removing enzyme in human erythrocytes. Blood 1997: 90: 49-4978.
7. Nagababu E, Chrest FJ, Rifkind JM. Hydrogen-peroxide-induced heme degradation in red blood cell the protective roles of catalase and glutathione peroxides. Biochim Biophys Acta 2003:1620: 211-217.
8. Chance B, Sies H, Bovaris A. Hydrogen peroxide metabolism in mammalian organs. Phys Rew 1979: 59: 527-615.
9. Diaz A, Horjales E, Rudino-Pinera, Arreola R, Hansberg W. Unusual Cys-Tyr covalent binding in a large catalase. J Mol Biol 2004: 342: 971-985.
10. Góth L, Eaton JW. Hereditary catalase deficiencies and increased risk of diabetes. Lancet 2000: 141: 1820-1821.
11. Góth L, Lenkey A, Bigler WN. Blood catalase deficiencies and diabetes in Hungary. Diab Care 2001: 2001: 24: 445-447.
12. Góth L, Tóth Z, Tarnai I, Bérces M, Török P, Bigler WN. Blood catalase activity in gestational diabetes is decreased but not associated with pregnancy complications. Clin Chem 2005: 51: 2401-2404.
13. Heales SJR. Catalase deficiency, diabetes, and mitochondrial function. Lancet 2001: 357: 414.

14. Evans JL, Maddux BA, Goldfine ID. The molecular basis for oxidative stress–induced insulin resistance. Antioxidant Redox Signalling 2005: 7: 1040-1052.
15. Gurgul E, Lortz S, Tiedge M, Jorns A, Lenzen S. Mitochondrial catalase overexpression protects insulin-producing cells against toxicity of reactive oxygen species and proinflammatory cytokines. Diabetes 2004: 53: 2271-2280.
16. Góth L, Rass P, Pay A. Catalase enzyme mutation and their association with diseases. Mol Diagn 2004: 8: 141-149 (Review).
17. Chystakov DA, Savastyanov KV, Turakolov RI, et al. The C1167T polymorphism of the catalase gene and polymorphic markers D11S907 and D11S2008 located in the vicinity are associated with diabetes mellitus type 2. Mol Biol 2000: 34: 733-736.
18. Wen JK, Osumi T, Hashimoto T, Ogata M. Molecular analysis of human acatalasemia. J Mol Biol 1990: 211: 383-393.
19. Casp CB, She JJ, McCormick WT. Genetic association of the catalase gene (CAT) with vitiligo susceptibility. Pigment Cell Res 2002: 15: 63-66.
20. Pant KPV, Tao H, Beilharz EJ, Ballinger DG, Cox DR, Frazer KA. Analysis of allelic differential expression in human white blood cells. Genom Res 2006: 16: 331-339.
21. Gavalas NG, Akhtar S, Gawkrodger DJ, Watson PF, Weetman AP, Kemp EH. Analysis of allelic variants in the catalase gene in patients with the skin depigmentation disorder vitiligo. Biochem Biophys Res Com 2006: 345: 1586-1591.
22. Banerjee, D., Madhousoodanom, U.K., Sharana, Bapasappa, M., Ghosh, S., Jacob, J.: Measurement of plasma hydroperoxide concentration by FOX-1 assay in conjuction with tryphenylphosphine. Clin Chim Acta 2003, 337, 147-152.
23. Vitai M, Góth L. Refrence ranges of normal blood catalase activity and levels in familial hypocatalasemia in Hungary. Clin Chim Acta 1997: 261: 35-42.
24. Góth L. A simple method for determination of serum catalase activity and

revision of reference range. Clin Chim Acta 1991: 196: 143-152.
25. Kishimoto Y, Murakami Y, Hayashi K, Takahara S, Sekiya T. Detection of a common mutation of the catalase gene in Japanese acatalasemic patients. Hum Genet 1992: 86: 487-490.
26. Xu L, Badr MZ. Enhanced potential for oxidative stress in hyperinsulinemic rats: Imbalance between hepatic peroxisomal hydrogen peroxide production and decomposition due to hyperinsulinemia. Hormone Metab Res 1999: 31: 278-282.
27. Sindhu RK, Koo JR, Roberts CK, Vaziri ND. Dysregulation of hepatic superoxide dismutase, catalase and glutathione peroxidase in diabetes: Response to insulin and antioxidant therapies. Clin Exper Hypertension 2004: 26: 43-53.
28. Houstis N, Rosen E, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 2006: 440: 944-948.

