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# Search for mitochondrial A3243G tRNA$^{Leu}$ mutation in Polish patients with type 2 diabetes mellitus

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## SUMMARY

***Background:*** *The influences of genetic and environmental factors form a clinical picture of type 2 diabetes mellitus. Genetic studies of type 2 diabetes mellitus become increasingly important. The knowledge of the molecular background of type 2 diabetes has been growing rapidly over recent years. One of the forms of the disease defined on the molecular level is maternally inherited type 2 diabetes mellitus. This diabetes, which is frequently accompanied by hearing impairment or deafness (maternally inherited diabetes with deafness-MIDD), was linked with sequence differences in mitochondrial DNA. The most frequent cause of MIDD is A3243G substitution in a mitochondrial tRNA$^{Leu}$ gene. While this mutation was identified in different races in several populations, it is still important and valuable to evaluate its prevalence in various ethnic groups. The aim of the project was to determine the prevalence of A3243G substitution in a mitochondrial tRNA$^{Leu}$ gene among Polish diabetic subjects.*

***Material and methods:*** *In total 129 individuals, with type 2 diabetes and 12 with gestational diabetes were selected for this study. Two techniques based on restriction fragment length polymorphism (RFLP) method were used to screen for A3243G mutation. In the first approach, non-radioactive PCR reactions of mitochondrial DNA region of interest were performed using DNA of the study participants. This was followed by Apa I restriction enzyme digestion of the PCR product. Subsequently an electrophoretic separation was done on 2% agarose gel with ethidium bromide staining. In the second, more sensitive, modification of RFLP, [$\alpha^{32}$P]dCTP was used for internal primer labeling and the electrophoresis was done on acrylamide gel. A positive sample was used to control the quality of the genotyping.*

***Results:*** *In both approaches none of the samples, except for the positive control, showed the evidence of the G variant.*

***Conclusions:*** *In summary, the A3243G mutation in mitochondrial tRNA$^{Leu}$ gene is not a frequent cause of diabetes in the Polish population. Further screening of enlarging study group is necessary to fully determine the prevalence of this mutation in our population. This, together with the search for other mitochondrial mutations, should allow to fully determine the prevalence of MIDD and its specific molecular background in the Polish population.*

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## BACKGROUND

A clinical picture of type 2 diabetes mellitus is created by joint influences of genetic and environmental factors. Two major pathophysiological defects coexist in this disease: impairment of insulin secretion by β-cells and decreased insulin sensitivity in peripheral tissues [1,2,3]. Genetic studies of type 2 diabetes mellitus become increasingly important due to their scientific, prognostic, prophylactic and also therapeutic significance, however the molecular background of the disease still remains mostly unknown. So far several different genes have been associated with autosomal dominant type 2 diabetes mellitus, whose early forms are known in literature as MODY (Maturity-Onset Diabetes of the Young) and are characterized by β--cells insulin secretion defect [4,5]. Autosomal dominant type 2 diabetes mellitus occurs in families in several subsequent generations with high penetrance among the carriers of the mutations. Its different forms show some evidence of clinical heterogeneity [4]. For example, mutations identified in glucokinase gene are responsible for relatively modest clinical form of the disease. Glucokinase is a key enzyme of β-cells metabolism which catalyses phosphorylation of glucose to glucose-6phosphate. It leads to ATP synthesis and eventually, through the changes in ionic channels, to the insulin output. More severe autosomal dominant type 2 diabetes is associated with mutations in transcription factors, which are directly or indirectly involved in the insulin gene expression. Mutations in HNF (hepatocyte nuclear factor)-1α were identified as the most frequent cause of MODY in several populations [4]. Recently, two susceptibility genes for the common forms of type 2 diabetes have been identified [6]. The first one is peroxisome proliferator-activated receptor-γ. The common Pro12Ala polymorphism in this gene was found to be associated γ with type 2 diabetes in several ethnic groups and may be responsible for as much as 25% of this disease [6]. The second gene is calpain 10, a member of a large family of intracellular proteinases [7]. They are essential for multiple cellular functions, since the processing activity of these enzymes makes it possible to modulate directly the activities and structural properties of other proteins [8]. The specific mechanism through which some polymorphisms in PPARγ and calpain 10 genes increase the risk of type 2 diabetes development must yet be discovered.

