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Relationship between the polymorphisms of catalase and GRB10 gene and the type 2 diabetes in Hungary

by Márta Vitai

Supervisor: Prof. László Góth

UNIVERSITY OF DEBRECEN
KÁLMÁN LAKI DOCTORAL SCHOOL OF THROMBOSIS, HAEMOSTASIS AND VASCULAR DISEASES

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Supervisor: Prof. László Góth M.Sc., Ph.D., D.Sc

Kálmán Laki Doctoral School of thrombosis, haemostasis and vascular diseases, University of Debrecen

Head of the **Examination Committee**: Prof. Dr. Muszbek László M.H.A.Sc.

Members of the Examination Committee: Prof. Dr. Anikó Somogyi D.Sc.
Dr. Ágnes Lakatos Ph.D.

The Examination takes place at the Lecture Hall of the 1 st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen
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Head of the **Defense Committee**: Prof. Dr. Muszbek László M.H.A.Sc.

Reviewers: Prof. Dr. Antal Szabó D.Sc.
Dr. Miklós Káplár Ph.D.

Members of the Defense Committee: Prof. Dr. Anikó Somogyi D.Sc.
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INTRODUCTION

The number of patients with type 2 diabetes (2DM) has been increasing insomuch that we can talk about diabetes pandemic. This epidemic, however, might be broken, stopped. Major international studies show that the 2DM is preventable, but only in the early stage when the glucose intolerance begins. This early stage is the IFG (Impaired Fasting Glucose) and IGT (Impaired Glucose Tolerance) phase, which is nowadays called in summary as prediabetic state, and the preceding period when solely the insulin resistance exists. During this period with lifestyle modifications, dietary restrictions and some of the insulin-sensitizing drugs the manifestation of type 2 diabetes can be prevented or shifted. The genomic studies, which are intensively carried on all over the world, day by day confirm relationships between gene differences and type 2 diabetics. On the one hand, the gene differences can help in the exploration of predisposition, on the other hand, if the differences cause changes in protein structure, they are of great promise to recognize the disease in the very early period.

By definition, insulin resistance is a condition in which normal amount of insulin results in subnormal response. In the majority of type 2 diabetics, the effect of insulin is lower than expected, the insulin sensitivity is decreased, and namely they are insulin resistant. There are large variations in insulin sensitivity also in the healthy population. Factors known to influence the actions of insulin (obesity, diet, physical activity, environmental factors, etc.) are responsible for only one-third of differences, indicating that genetic factors influence the insulin sensitivity decisively.

Insulin resistance can practically hardly be diagnosed. The internationally accepted method of measuring insulin sensitivity, the "gold standard", is the hyperinsulinemic-euglycemic clamp (HEC) technique. This method requires a lot of time, it is invasive and expensive, so this test is not suitable for diagnostic screening.

The alternative solution would be using the HOMA value, which can be calculated from fasting blood glucose and insulin levels (HOMA-R = fasting glucose (mmol/l) x fasting insulin (μE/ml) x 22.5^{-1}). However, this widely used parameter shows slight relation with the value M obtained by HEC technique (M = mg glucose/min/kg body, muscle or fat weight). There are several more complicated variants of HOMA-R parameter, in which it has been rather unsuccessfully tried to improve the applicability of this method by measuring free fatty acid, fat parameters and adipokines. Molecular genetics also shows on alternative solution to these problems.

The insulin resistance is not only the preceding and characteristic state of 2DM but also clinical symptom of several diseases, such as metabolic syndrome, obesity, starvation, pregnancy, acromegaly, sepsis, burn trauma, carcinoma, cachexia, states when the presence of oxidative stress and reactive oxygen species (ROS) presumably or even confirmedly plays a role in pathomechanism. The catalase enzyme, which transforms H_2O_2, is of great importance in this process. The catalase gene has some polymorphisms, which can be associated with different diseases but there are several mutations known that do not influence protein expression, do not affect enzyme activity and do not associate with diseases (benign polymorphisms). Oxidative stress may play a role in the development of osteoporosis, as well. Numerous data show that postmenopausal osteoporosis do not reflect only the change in hormone state since trabecular bone loss appears even at the age of 20 in young women and men without any hormonal change. The greatest risks of postmenopausal osteoporosis which is independent of sex steroids are the age, the oxidative stress which increasing continuously with metabolism deviations and the weakening defence against oxidative stress. The bone-protecting effect of estrogens and androgens may be partly ascribed to the direct antioxidant impact of these hormones. The rate of bone loss accelerates in ovariectomised animals because glutathione and thioredoxin content of bones decreases, which can be, however, set back by giving external oestrogen and/or antioxidants.