Figure Legend

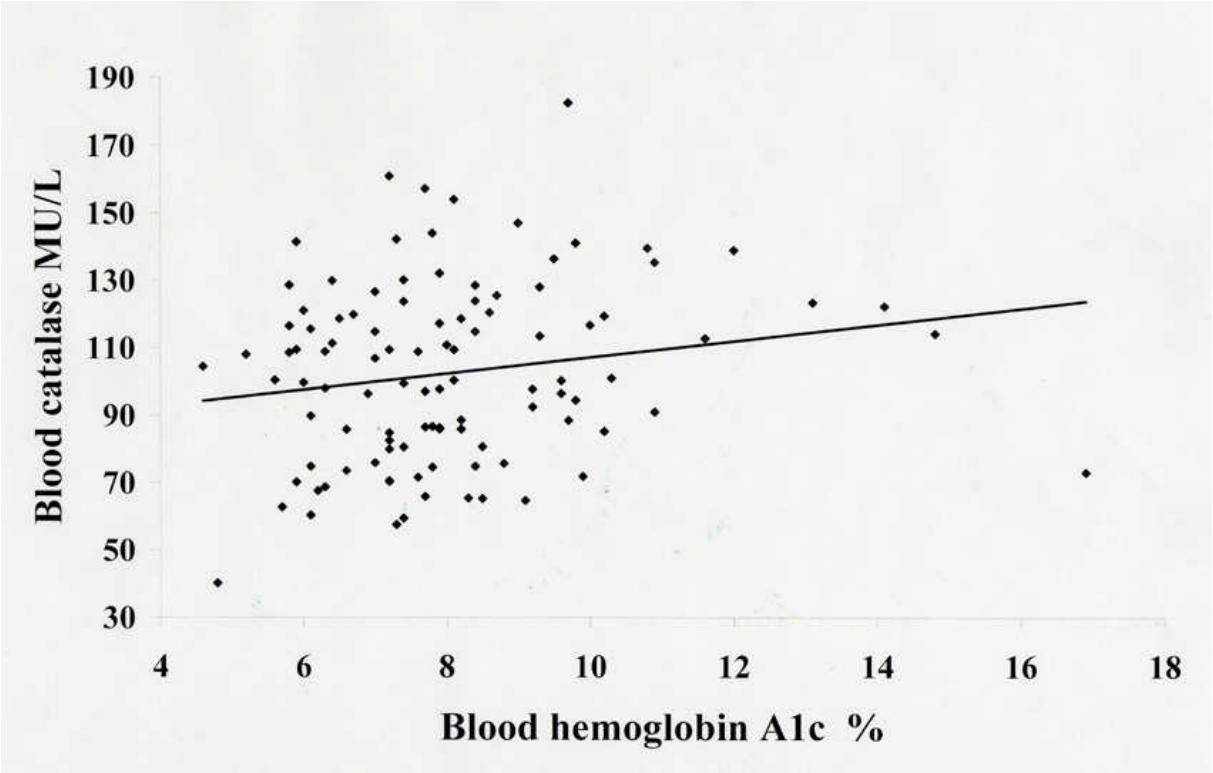


Fig.1. Blood hemoglobin A1c and blood catalase in type 1 diabetes mellitus (y=2.412 x + 83.1, r=0.182, n=106)

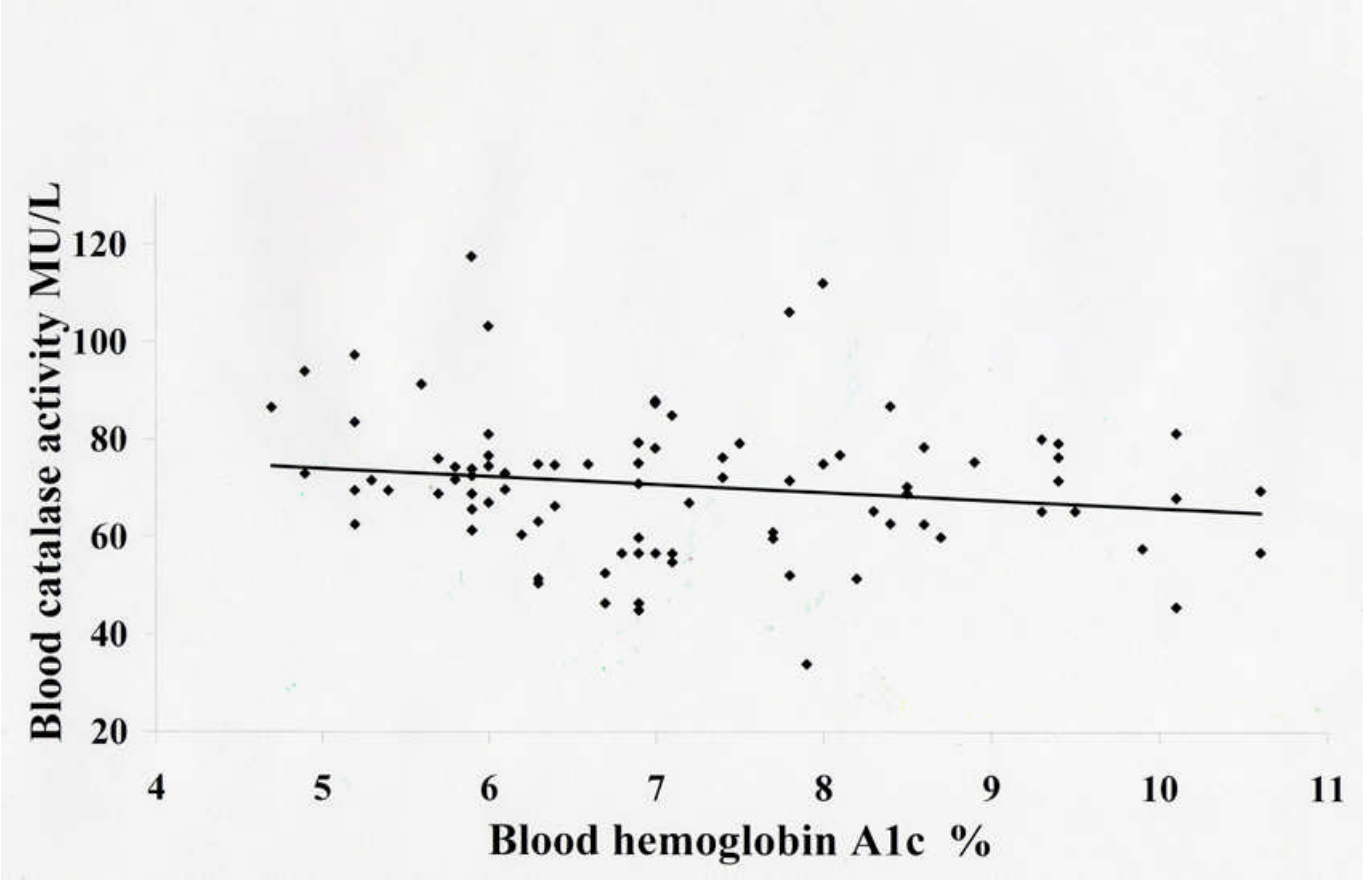


Fig 2. Blood hemoglobin A1c and blood catalase in type 2 diabetes mellitus (y= --0.309 x + 73.4, r=0.036, n=100)