Another form of the disease defined on the molecular level is maternally inherited type 2 diabetes mellitus. This form of diabetes was linked with sequence differences in mitochondrial DNA [9]. It is characterized by early age at diagnosis, usually in 3–5 decade of life, and impairment in insulin secretion. This form of diabetes is frequently accompanied by impaired hearing or deafness (maternally inherited diabetes with deafness-MIDD) [10,11]. There were plural mutations described in mitochondrial DNA that resulted in a diabetic phenotype. The most frequent one is A3243G substitution in a mitochondrial leucyl tRNA gene [11]. This mutation was originally identified in MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke like episodes) syndrome and it was later linked with different phenotypes, including type 2 diabetes among the others [12]. While this mutation was described in different races in several populations [13–19], it is still important and valuable to evaluate its prevalence in various ethnic groups.

The aim of our study was to determine the prevalence of A3243G substitution in a mitochondrial tRNA$^{Leu}$ gene in Polish diabetic subjects.

## MATERIAL AND METHODS

### Subjects

In total 139 individuals were selected for this study. Of them, there were 127 patients with type 2 diabetes and 12 with gestational diabetes. The current WHO criteria and definitions were used [20]. Subjects were classified as having type 2 diabetes if they were initially treated with diet only or oral hypoglycemic agents for a minimum of 2 years. Patients answered a questionnaire including age at diagnosis of diabetes, diabetes therapy, family history of diabetes, and accompanying diseases. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Jagellonian University, Krakow, Poland.

### Detection of the A3243 mutation in the mitochondrial tRNALeu gene

DNA was isolated from peripheral blood lymphocytes of the patients selected for the study. To isolate DNA a novel guanidine-detergent procedure (DNAzol Reagent, GIBCO) was used [21]. The extraction solution was used for this purpose according to manufacturer's guidelines.

Primers for mtDNA region of interest were used as previously published [10] and PCR reactions were

performed using DNA of the study participants. Two different techniques based on restriction fragment length polymorphism (RFLP) method were used to screen for A3243G mutation. In the first approach, the volume of PCR reaction was 20 microliters. Each PCR reaction contained 50 ng of genomic DNA, 20 pmol of each primer, 200 μM dNTP, 1.0 mM $MgCl_2$ and 0.5 units of Display Taq (Display Systems Biotech) with 2.0 μl of 10x PCR buffer. The following conditions were used on 2400 Perkin-Elmer Thermocycler: after 5 min of a denaturation step at 94°C, samples were subjected to 39 cycles of 1 min at 94°C for denaturating, 1 min at 55°C of annealing temperature, and 1 min at 72°C for elongation. This was followed by Apa I restriction enzyme digestion of the PCR product at 25°C for 8 h. Subsequently an electrophoretic separation was done on 2% agarose gel with ethidium bromide staining.

In the second technique, the volume of PCR reaction was 20 microliters. Each PCR reaction contained 10 ng of genomic DNA, 1.5 pmol of each primer, 2 umol of dATP, dGTP, dTTP, 0.025 umol dCTP, 0.1 mmol/l $MgCl_2$, 0.07uCi [$\alpha^{32}$P]dCTP (800Ci/mmol) for internal primer labeling and 0.3 U of Taq polymerase. The cycles were done on a model PTC-100 Thermal Cycler (MJ Research, Waltham, MA, USA). The initial denaturation step at 95°C (5 minutes) was followed by 34 cycles of denaturation (45 s at 95°C), annealing (45 s at 59°C) and extension (60 s at 72°C). The PCR product was digested with Apa I restriction enzyme at 37°C for 8h. Gels were then dried and exposed to films at –70°C for 1 day. After the digestion of PCR product, 2 volumes of sequencing stop solution were added to the PCR product. The mixture was heated at 95°C for 5 min, and 5 ul of the denaturated product was loaded onto 5% polyacrylamide DNA sequencing gels and resolved electrophoretically in 0.6xTBE at 100 W constant current. Dried gels were exposed to X-ray films for 12 h without intensifying screen. Positive sample was used to control the quality of the genotyping.

## RESULTS

On average the diabetic patients were 44.7±11.6 years old at the time of the diagnosis and the mean duration of their disease was 13 years. Of 139 individuals, 61% had a positive family history of diabetes, including 30% with maternal history of diabetes, 14% with paternal history of diabetes. Both parents being diagnosed with diabetes was reported by 1% of the participants.

15 examined patients were probands from the families that fulfilled MODY criteria: diabetes in three generations and at least one patient with age at onset of < 25 years. In one family with maternal pattern of diabetes inheritance two individuals reported the diagnosis of the hearing impairment.