Several in vitro and animal experiments confirm that oxidative stress decreases bone formation and stimulates bone resorption because differentiation and lifetime of osteoblasts are reduced by the stress. In individuals with reduced catalase activity, dislipidemie may develop also because of increased oxidative stress since mitochondrial H_2O_2 emission rises due to the decreased antioxidant capacity. This happens when consuming high fat diet, when increased lipid oxidation causes oxidative stress. Oxidative stress activates the NF-κB gene and through this, the angiotensin-II activity and the production of insulin signalling inhibitor (TNFα) cytokine, causes increased expression of lipoxygenase enzyme together with concurrent reduction of WNT signalling system, which associates with decreasing bone formation and development of insulin resistance. Korean authors examined the C→T polymorphism (+22348C>T, RS769217) at the position 111 of exon 9 of catalase enzyme and found relation between genotypes, bone status parameters (Bone Mineral Density, BMD) and serum osteocalcin concentration. Osteocalcin (bone-γ-carboxyglutamic acid protein, BGP) is a bone-specific protein containing 50 amino acids. Osteocalcin is produced by osteoblasts and after secretion from osteablasts it gets partly into the bone matrix, partly into the blood stream, hence the quantity of osteocalcin in serum depends on bone remodelling (formation-

resorption) processes. In absence of osteocalcin not only bone formation decreases in mice but also proliferation of pancreatic ß-cells and expression of insulin gene in ß-cells and adiponectin gene expression in fat cells are also reduced, suggesting the connection between bone status and energy metabolism. In healthy, postmenopausal women the relationship between glucose metabolism, insulin resistance and bone status has already be confirmed. This relationship disappears, when glucose tolerance deteriorates and insulin resistance develops. Several metabolic parameters are independent and significant predictors of serum osteocalcin level. In the case of women these are, among others, fasting glucose levels, total body and muscle tissue glucose consumption, metabolic clearance rate of glucose and LDL cholesterol concentration, while in the case of males serum calcium levels, area under OGTT glucose curve and free fatty acid levels.

The search for "diabetes candidate gen" is the process when the expression levels of genes are compared between healthy and diabetic subjects. than the structure of the differentially expressed genes are determined and the connection between altered gene structures and metabolic parameters are investigated. One of these genes is the GRB10 (Growth Factor Receptor Binding Protein).

Numerous in vitro verified that the binding of insulin receptor (IR) to GRB10 reduces the effect of insulin. Overexpression of GRB10 decreases insulin-stimulated PI-3 (phosphatidylinositol) kinase-, Akt/PKB (protein kinase B)-, MAPK (mitogen-activated protein kinase)- activation, glycogen synthase activity and glucose uptake. Tests carried out on GRB10 deficient (knocked out) mice confirmed also in vivo that the absence of GRB10 unequivocally enhances the effect of insulin, and phosphorylation of insulin-stimulated Akt and MAPK. Hyperinsulinemic-euglycemic clamp tests proved that reduction of GRB10 expression in peripheral tissues (muscle and fat) increased insulin sensitivity, while insulin and fasting glucose concentration did not change. These results suggest that GRB10 is a negative regulator. GRB10 knock out mice have significantly higher postnatal weight gain than wild type ones, which can be explained by suppressor effect of endogenous GRB10 on growth factors. A special role is also attributed to GRB10 in signalling, namely that it is able to integrate and collect (scaffold protein) incoming external signals. In the signalling complex the special role of GRB10 depends on the quantity and affinity of mediator proteins connected to it. Hence GRB10 is able to be a positive and negative regulator as well, while its quantity is constant.

Numerous single point polymorphisms (SNP) of the GRB10 gene are known, several of which are related to type 2 diabetes. It has been demonstrated that in the presence of

RS2237457 polymorphism (+11275G>A) the GAUC value (Glucose Area Under Curve; area under curve obtained by oral glucose tolerance test) of genotype variants significantly differs in non-diabetic individuals. The frequency of RS494710 polymorphism (1209A>G) in the type 2 diabetic patients' group is significantly higher, and the development of diabetes in individuals with A allele has lower likelihood.