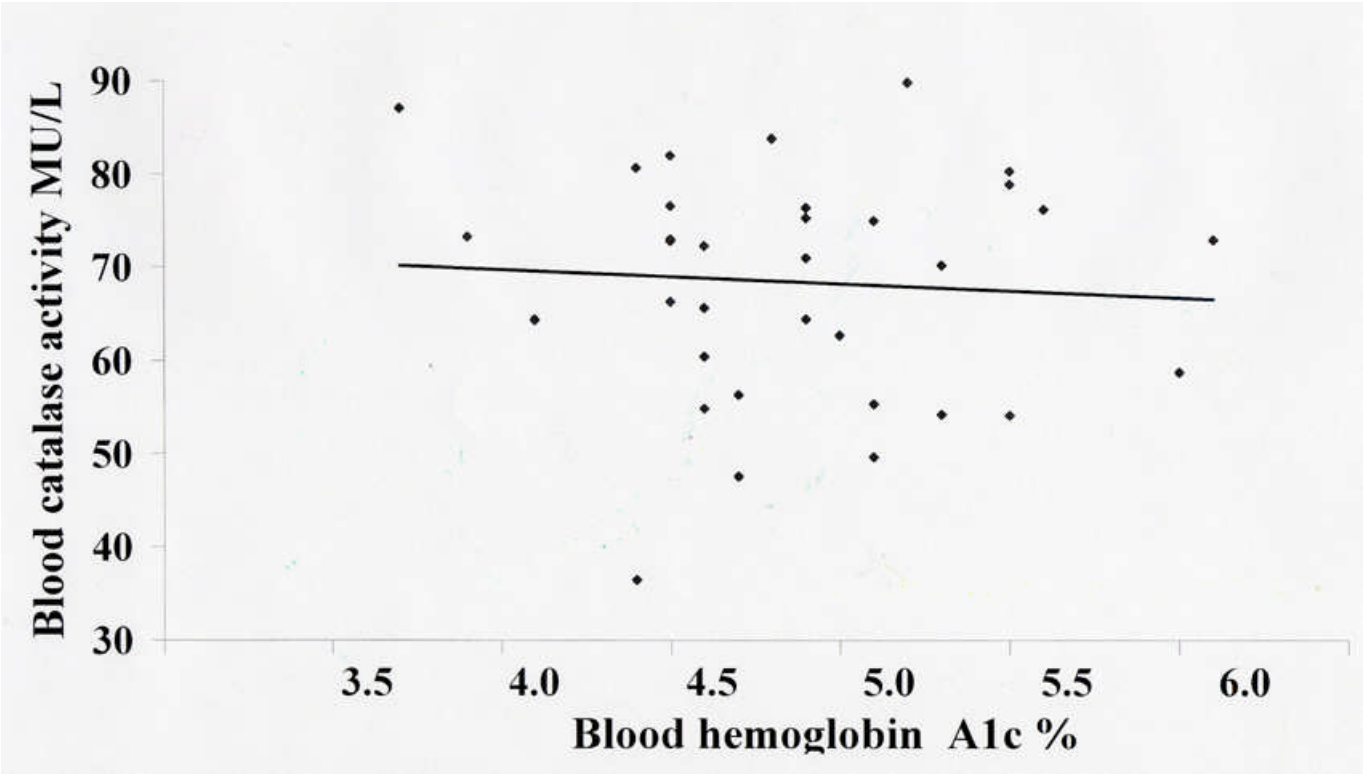


Fig 3. Blood hemoglobin A1c and blood catalase in gestational diabetes mellitus (y=--1.537 x + 75.9, r=0.067, n=33)