None of the patients, except for positive quality control sample, showed the evidence of the G variant. This applies to both techniques used in this study: non-radioactive PCR with subsequent electrophoresis on the ethidium bromide stained agarose gel and the confirmation by more sensitive approach: radioactive PCR with electrophoresis on the acrylamide gel.

## DISCUSSION

In this paper, we report the results of the search for A3243G mutation in mitochondrial $tRNA^{Leu}$ gene in subjects with type 2 and gestational diabetes in a Polish population. None of the individuals was identified as a carrier of this mutation. Below, we discuss the contribution of this mutation to diabetes in different populations and the methods, which can be used to screen for A3243G mutation.

The knowledge of the molecular background of the type 2 diabetes has been growing rapidly over last decade. One of the first sequence differences linked to this disease were mutations in mitochondrial DNA [9]. The mechanism of the mitochondrial mutations affecting glucose homeostasis is uncertain. Most likely, the glucose sensoring function of the β cells or their ability to produce insulin are affected. The impairment of insulin secretory capacity assessed by the oral glucose tolerance test, glucagon test and C-peptide excretion was described in patients with MIDD [22]. This observation was confirmed by hyperglycaemic and euglycaemic clamp studies [23,24]. Early failure of sulphonylurea therapy and a clinical phenotype similar to type 1 diabetes frequently observed among patients with MIDD suggest the reduction of β-cell mass in these patients. Autopsy data showed the atrophy of pancreatic islets and the reduction of the number of β cells among patients with MELAS [11]. In general, the additional clinical features that may suggest a defect in mitochondrial DNA include multiorgan disorder (ECG abnormality, proteinuria, neuromuscular signs), and elevated lactate or lactate/puryvate ratio [11]. Cardiovascular problems, hypertension, nephropathy and spontaneous abortions have been observed among these patients. [12,26–28]. However, it is not clear, whether these

are true diabetic complications or clinical features related to the 3243 mutation itself.

The majority of MIDD cases were associated with A3243G mutation in tRNA$^{Leu}$ gene [11]. The frequency of this mutation reported in different populations varies. The prevalence above 1% of unselected type 2 cases was found in Japanese and Dutch populations [13,16,17], while in Australian ethnically mixed population it reached about 0.5% [18]. The data coming from a large British UKPDS study suggest low frequency of G variant (about 0.2%) among type 2 diabetes patients in this population [15]. On the other hand, in selected Scandinavian groups of patients with early onset diabetes (age at diagnosis< 40) and positive family history, A3243G mutation represented the cause of the disease in 2.6% [19].

We screened a large group of Polish type 2 diabetic patients together with a small number of gestational diabetes patients. It is well known that women who were diagnosed with gestational diabetes are at high risk of type 2 diabetes, so the inclusion of this subgroup seems to be well justified [20]. Our results suggest that A3243G mutation is not a frequent cause of diabetes in the Polish population. Further screening is required to fully determine whether this mutation contributes to the existence of diabetes in our population. This, together with the search for other mitochondrial mutations, will allow to fully determine the prevalence of MIDD and its specific molecular background in the Polish population. In our study group, at least one family appears clearly to have diabetes associated with mitochondrial DNA mutation, since it shows maternal pattern of inheritance and in addition two diabetic patients were diagnosed with hearing impairment.

There are several possible methods of screening for this mutation. In the first report of van den Ouweland et al. direct sequencing was initially used. Since G variant creates a new restriction site for Apa I, techniques based on RLFP were subsequently developed [10]. The simplest approach is to separate the digested PCR product on the agarose gel with etidium bromide staining. However, this may not be sensitive enough to detect the mutation if the percentage of mutant mitochondrial DNA is low. It was reported that this method might omit as much as one third of the heterozygous samples in comparison to more sensitive acrylamide electrophoresis followed by silver staining [21]. However, the „gold standard" remains radioactive PCR and electrophoresis of digested PCR product on acrylamide sequencing gel [10,19]. It seems very likely that, at least in part, the discrepancies in frequency of investigated variant of leucyl tRNA gene between the populations may be attributed to different sensitivity of screening methods. In our study however, the initial negative results of the less sensitive method were confirmed by reference modification.

## CONCLUSIONS

In summary, the A3243G mutation in mitochondria tRNA$^{Leu}$ gene is not a frequent cause of diabetes in the Polish population. Further screening of enlarged study group is necessary to fully determine the prevalence of this mutation in our population. This, together with the search for other mitochondrial mutations, will help to fully determine the prevalence of MIDD and its specific molecular background in the Polish population.

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