OBJECTIVES

- **To determine reference range of blood catalase enzyme activity, to define hypocatalasemia and acatalasemia.**

The reference range of blood catalase enzyme activity did not exist in that geographic area we studied. First we determined the reference range of blood catalase enzyme activity in order to define hypocatalasemia and acatatalasemia. Furthermore, we examined its relation to gender and age.

- **The screening for hypocatalasemic and acatalasemic families, to establish DNA bank for genetic studies.**

We looked for hypocatalasemic families and determined the frequency of hypocatalasemia by using almost 5000 blood samples' catalase enzyme activity value.

- **To study known mutations of catalase gene in Hungarian subjects.**

We found two acatalasemic sisters who were similar - based on molecular weight and electrophoretic mobility of the catalase enzyme - to Japanese-type acatalasemia hence our study started with using the mutations found in Japan. We looked for A→T mutation in the promoter region at position -21.

- **To study the relationship between catalase gene polymorphisms and type 2 diabetes.**

Eight Hungarian patients with inherited catalase deficiency revealed type 2 diabetes (2 of them were acatalasemic and 6 hypocatalasemic); while nobody with normal catalase activity had this disease. To find the cause of this difference, we start investigating the Hungarian population with recently published mutations in exon 2 and its proximate environment.

- **To establish gene bank for studying gene polymorphisms in Hungary.**

To study gene differences published in literature and to find new polymorphisms we need to establish a DNA bank representing the Hungarian population.

- **To study the relation between the +22348C>T polymorphism of catalase gene and glucose and bone metabolism.**

Korean authors found relation between +22348C>T polymorphism in exon 9 of catalase gene and serum osteocalcin concentration hence we analysed also in Hungarian population in what extent relations obtained correspond with literary data. We broadened the field of study besides osteocalcin to other bone markers and glucose metabolism parameters.

- **To look for differentially expressed genes in animal models.**

Nowadays genetic tests belong to the investigation of effect mechanism of drug candidate molecules. We used differential display technique to find differentially expressed genes as an effect of the drug molecule. Since we examined the effect mechanism of a molecule developed for prevent complications of type 2 diabetes, we used diabetic animal models for confirming expression of candidate genes.

- **To analyse polymorphism of GRB10 protein in Hungarian population and its relation to glucose metabolism.**

Earlier population genome studies revealed relationship between GRB10 as a candidate gene and type 2 diabetes. Therefore, we analysed the frequency of +11275G>A polymorphism of GRB10 gene in healthy and diabetic population and looked for relationship between polymorphism and glucose metabolism parameters.

MATERIALS AND METHODS

1. Determination of reference range of blood catalase enzyme activity

For determining reference activity we used 880 female and 876 male (age of 14-90 years) samples from Sümeg (Hungary) neighbourhood (7 settlements, no industrial areas). For characterising hypocatalasemia we used blood catalase enzyme activity results of 3300 healthy persons and 1630 in-patients (from Sümeg and its neighbourhood).

We measured blood catalase activity – by measuring the decrease of H_2O_2 substrate concentration per time unit – with simple spectrophotometric assay.

2. Isolation of DNA

We isolated DNA from lymphocytes. First we used a standard method, which is known as salting out technique, then we applied commercial kits for DNA isolation.

3. Analysis of HinfI polymorphism on exon 2 of catalase gene

We analysed 87 samples (52 hypocatalasemic individuals and 35 normocatalasemic control subjects). After PCR reaction we digested the products with HinfI restriction enzyme and applied RFLP (Restricted Fragment Length Polymorphism) analysis on poly-acrylamide gel.

4. SSCP (Single Strand Conformational Polymorphisms) and PCR heteroduplex analysis in exon 2 of catalase enzyme

We analysed 308 diabetics subject from different regions of Hungary with ages of 45-70 years (mean±SD: 58.4±6.5 years). We looked for mutations by using SSCP analysis and detected GA and G insertions of catalase gene in exon 2 by PCR heteroduplex analysis.