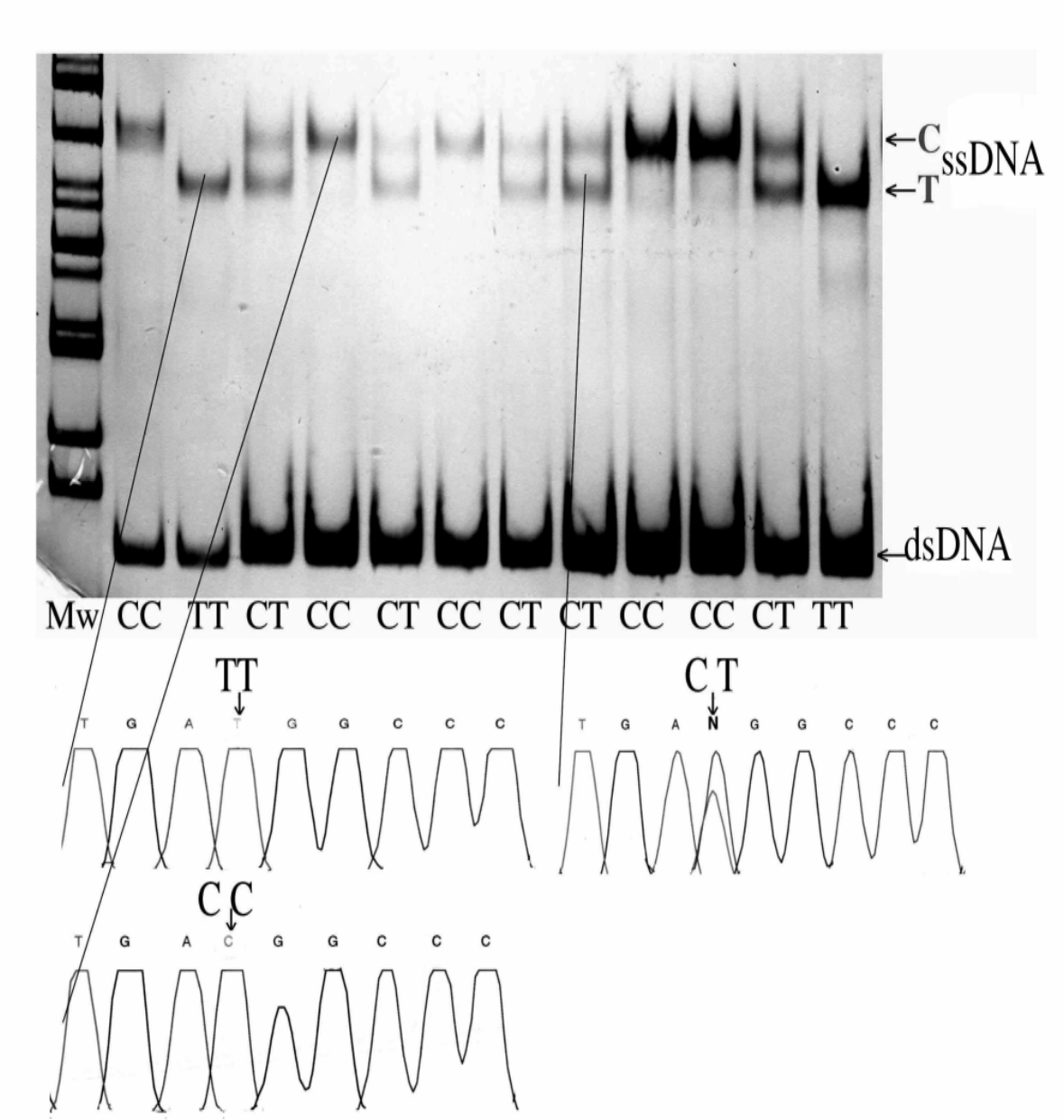


Fig. 4. C111T polymorphism in exon 9 of the catalase gene (ds: duble stranded DNA, ssDNA: single stranded DNA) and nucleotide sequence analysis of CC, CT and TT genotypes.

A new SSCP method and identification of a novel catalase mutation (Hungarian type E)

Góth L.

Introduction

High concentrations (above 50 µmol/l) of hydrogen peroxide are toxic for human cells and tissues [1] but an increasing body of evidence indicates that low concentrations of hydrogen peroxide function in intracellular signaling for modulation of inflammatory processes, expression of adhesion molecules, control of cell proliferation, apoptosis and platelet aggregation [2,3]. The enzyme catalase is the main regulator of hydrogen peroxide metabolism, especially in erythrocytes [4,5]. It decomposes hydrogen peroxide into oxygen and water. Catalase activity is dependant on the concentration of hydrogen peroxide. At high substrate concentrations the rate of reaction is rapid; at low concentrations catalase activity is minimal. Explanations of this phenomenon have been based on the unique reaction mechanism and structure of catalase [6,7].

Inherited human catalase deficiency is associated with disorders [3] such as diabetes mellitus [8,9], vitiligo [10], dyslipidemia [11] and abnormal erythrocyte metabolism[12]. To date 114 cases of acatalasemia (homozygous inherited catalase deficiency) have been diagnosed in 61 families from 12 countries [3]. Catalase gene mutations have been reported from Japan (one missense in exon 4 and one splicing in intron 4), from Canada (one missense in exon 9), from Austria (one missnense in exon 3) and from Hungary (two insertions in exon 2, one splicing in intron 7, one missense in exon 9) [3,13,14,15]. One silent substitution in exon 9 has been associated with vitiligo susceptibility [10]. These reports indicate that decreased catalase activities are associated with mutations, in both exons and introns of the catalase gene, and that more than one third are localized in exon 9.

We used simple, cheap methods to screen for catalase mutations - single stranded conformational polymorphism (SSCP) and heteroduplex analyses. To confirm the results from screening we used nucleotide sequencing. Using these simple techniques we detected 12 novel polymorphisms and four novel muations, which are responsible for inherited catalase deficiencies [3,15]. Other mutation screening methods require complicated techniques and expensive instruments.

The aim of the current study was to evaluate a new loop enhanced single strand conformational polymorphism technique for mutation screening. Using this method we screened samples from patients with various disorders associated with decreased blood catalase (gestational and non-insulin dependent diabetes mellitus, and vitiligo [9,10]) for mutations in exon 9 of the catalase gene.

Materials and Methods

Blood catalase activity was measured by spectrophotometric determination of hydrogen peroxide. The reference mean (SD) and the range of blood catalase activity are 113.3 (+/-16.5) MU/L and 80.3 – 146.0 MU/L (n=1756) [16], respectively. Samples were from the following subjects: inherited catalase deficiency (n = 23; mean and SD of blood catalase activity 56.7±13.2 MU/L), gestational diabetes (n = 43; 78.6±14.0 MU/L), non-insulin dependent diabetes (n = 78; 85.3±19.6 MU/L), vitiligo (n = 27; 102.4±19.8 MU/L) and controls (n = 22; 110.6±15.6 MU/L).

Genomic DNA was extracted from leukocytes using a QIAmp Blood Kit from Qiagen (Hilden, Germany). PCR and primers were the same as described by Kishimoto *et al.* [17]; the region amplified included 139 nucleotides in exon 9, plus 56 nucleotides of intron 8 and 43 nucleotides of intron 9. Reagents (ReadyMix REDTaq with $MgCl_2$) and primers were purchased from Sigma (Sigma-Aldrich, St. Louis, Missouri, USA). Amplifications were performed in total volumes of 12.0 μL. The mixture of 5.0 μL H_2O, 1 μL of each primer (10

μmol/L), and 2.0 μL of genomic DNA (0.2 μg/μL) was incubated at 94°C for 5 min. After that 5.0 μL ReadyMix RED Taq (20 mmol/L Tris-HCl pH:8,3, 100 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.002 % gelatine, 0.4 mmol/L dNTP mix, 60 U TaqDNA) was added. Thirty amplification cycles (94ºC, 55ºC, and 72ºC for 0.5 min, 0.5 min, and 1.0 min, respectively) were performed in a DNA thermal cycler (TC1, Perkin Elmer-Cetus, Norwalk, CT, USA).

