A3243G mitochondrial DNA mutation in Tunisian diabetic population

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RÉSUMÉ

Prérequis : Un excès dans la transmission maternelle du diabète est observé dans la population Tunisienne, en effet, elle est significativement plus importante que la transmission paternelle (p<10-6). Cet excès de transmission du diabète du côté maternel pourrait être le résultat d'un défaut touchant l'ADN mitochondrial qui se transmet exclusivement de la maman à l'enfant.

But : Evaluer l'atteinte du ADN mitochondrial dans une population tunisienne du diabéttique et la prévalence du A3243G.

Méthodes : Dans la présente étude nous avons recherché la mutation mitochondriale touchant le gène de l'ARNt Leu à la position 3243 du génome mitochondrial chez 280 diabétiques Tunisiens.

Résultats : Les résultats montrent que la fréquence de la substitution A/G de l'ARNt Leu est de l'ordre de 1.07%. Une fréquence comparable à celle retrouvée dans les populations japonaise, allemande et française.

Mots-clés

Diabète, population tunisienne, ADN mitochondrial, polymorphisme 3243

SUMMARY

Background: An excess of maternal transmission of adult onset diabetes mellitus has been observed in the studied Tunisian patients, in fact, diabetic patients with affected mother are significantly more important than those with affected father (p< 10-6) There is increasing evidence that mtDNA mutations may be involved in this disease, since mitochondrial transmission offers a plausible explanation for a proportion of this maternal excess comparing to paternal transmission.

Aim : The aim of the present study was to investigate the mitochondrial DNA involvement in the inheritance of diabetes in Tunisian population and to evaluate the frequency of substitution A3243G in these patients.

Methods : In the current study we investigated for the first time, the 3243 mtDNA in 280 Tunisian diabetic patients.

Results : Results showed that the frequency of this substitution in tRNALeu is about 1,07%. This percentage is similar to those reported in Japanese, German and French populations.

Key-words

Diabetes, Tunisian population, mitochondrial DNA, 3243 variant

Diabetes mellitus covers a wide spectrum of diseases with hyperglycaemia as the hallmark. Several studies have suggested that a maternal history of diabetes may be more important in determining the incidence of diabetes in offspring, than a paternal history (1-3). In Tunisian population, Arfa et al, (2007) reported recently a positive familial aggregation of the type 2 diabetes (T2D), with an excess transmission on the maternal side (4). Many hypotheses may explain this inheritance pattern:
1) a possible contribution of the foetal environment in development of T2D later (5).
2) mitochondrial DNA (mtDNA) could be an obvious candidate for genetic susceptibility of this metabolic disturb, given the central role played by mitochondria in insulin secretion pathway. Indeed, Oxidative mitochondrial metabolism is extremely important in the regulation of insulin production, in the pancreatic beta cell.
Besides, in 1992, a subtype of diabetes, maternally inherited, associated with deafness (MIDD) and more generally with neurological defects, was reported to co-segregate with a point mutation taking place on mitochondrial genome (6, 7). Patients with MIDD are young at diabetes onset and present a normal or low body mass index. Since mitochondria passed exclusively down the maternal line (8, 9), it was postulated that mtDNA defects might contribute to the excess maternal transmission.
Human mtDNA is circular and consists of 16569 bp, it is exclusively maternally inherited (8) and encodes 13 sub-units of the respiratory chain and oxidative phosphorylation enzymes, 22 tRNA and 2 ribosomal RNAs (10). Deletions, duplications and point mutations in mtDNA have been reported to be associated with a variety of diseases which affected tissues with a high need for energy (11,12). Pathogenic mtDNA usually exists in heteroplasmic form, with the existence of both mutant and wild-type mtDNA in affected cells and the degree of heteroplasmy varies considerably among different tissues and individuals (13, 14). A number of mtDNA defects have been implicated in diabetes risk (6).
Recently, several case reports (15-17) suggested that mutations in mtDNA, especially one involving the substitution of guanine for adenine (A to G) at position 3243 of leucine tRNA gene (16, 17), may cause MIDD. This point mutation occurs within the mtDNA binding site for a protein factor that promotes the termination of transcription at the boundary between the 16S ribosomal and tRNA Leu (UUR) genes. It appears to interfere not only with the synthesis of tRNA Leu (UUR) but also with the binding of the transcription termination factor, thereby causing defects in synthesis of mitochondrial proteins (18).
The aim of the present study was to investigate the mitochondrial DNA involvement in the inheritance of diabetes in Tunisian population and to evaluate the frequency of substitution A3243G in these patients.