5. Foundation of Korányi András Transdanubian Diabetes Gene Bank

In this gene bank type 2 diabetic patients and their families' samples were collected if at least 6 or more first-degree relatives (grandparents, parents, and children) had been reached. Persons' phenotype data, genomic DNA, plasma and mononuclear cell samples are stored in the bank. Here, we also stored samples from those subjects who underwent the hyperinsulinemic-euglycemic clamp study. Samples from early (perimenopausal) osteoporotic women and their family members were also collected and stored.

6. Determination of glucose metabolism characteristics

We measured insulin sensitivity, glucose utilization of the whole body and its compartments (fat and muscle tissue, fat free weight) with hyperinsulinemic-euglycemic clamp method (DeFronzo et al. 1979). Insulin sensitivity was characterised by M value (M = glucose disposal per total body, or different tissue compartment) and HOMA index (HOMA – IR = G_o x I_o / 22.5, where I_o = fasting insulin (μU/ml) and G_o = fasting glucose (mmol/l), HOMA-IS = 1/ HOMA-IR). Body composition was measured by DEXA (DPX- MD+, GE-Lunar, USA) equipment. General clinical laboratory parameters related to glucose and bone metabolism were determined by Cobas Mira laboratory automats (Roche Diagnostics, Germany) by using Roche tests. Hormones and bone markers were measured by Roche Elecsys 2010 with electro-chemiluminescent immunoassays or were determined by ELISA method.

7. Analysis of +22348C>T polymorphism of catalase gene and +11275G>A polymorphism of GRB10 gene

For the study we used DNA samples of 141 persons who took part in hyperinsulinemic-euglycemic clamp study. We sorted the individuals studied based on results of oral glucose tolerance test (75g glucose OGTT) according to American Diabetes Association criterions. We used a simple screening method to determine +22348C>T polymorphism of catalase gene. Exon 9, the site of this polymorphism was multiplied with the PCR, and then PCR products were run on 6 % acrylamide gel and made visible by silver staining. Since these single stranded PCR products due to their internal loop formation did not become totally double-stranded and they could be separated on polyacrylamide gel without denaturation. We used target-specific FRET probes to analyse the +11275G>A (RS 2237457) polymorphism of GRB10 gene and detected with Roche LightCycler 2.0. The Hungarian allele frequency of +11275G>A polymorphism was analysed in DNA samples of 77 healthy and 85 type 2 diabetic individuals.

8. Differential display

We treated spontaneously hypertonic rats with a diabetic drug candidate molecule (Bimoclomol, 20 mg/kg/day; treated group), and with physiological saline solution (control group) for 3 months. After the treatment the animals were narcotized and their thoracic aortas removed and RNA isolated for the differential display analysis. We carried out differential display analysis according to descriptions by Liand P. et al. (1992). To confirm the results of differential display experiment with real-time PCR, Wistar rats with streptozotocin induced diabetes (insulin deficit, a model of type 1 diabetes) and genetically diabetic rats (GK, a model of type 2 diabetes) were used. To analyse toxic effect of glucose we used L8 (myoblast→myotube) cultures differentiated in normal and high glucose media. We performed the real-time PCR confirmations with TaqMan probes. The extent of gene expression levels were calculated on the basis of internal controls

9. Statistical analysis

Differences between groups (female/male, healthy control/diabetic, genotypes, etc.) were calculated by using Student's two-sample t-test (MS Excel). The allele frequency differences were characterised by using χ^2 test and odds ratio (www.meta-numerics.net/Samples/ContingencyCalculator.aspx).

Correlations between metabolic and antropometric parameters were determined by single-variate correlation analysis (MS Excel), multivariate linear regression and Sperman's rank correlation analysis (STATISTICA Trial Version 2010 (StatSoft Inc.). Significance level was in general $p<0.05$.

RESULTS AND CONCLUSIONS

We determined the reference range of blood catalase enzyme activity based on ~ 1700 activity results. We studied female and male samples separately, and for women we got significantly ($P<0.001$) lower values (107.7±14.4 MU/l) than for men (117.9±16.9 MU/l). Those individuals were considered as hypocatalasemic whose catalyse enzyme activity was reduced by 50 % compared to reference range.

After analysing 5000 samples 9 families were found with decreased catalase enzyme activity. We determined hypocatalasemia frequency as well. Among the family members 37 persons were hypocatalasemic (57.5±11.7 MU/l) and 47 normocatalasemic (98.6±17.9MU/l). Two sisters in a family were qualified to be acatalasemic. The hypocatalasemia frequency is 0.18 %, which is similar to that found in Japan and Korea but less than 5 % measured in Iran. DNA from these families' blood samples was isolated and this collection was used for mutation analysis.