For SSCP analyses 5 μL of PCR product and 5 μL of 99 % formamide, 20 mM/L EDTA, 0.05 % bromphenol blue and 0.05 % cylene cyanol were mixed, heated at 96ºC for 6 min and cooled on ice.

For the new loop enhanced SSCP method 10 μL of PCR product was mixed with 4 μL of loading dyes (0.05 % bromphenol blue and 0.05 % cylene cyanol in glycerol) and loaded directly into the gel.

For heteroduplex formation 2 μL of PCR product was heated to 94ºC, cooled slowly [18] and then mixed with 5 μL of loading dye (0.0125 % bromphenol blue and 0.0125 % cylene cyanol in 20 % glycerol).

Electrophoresis was performed in 6 % polyacrylamide (Acrylamide for molecular biology from Sigma-Aldrich and 5xTAE puffer) gel (175x160x1.5 mm) at 170 V and room temperature for three hours. DNA bands were visualized by silver staining and the bands were quantified with Gel Doc 1000 from Bio-Rad.

For DNA sequence analyses, the PCR products were purified by agarose gel electrophoresis. Sequencing reactions were carried out using Taq Dye-Deoxy Termination Cycle Sequencing Kits and DNA fragments were separated and detected by capillary electrophoresis (3100-Avant, Genetic Analyzer, ABI PRISM, Applied Biosystems). Sequencing was performed for all samples in which screening detected polymorphisms. Sequencing reactions were performed in both directions.

Predictions of the secondary structure of DNA utilized the Mfold web server of Zuker [19].

Results and discussion

For SSCP analyses double stranded PCR products were denatured with formamide and then separated in polyacrylamide gels. Single strand PCR products required strict denaturing conditions and showed only slight differences in their mobilities.

The simple screening method, which was used successfully to detect heteroduplex formation for exon 2 mutations of the catalase gene [17], yielded no detectable heteroduplexes for exon 9 (one band only at molecular mass of 240 bp) for both controls and patients.

When the incubation at high temperature, which is required for formation of heteroduplexes, was omitted and the PCR products were loaded directly onto the gel with buffer and dyes, silver staining revealed two or more separated bands in the 600-800 bp region. (Fig.1). The positions of these bands were similar to the SSCP bands detected using regular PCR-SSCP analyses. Furthermore, these bands were not visualised by ethidium-bromide stain but were detected when the single strand stain SYBR-Green II was used. Quantitation (n=74) revealed that 37±13 % of the silver stained material migrated as 600-800 bp products and 63±15 % migrated like double stranded bands at 240 bp.

The Casp method [10] was used to test for T111C polymorphisms in PCR products. In this method, BstX 1 enzyme cleaves the 239 bp TT type PCR product into two faster migrating fragments (191 bp and 47 bp) but does not cleave the CC type. Evaluation of samples from 72 subjects found that 33 were homozygous for the wild type TT, 31 were heterozygotes for type TC and 8 were homozygous for type TT. These findings were in full agreement with the PCR-DNA sequence analyses.

In the 600-800 bp region (Fig.1) the new SSCP method yielded distinct patterns associated with the T111C polymorphism, i.e., different patterns for the

TT, TC and CC types. The patterns were: homozygous CC samples (n=8) - only the (D) band, e.g., lane 3,5,8,11; heterozygous TC samples (n=31) - both bands (A) and (D), e.g., lane 2,9, and wild type homozygous TT samples (n=33) - only (A) bands, e.g., lane 7,10.

Lane 6, Fig.1 illustrates a heterozygous state with another slow band, (B). Nucleotide analysis revealed a C to T substitution at position 5 of exon 9. This mutation is responsible for Hungarian type D acatalasemia [15].

The sample in lane 4 (Fig 1) yielded a heterozygous state with an intermediate band (C). Nucleotide analysis revealed a C to T substitution at position 36 of exon 9. This new mutation was found in two sisters who had abnormally low blood catalase activities (71.9 MU/L and 61.9 MU/L). Their brother and mother had normal blood catalase activities (110.1 MU/L and 106.3 MU/L); their father had died (Fig 2). This nucleotide substitution causes an Arg to Cys change at position 365 of catalase. Arg 365, Arg72 and Arg 112 are important in the neutralization of the charge distribution at Tyr 358, which is essential for the reactivity of catalase's heme containing active site [6,7]. This nucleotide substitution may be denoted as type E Hungarian acatalasemia.

For explanation of the new SSCP electrophoretic patterns we used Zuker's mfold method [19] for prediction of secondary structures in single stranded nucleic acids, which have loops and double stranded regions (Fig 3). Some single stranded PCR fragments are prone to internal base pairing and the result is folding which may persist and block renaturation to double strands. Due to its conformation such loop single stranded DNA moves slower than double stranded DNA during electrophoresis.

The different mobilities of these bands (A,B,C,D) of single stranded DNA may be due to the uniquely folded structures caused by their distinct internal base pairing. Predicted folded structures, dG(kcal/mol) and T_m(°C) values, which may explain the different migration rates, are shown in Table 1 and Fig 3.