PATIENTS AND METHODS

Subjects

The studied population includes 200 patients with T2D and 80 patients with type 1diabetes (T1D). all patients were Tunisian, they were recruited from the Endocrinology Department in Charles Nicolle's hospital in Tunis.
Glycemic status was defined according to American Diabetes Association criteria 1997: normoglycemia, defined as fasting glucose < 6.1 mmol/l without hypoglycemic treatment; T2D, defined as fasting plasma glucose > 7.0 mmol/l and/or treatment by antidiabetic agents. Genomic DNA was extracted from peripheral blood by phenol-chloroform standard procedure.
Personal and familial history of diabetes in first and second degree relatives were reported by participants who were questioned about. Other helpful characteristics in identifying patients with MIDD are reported in table 1.

Tableau 1 : Clinical features of the studied cohort

Clinical features		T1D	T2D
GENDER	Men/	30(37,5%)/	84(42%)/
	Women	50 (62,5%)	116(58%)
AGE	Age <25years	43 (53,75%)	3 (1,15%)
	25<Age <35	16 (20%)	24 (12%)
	Age>35	21 (26,25%)	173(86,50%)
OBESITY	Non obese	68 (85%)	114 (57%)
	Over weigth and obese	12 (15%)	86 (43%)
Diabetes inheritance	absence of inheritance	32 (40%)	122 (61%)
	familial diabetes	48 (60%)	78 (39%)
neurological defects (deafness and neuro-muscular defects)	Presence	9 (11,25%)	10 (5%)

Values are expressed as effectives (frequency %), non obese have a Body mass index (BMI) <25Kg/m^2, overweight and obese have a BMI> 25Kg/m^2

Sample preparation

Venous blood samples were collected in EDTA and plain tubes were centrifuged at 3000 rpm to separate plasma and serum, the buffy coat and red blood cells pellet were used for DNA extraction. Leukocytes DNA was extracted by standard method involving proteinase K and Phenol/ Chloroform.

Genotyping

For each subject, fragment of the human mtDNA encompassing the polymorphic site was amplified by PCR (94°C for 10 minutes, followed by 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute) using the forward primer 5'AAGGTTCGTTTGTTCAACGA3'and the reverse primer 5'GGCCTAGGTTGAGGTT- -GACC3'. The PCR reactions mixture were carried out in 50 μl containing 30 ng of genomic DNA, 5 μl of PCR Buffer10X with MgCl2, 0.5μl of dNTPs (10μg/μl), 1,25 μl of each primer (10ÌM) and 0,125 μl of Taq DNA polymerase (5U/μl).
PCR products were digested with ApaI restriction endonuclease for two hours at 37°C. The reaction was carried out in 25μl containing 10μl of PCR product, 2.5 μl of ApaI buffer10X and 0.2μl of ApaI endonuclease. Digested products were analysed by electrophoresis through 1% agarose-gel and fragments were visualized using UV fluorescence.

In order to verify the presence of the mutation, PCR product of suspected patients were purified using the kit Quiagen column and sequenced according to the following program: 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds, and 60°C for 60 seconds. Then sequencing reactions were performed using Automated Dye Terminator Sequencing.

RESULTS

The studied population includes 200 patients with T2D and 80 with T1D. General clinic features of these patients are reported in table 1. In fact; we noticed that patients with T1D are younger than those with T2D, obesity is rather significantly associated with T2D than T1D (p=0,001) and familial aggregation is important in both T1D and T2D, thus 60% of T1D cases versus 39% of T2D have a familial history of diabetes. Neurological diseases are not commonly associated with diabetes.

Based on a familial history, we reported in table 2 the maternal and paternal inheritance of diabetes in the studied Tunisian sample. Transmission of the disease from the mother is significantly more important than that from the father. This result has been obtained for both T2D and T1D group of patients with p=1.10-7 and 5.10-7 respectively.

Tableau 2 : Significant difference between maternal and paternal transmission of diabetes in Tunisian studied sample

Diabetic patients effectives	Maternal Transmission (MT)	Paternal transmission (PT)	sporadic cases	P value (MT versus PT)
T1D n= 80	45 (53,75%)	5 (6,25%)	30 (37,5%)	5.10-7
T2D n=200	60 (30%)	10 (5%)	122 (61%)	1.10-7

Comparing clinical features of the studied cohort and those characterizing MIDD, we revealed 4 % of patients with at least three features of MIDD including most commonly maternal inheritance of the disease, absence of obesity and an age at diabetes onset varying between 25 and 35years. Neurological disturbs are scarcely meeting in our studied sample.

PCR/ RFLP were realized for all subjects. The presence of 3243mt mutation allows the 581bp amplified fragment to be cleaved into 367 and 214 bp fragments when digested with ApaI restriction endonuclease. On agarose-gel we visualize either only the 581bp fragment corresponding to the amplified mtDNA that haven't been cut by the endonuclease or three fragments 581, 367 and 214 bp because of the heteroplasmy of the mutation.