We begun first the genetic characterisation of acatalasemic and hypocatalasemic individuals with the known Japanese mutations, the A → T mutation in the promoter region at position -21 was investigated**.** An unexpectedly high number of mutations were found in the 87 persons, which were confirmed later by sequence analysis. Besides A → T substitution at position -21 two novel point mutations, not known before in Hungarian population were found: C→T mutation at position -18 and C→A mutation at position -20. The prevalence of A→T mutations was significantly ($p<0,001$) higher in hypocatalasemic individuals than in normocatalasemic ones (2/36 vs. 14/18). This mutation was identical to that found in Japan and our two acatalasemic Hungarian sisters were also A→T homozygous mutants. However, since this mutation is also present in the control group, therefore, A→T mutation at position -21 cannot be an acatalasemia mutation. The two new mutations do not seem to affect the catalase enzyme activity, the first due to its low frequency (-20 C→A, 3/35 vs. 0/32) in hypocatalasemic individuals, the other because of the high control frequency (-18 C→T, 18/20 vs. 20/12).

In diabetic group (308 patients) SSCP analysis resulted in 5 new point mutations in exon 2 of catalase gene. Two of them, the T→G (position 96) and the G→C (position 135) mutation were missense mutations, which changed the amino-acid sequence, what may result in decreased catalase activity. The two frameshift mutations (GA and G insertion on exon 2) caused stop codons at amino-acid positions 134 and 57 (instead of 517amino acid) resulting truncated catalase proteins, which can be also responsible for the decreased catalase enzyme

activity. The decreased activity may induce increased H_2O_2 concentration and continuous intracellular oxidative stress, which can contribute to development of type 2 diabetes.

Examining the +22348C>T polymorphism of catalase gene, changes in femur neck bone density were detected in the different male genotypes. Individuals with C allele (wild*CAT*) had higher BMD than those with mutant*CAT* genotype (CT+TT=mutant*CAT*).

In accordance with published data, bone density was significantly different in the two genders ($p<0,001$). However, when genotypes were also taken into account, the BMD-F differences in mutant*CAT* genotypes were equalized (BMD-F male mutant*CAT* vs. female mutant*CAT*: p=0,247; BMD-L_{1-4} male mutant*CAT* vs. female mutant*CAT*: p=0,279). The change of C nucleotide to T at position 22348 of catalase gene caused significant decrease of osteocalcin serum concentration and increase of bone density (BMD-F and BMD L_{1-4}) in Korean postmenopausal women (Oh B. et al. 2007). In Hungarian population we could not find this relationship, which attracts attention to that genetic studies can have population-specific results, hence it is necessary to examine all polymorphisms on domestic population as well. We analysed the relationship of +22348C>T polymorphism of catalase gene to osteocalcin concentration, whole body - (M1), muscle tissue - (M2) and fat tissue - (M3) glucose utilization. Our preliminary results showed that the significant correlation between glucose disposal and osteocalcin level ceased in both genders when insulin sensitivity deteriorated. However, if we analyse this correlation as a function of +22348C>T polymorphism, in the case of women we can find a positive correlation in wild type (wild*CAT*) subjects between osteocalcin and M values. In mutant*CAT* genotype, however, this correlation ceases. In the case of males the reverse is true. The +22348C>T nucleotide substitution in exon 9 does not cause amino-acid change, hence this polymorphism may be regarded as a benign one which could not contribute to catalase activity change, or to transcription. As these relations were not obtained from a great number of samples, it would be premature to claim that the catalase gene polymorphism may predestinate the relation between bone and glucose metabolism but it can give cause to broaden this analysis.

In women with mutant*CAT* gene (T allele) the changing metabolic (adiponectin, leptin – M-2) and bone (adiponectin – BMD) relationships did not cause significant bone density changes. In men the appearance of T allele caused density loss on femur maybe because of the changing adipokine − metabolism (M-2) relations. Data of multiple correlation analysis (the leptin/adiponectin ratio influenced the femur in women, and the L1-4 BMD value in men and these relations ceased when T allele appeared), the disappearing negative leptin - metabolism

effect with the presence of T allele and the also disappearing positive leptin-DHEAs and DHEAs-femur BMD relations, suggest the role of leptin, with its known gender differences.