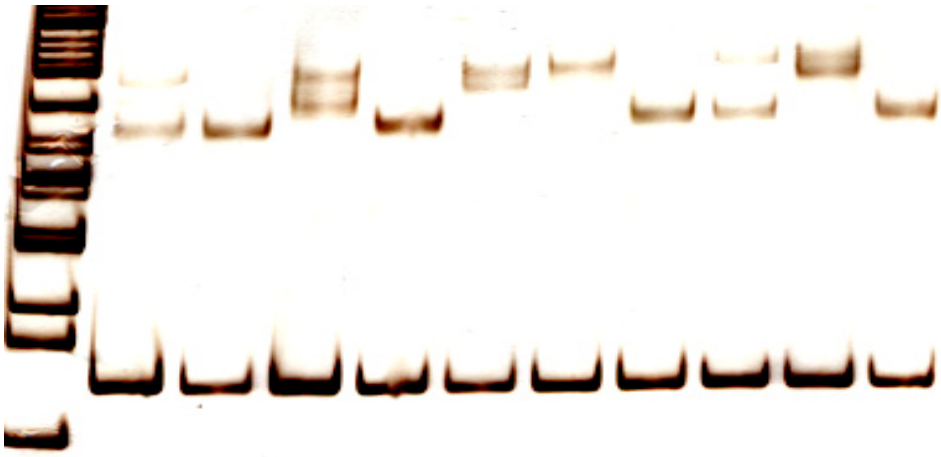
In conclusion, a simple and inexpensive loop enhanced SSCP method was developed for nucleotide mutation screening of exon 9 of the catalase gene. Amplified DNA from exon 9 yielded 37 % single stranded PCR products, which migrated as bands in the region corresponding to 600-800 bp of double stranded DNA. Polymorphisms in exon 9 were detected as specific bands. This simple sensitive method verified the known T111C and C5T mutations and yielded one new catalase gene mutation (Hungarian type E), which is associated with decreased blood catalase activities.

References

1. Eaton JW. Hydrogen peroxide a friend of the faux blonde and foe of the cell. Redox Report 1999: 4: 133-134.
2. Halliwell B, Veronique M, Lung LH. Hydrogen peroxide in human body. FEBBS Lets 2001: 486: 10-13.
3. Góth L, Rass P, Páy A. Catalase enzyme mutations and their association with diseases. Mol Diagnosis 2004: 8: 141-149.
4. Gaetani GF, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HN. Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. Blood 1996: 87: 1595-1599.
5. Mueller S, Riedel HD, Stremmel W. Direct evidence for catalase as the predominant H_2O_2 removing enzyme in erythrocytes. Blood 1997: 90: 4973-4978.
6. Putnam CD, Arvai AS, Bourne Y. Active and inhibited human catalase structures: ligand and NADPH binding and catalase mechanism. J Mol Biol 2000: 296: 383-393.
7. Diaz A, Horjales E, Rudina-Pinera E, Arreola R, Hansberg W. Unusual Cys-Tyr covalent bond in large catalase. J Mol Biol 2004: 342: 295-309.
8. Góth L., Eaton J.W.: Hereditary catalase deficiences and increased risk of diabetes. Lancet 2000;356:1820-1821.
9. Góth L. Lenkey Á, Bigler NW. Blood catalase and diabetes in Hungary. Diabetes Care 2001, 10, 1839-40.
10. Casp CB, She JX, Mccormack WT. Genetic association of the catalase gene (CAT) with vitiligo susceptibility. Pigment Cell Res 2002: 15: 62-66.
11.Góth L.: Lipid and carbohydrate metabolism in acatalasemia. Clin Chem. 2000;46:564-566.
12.Góth L, Vitai M: The effects of hydrogen peroxide promoted by homocysteine and inherited catalase deficiency on human hypocatalasemic patients. Free Rad Biol Med 2003: 35: 882-888.
13.Parboosingh JS, Rousseau M, Rogan F, Amit Z, Cherkow H, Johnson WG,

Manganaro F, Schipper N, Curran TJ, Stoessel J, Rouleau GA. Absence of mutations in superoxide dismutase and catalase genes in patients with Parkinson's disease. Arch Neurol 1995: 52: 1160-1163.
14.Walley V, Buchroithner B, klausegger A, Laimer M, Hintner H, Bauer JW. Acatalasemia: Identification and characterization of a novel missense mutation. J Invest Dermatol 2004: 122: A85-A85 506.(meeting Abstract).
15.Góth L, Vitai M, Rass P, Sükei E, Páy A. Detection of a novel familial catalase mutation (Hungarian type D) and the possible risk of inherited catalase deficiency for diabetes mellitus. Electrophoresis 2005: 26: 1646-1649.
16.Vitai M, Góth L. Reference ranges of normal blood catalase activity and levels in familial hypocatalasemia in Hungary. Clin Chim Acta 1996:261: 35-42.
17.Kishimoto Y, Murakami Y, Hayashi K, Takahara S. Sugimura T, Sekiya T. Detection of a common mutation of the catalase gene in Japanese acatalasemic patients. Hum Genet 1992: 88: 487-490.
18.Góth L, Gorzsás A, Kalmár T. A simple PCR-heteroduplex screening method for detection of a common mutation of the catalase gene in Hungary. Clin Chem 2001:46: 1199-1200.
19.Zuker M. DINAMelt web server for nucleic acid folding and hybridization prediction. Nucleic Acid Res 2003: 31: 3406-3415.
http.//www.bioinfo.rpi.edu//applications/RNA&DNA folding/new version

Figure legend



1 2 3 4 5 6 7 8 9 10 11

Fig 1. PCR-SSCP pattern of catalase exon 9. SSCP bands (A,B,C,D) with different migration (top), double strands at the bottom.
Lane 1: Mw markers, Lane 2-11 - patients' samples. , Lanes 3,5,8 & 11 homozygotes-Type TT for C111T polymorphism, with D bands. Lanes 7&10: homozygotes-Type CC for C111T polymorphism, with A bands, Lanes 2 & 9: heterozygotes-Type TC for C11T polymorphism, with A and D bands. Lane 4: C36T polymorphism hetrozygote, with A and C bands. Lane 6: C5T polymorphism hetrozygote, with B and A bands