For 277 diabetics, agarose-gel profile showed only the 581bp fragment, which suggests absence of ApaI restriction site and therefore the absence of 3243 mutation in mtDNA of these subjects. Only three diabetic patients seem to have the 3243mt mutation. Among them, one has T1D and the two others have T2D, while all of them have an insulin therapy. All of these three patients have a mother with diabetes, were young at the time of diagnosis (age range from 25 to 35 years) and were not obese at diabetes onset. Two of them have developed either neuromuscular dysfunction or hearing loss.

DISCUSSION

In this paper, our interest concerned A3243G mitochondrial substitution known for its association with diabetes in several populations. An excess of maternal transmission diabetes mellitus has been observed in the studied Tunisian patients, in fact, diabetic offspring having a diabetic mother are significantly more important than those having diabetic father (p< 10-6). Moreover, maternal transmission of diabetes can spread through 2 or 3 generations but paternal one is observed only through 2 generations. (Table 3) This last result underlines the importance of maternal inheritance of diabetes. Given the role of mitochondria in insulin secretion and its inheritance pattern, mtDNA offers a plausible explanation for a proportion of this maternal excess comparing to paternal transmission (? seven times more important than paternal one). However, epigenetic factors such as maternal auto-antibodies or parental imprinting could explain this situation particularly in T1D for which maternal inheritance is about 9 times more frequent than paternal inheritance. The high rate of maternal transmission of diabetes in Tunisia may also, be explained by certain mitochondrial haplogroups predisposing the individuals to type 2 diabetes.

Tableau 3 : Diabetes transmission in the Tunisian studied sample

Diabetic patients effectives	sporadic cases	familial cases			
		1G touched	MT through 2G	MT through 3G	PT through 2G
T1D n= 80	30 (37,5%)	0	34 (42,5%)	9 (11,25%)	5 (6,25%)
T2D n=200	122 (61%)	8 (4%)	50 (25%)	10 (5%)	10 (5%)

Values are expressed as effectives (frequency %), T1D: type1diabetes mellitus, T2D: type2 diabetes mellitus,
G: generation, MT: maternal transmission, PT: paternal transmission

A3243G mitochondrial mutation was reported to co-segregate with diabetes in several populations. Patients having the substitution were reported to have a special clinical profile (11,12). In fact, diabetes of these patients is maternally inherited, generally associated with neurological defects, individuals are young at diabetes onset with a normal or low body mass index (BMI).

Given the clinical table characterizing MIDD patients, the diagnosis would de based on either maternal inheritance of the disease or the presence of neurosensorial muscular signs associated with glucose intolerance. However, confirmation of the diagnosis is only provided by identification of genetic defect through molecular biology techniques. The molecular characterization of subjects with MIDD is very useful because patients with mitochondrial diabetes need a special taking care

of. Vialettes et al (2001) (19) reported that this diabetes form imposes specific check-up and therapy comparing with other diabetes type. In fact, patient with MIDD needs usually neurological and cardiac controls. Besides, drugs based on Metformin are misadvised for the treatment of these patients, because they inhibit neoglycogenosis, contrary to Glitazone which preserve mitochondria morphology in , cells. In our population 4% of diabetic patients presented MIDD profile and three among them carried the 3243 mutation.

In the current study, the 3243 mutation in Tunisian diabetic population, appears with a frequency of 1,07%. This percentage is similar to those reported in Japanese, German and French populations (20-22). Tunisian patients having the substitution presented maternally inherited diabetes, were young and present a low BMI at diabetes onset and two among them suffer from neurological defects in association with diabetes. These clinical characteristics are in agreement with those proposed for mitochondrial inherited diabetes and deafness (MIDD) by Maassen et al (1997) (23). Moreover, the A>G mutation of 3243 mtDNA may be associated with either type 1 or type 2 diabetes mellitus and type 2 diabetic patients become rapidly insulin dependant. These results confirm those reported by Kadowaki et al (1994) (24).

However, among 11 subjects with MIDD profile only 3 were positive for 3243 mitochondrial DNA mutation, this may be related to the methodology used in our study using blood leucocytes. Since 3243 mitochondrial DNA mutation is heteroplasmic, the amount of mutant mt3243 may vary from one tissue to another. In leucocytes this amount is low comparing with fraction of non mutated mtDNA leading to a weak sensitivity of the technique. In order to confirm this mutation, sequencing of the concerned region was performed. But low level of mutated mtDNA did not allow showing the presence of 3243 substitution. In order to improve our approach and confirm this result, it is proposed to start from other tissues and to use DHPLC as genotyping method.

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