The aim of using differential display was to analyse the way of action of drug candidate molecules, genes with altered expression in certain pathologic models were searched and drug candidate molecules were tasted if they were able to compensate these expression differences. As a result of this analysis we found three genes that we continued to analyse confirming the observed expression differences. The most promising gene was the GRB10. We proved that it had different expression both in type 1 and 2 diabetic animal models, high glucose concentration also increases the quantity of mRNA.

In Hungaraian subjects we examined the occurrence of the GRB10 RS 2237457 (+11275G>A) allele frequency in healthy and untreated 2DM patients and relation of allele types to biochemical parameters, such as glucose metabolism indicators, hormone, adipokine and cytokine levels and to whole body glucose utilization reflecting insulin sensitivity. In Hungarian 2DM patients there are no allele frequency difference in GRB10 gene polymorphism (+11275G>A) compared to control group, which is in accordance with data found in Scandinavia and in several ethnic groups of America but it differs from those found in American Amish group (Hungarian population: healthy G: 62% vs. 2DM G: 70 %; Amish community: healthy G: 68 % vs. 2DM G: 53 %). Analysis of the American Amish community showed tight correlation between the +11275G>A polymorphism of GRB10 gene and glucose AUC value calculated from oral glucose tolerance test of non-diabetic individuals ($p = 1.07 \times 10^{-5}$). In our study "prediabetic" persons – spanning from healthy to IFG, IGT and untreated 2DM individuals – were analyzed. In this population no relations were found between genotype and glucose AUC, however the higher insulin secretion in male wild type suggests that the polymorphism of the gene influences insulin signalling in accordance those animal experiment data connecting GRB10 to insulin secretion. Since this phenomenon cannot be observed during intravenous glucose tolerance test, it can be assumed that the effect of GRB10 on insulin secretion related to incretin signal transduction. To confirm our results higher number of cases would be necessary but the high cost of clamp analysis makes it difficult. Based on allel variation dependent leptin/adiponectin ratio and lipid profile it could be assumed GG homozygotes are alleles with stronger function. The more active GG allele is able to influence the fat accumulation (assisting fat cell polyferation on MAP kinase pathway) and metabolic insulin signalling (on IR-IRS1 level) in postnatal phase, hence it may play a role in development of insulin resistance and 2DM together with other polygenic and environmental effects. Based on our study it is clear that there are gender differences in

glucose utilization, insulin sensitivity and adipokine connections. Although the number of our cases is low, these gender-specific relations seem not to be influenced by polymorphism of GRB10 gene.

SUMMARY

We determined the reference range of normal blood catalase activity in female and male Hungarian subjects.

We detected the Japanese catalase mutation (A → T substitution in the promoter region at position -21) also in Hungarian hypocatalasemic patients, and two new mutations at the position -20 and -18.

Five novel and two previously reported point mutations were detected in the second exon and its exon/intron boundaries in diabetic patients. Two novel missense mutations on exon 2 (position 96 and 135) cause amino acid substitutions (position 53 and 66) in catalase protein. These changes may be responsible for activity losses. The two previously reported frame shift mutations (GA or G insertion in exon 2) cause early stop codon. This leads to production of truncated catalase proteins, which may be responsible for decreased catalase activity.

We found that the effects of +22348C>T polymorphism of catalase gene on glucose metabolism and bone status were gender specific. The appearance of T allele had protective metabolic effect, which was disadvantageous for the status of the femur in men due to the evolutionary negative relationship of energy and bone metabolism. The gender specificity of catalase gene polymorphism may be related to the distribution of fat (different ratio of visceral and subcutaneous fat) and the differences in adipokine (leptin) effects. Our results did not confirm the observation obtained in Korean postmenopausal women that the T allele associated with lower osteocalcin level and higher femur density.

We detected differentially expressed genes with “differential display analysis” in diabetic models, of which importance was later also demonstrated by genome studies.

We could not find any significant differences in the GRB10 +11275 G>A polymorphism between in control and diabetic groups in the Hungarian population. Based on gender-specific body fat, insulin sensitivity, leptin/adiponectin ratio and the relationship between allelic variants it can be assumed that the GG alleles may play a role in the development of type 2 diabetes.

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