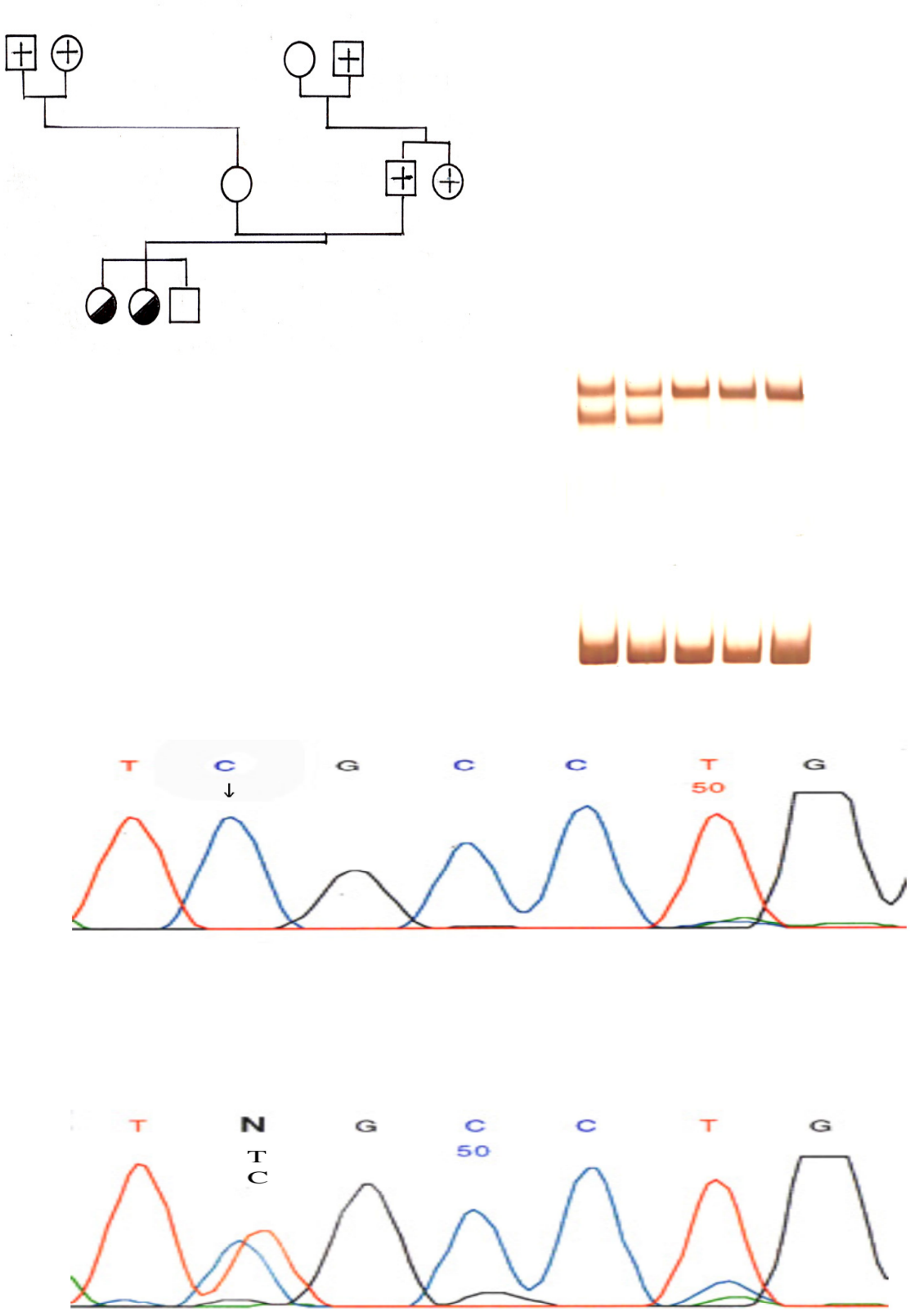


Fig 2. Pedigree (A), loop enhanced SSCP pattern (B), nucleotide sequence analyses (C, left: mutant, right: wild type) of new acatalasemic family in Hungary

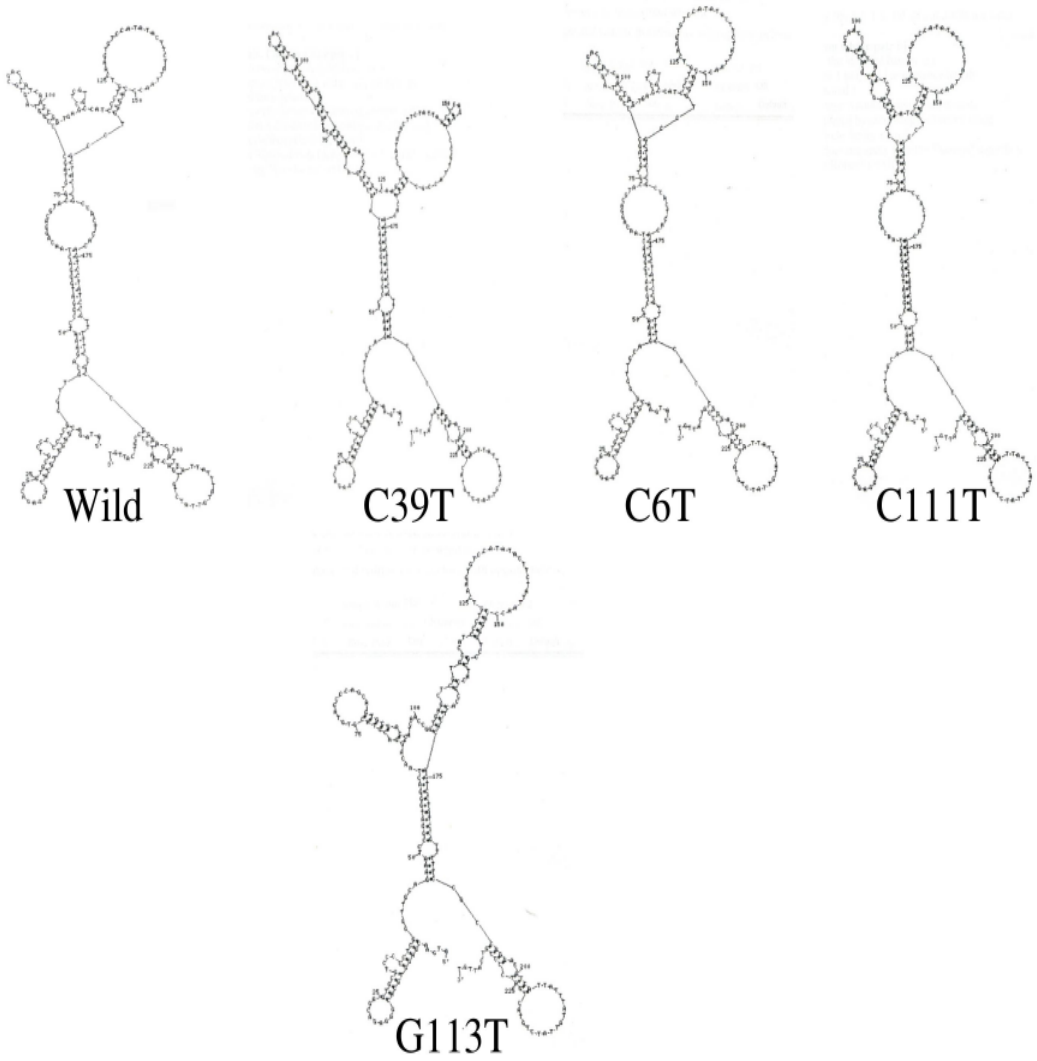


Fig 3. Predicted structures of single strand DNA fragments with different mutations in exon 9 of the catalase gene

V. Levelező PhD hallgatók közremükődése

Vitai Márta levelező PhD hallgató már elkészítheti PhD értekezését.
Rass Péter, Tarnai Ildikó és Sükei Eszter levelező PhD hallgatók a szükséges krediteket megszerezték közleményekben társszerzők és további közleményeket kell irniuk kutatásaik alapján.

VI. A témavezető munkáira történő hivatkozások

A témavezető munkáira történő 17 hivatkozást az utóbbi 10 évben (1992-2002) az egyik bíráló kevésnek tartotta.

A pályázat ideje alatt 2002- 2007-ig a témavezető 22 közleményére a nemzetközi szakirodalomban (Web of Science) adatai alapján 145-ször hivatkoztak.
Az alábbi összesítés az egyik leggyakrabban idézet közleményének adatait mutatják, vastag betűvel kiemelve azon folyóiratok Impakt faktorát, amelyek értéke 10 feletti, a 2005-ös IF lista alapján.

Góth L, Eaton JW. Hereditary catalase deficiencies and increased risk of diabetes. ***Lancet*** *2000: 356: 1820-1821* ***IF: 23,407***

LANCET 2001;357:31 **IF: 23,407**

BIOCHEM BIOPHYS ACTA GENE STRUC EXPRESSION 2001: 1522: 217-220 IF: 2,836

PIGMENT CELL RESEARCH 2002:15:62-6. IF: 3,000

ENDOCRINE REVIEWS 2002: 23: 599-622**.** **IF: 22,538**

DIABETOLOGIA 202:45:1550-1561 IF: 5,583

METABOLIC ENGINEERING 2003:5: 177-182.

CURRENT MEDICAL RESEARCH AND OPINION 2003: 19: 581-586. IF: 2,928

JOURNAL OF BOIOLOGICAL CHEMISTRY 2003: 278: 42495-42504. IF: 6,355

DIABETES RESARCH AND CLINICAL PRACTICE 2004: 64: 181-183. IF: 1,730

AMERICAN JOURNAL OF PHYSIOLOGY-REANAL PHYSIOLOGY 2004: 286: 1030-1038 IF: 4,354

JOURNALS OF GERONTOLOGY SERIES A-BIOLOGICAL SCIENCIES AND MEDICAL SCIENCIES 2004: 59: 886-889. IF: 4,122

AGEING RESEARCH REVIEW 2005: 4: 1-22. IF: 4,953

ROMANIAN JOURNAL OF INTERNAL MEDICINE. 2004: 42: 423-429.

KIDNEY INTERNATIONAL 2005: 68:1018-1031 IF: 4,729

CELL BIOCHEMISTRY AND BIOPHYICS 2005: 43: 289-330. IF: 1,945

FREE RADICAL RESEARCH 2005: 39: 1345-1350. IF: 2,744

PHYSIOLOGICAL RESEARCH 2005: 54: 527-532. IF: 1,140

CLINICAL CHEMISTRY AND LABORATORY MEDICINE 2005: 43: 1346-1350 IF: 1,685

TRAFFIC 2006: 7: 907-107. IF: 7,18

PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 2006: 103: 4952-4957. **IF:10,452**

PEDIATRIA POLSKA 2006: 81: 176-179.

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. 2006: 345 (4): 1586-91 IF: 2,904

NATURE 2006: 440: 944-948. (Supplementary reference) **IF: 32,182**

BIOCHEMICA ET BIOPHYSICA ACTA-CELL MOL RES 2006: 1763: 1749-54 és 1697-1706 IF: 4,844