Full Length Research Paper

The search for mitochondrial tRNA[Leu(UUR)] A3243G mutation among type 2 diabetes mellitus patients in the Nigerian population

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The study aimed to compare the incidence of the pathogenic point mutation A3243G in the gene tRNA[Leu(UUR)] indicating sub-type 2 diabetes mellitus conducted within the Nigerian population with that reported in other populations. 112 patients diagnosed with type 2 diabetes (T2D) mellitus according to the World Health Organization criteria were selected based on family history and re-evaluated for associated disorders from the diabetic clinics in the Northern part of Nigeria. The mtDNA of these patients was extracted and the tRNA[Leu(UUR)] gene screened for A3243G by PCR-RFLP method. Probands with maternal history were further investigated for other mutations using PCR-sequencing methods. None of the 112 patients were found to carry the A3243G mutation in the mitochondrial tRNA[Leu(UUR)] gene in the homoplasmic or in the heteroplasmic form. However, C3254T was identified in two of our patients. This mutation was reported to be associated with gestational diabetes and linked with population from sub-Saharan Africa. The A3243G mutation in mitochondrial tRNA[Leu(UUR)] is not a frequent cause of maternal diabetes in the Nigerian population contrary to other reported populations. However, further screening of an enlarged selected study group is necessary to fully determine the prevalence of this mutation in this population. This further search will help to fully appreciate the prevalence of maternal inheritance and diabetic deafness (MIDD) as extensively reported in other populations.

Key words: Maternal diabetes, mitochondrial gene, maternal Inheritance and diabetic deafness, Nigeria, sub-Saharan Africa.

INTRODUCTION

The mitochondrial tRNA[Leu(UUR)] gene is a hot spot for pathogenic mutations and they are associated with mitochondrial diseases in various clinical features (Emna et al., 2007). The landmark discoveries of mitochondrial diabetes were the identification of a 10.4 kb deletion in mitochondrial DNA (mtDNA) associated with diabetes and deafness syndrome and the tRNA[Leu(UUR)] A3243G mutation associated with diabetes and deafness in a large pedigree (Wei et al., 2006). Since then, numerous point mutations, deletions and duplications in mtDNA have been detected to associate with diabetes worldwide. Of these, the A3243G mutation appears to be the most common diabetes associated mtDNA mutation, accounting for between 0.5 and 3% of all type 2 diabetes (T2D) in various populations (Walker and Turnbull, 1997; Takashi et al., 1994; Wallace, 2005). This mutation, which is present in heteroplasmic form in patient cells, exhibited a remark-able variability of its clinical manifestation. When 70% of total mtDNA in cells carried

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the A3243G mutation, this mutation does not cause diabetes, but instead causes more severe symptoms, including short stature, cardio-myopathy, mitochondrial encephalomyopathy, lactic acid and stroke-like episodes (MELAS) syndrome (Yu-ichi et al., 1990). When present at relatively low levels (10 to 30%) in the patient's blood, the patients may manifest only diabetes with or without sensorineural hearing loss and it is now associated with maternal inheritance and diabetic deafness (MIDD) (Van den et al., 1992; Maassen, 2002; Ohkubo et al., 2001), also known as maternal diabetes. The age at onset of diabetes in patients with the A3243G mutation varies, with average at the age of 35 years (Maassen et al., 2002; Ohkubo et al., 2001).

There is a growing body of evidence that the mito-chondrial dysfunction plays a crucial role in the pathogenesis of type 2 diabetes. Also, evidences have shown that mtDNA abnormalities account for appro-ximately 1% of all cases of diabetes (Maassen et al., 2001). Point mutations in mtDNA (mitochondrial tRNA genes) are particularly frequent since over 100 pathogenic mtDNA mutations were described in various tRNA genes. (MITOMAP:

Human Mitochondrial Genome Database, www.mitomap.org). While this mutation (A3243G) has been described in different populations with Caucasians as the majority of the study groups, there has been no documented evidence of this mutation among an unmixed population from the Sub-Saharan in Africa. This observation therefore prompted this study to test for this hypothesis: incidence of the pathogenic point mutation A3243G in the gene tRNA$^{Leu(UUR)}$ indicating type 2 diabetes mellitus is comparable within the Nigerian popu-lation to that determined in other reported Caucasian population. Hence, this study to our knowledge may be the first documented evidence of A3243G mtDNA mutation from this part of the world.

MATERIALS AND METHODS

Study population

112 type 2 diabetic patients according to the World Health Organization criteria (WHO consultation, 1999) with parental history only were selected from diabetic units of three hospitals located in the Northern part of Nigeria. Those that could not recall or were not sure of parental history of diabetes were excluded. Subjects identified for the study signed an informed consent form before they were allowed to participate. Ethical approval was gained from these hospitals- Sir Yaya Memorial Hospital, Yauri General Hospital and Apex Clinic. The selected patients were also re-evaluated for the following clinical characteristics: body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), neurosensorial deafness, cardiomyopathy, stroke like episodes, retinopathy, foot disease and neuropathy.

DNA extraction

MtDNA was isolated from 3 ml of whole blood according to the

published protocol (Shazia et al., 2007). The extracted DNA samples were stored at -20°C before analysis.

Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP)

The method and primers used to perform amplification of the gene of interest (tRNA$^{Leu(UUR)}$) and part of NADH dehydrogenase subunit 1 (ND1) located on mtDNA are the same as described by Emna et al. (2007) with the following modifications: the total reaction volume of 20 µL containing an average of 200 ng mtDNA, 10 pmol of each primer, 1.25 mM dNTPs, 5 × PCR buffer (GO TAQ^{TM}, Promega Corporation, USA), 5 U of Taq DNA polymerase (GO TAQ^{TM}, Promega Corporation, USA) and molecular grade water was amplified using Peltier thermal cycler (PTC-200, MJ Research Inc., Canada).

The reaction generated PCR products of the gene tRNA$^{Leu(UUR)}$ of 528 bp only. Amplified products were confirmed by performing agar gel electrophoresis. The PCR products and unmethylated DNA lambda were digested with endonuclease restriction enzyme, Apal separately. The total reaction volume of 20 µl containing Apa I (10 U /µL), 10 × restriction buffer, PCR products or DNA lambda and molecular grade water was incubated at 37°C for 1 to 2 h for the digestion to occur. The reaction with the endonuclease enzyme and DNA lambda was the positive control and the one without the enzyme served as negative control. Furthermore, the sample and control digests were resolved on 2 and 0.8% agarose gel, respectively. Gels stained with ethidium bromide were placed on a UV light transilluminator and PCR and digested products were visualized using Quantity One software (CHEMIDOC, Bio-Rad, USA).

PCR sequencing

Approximately, 10 ng of the purified PCR product from probands with maternal history only (using GENELUTE^{TM} PCR Clean-up Kit, Sigma-Aldrich, Inc.) was amplified in a total reaction volume of 20 µL containing the Big Dye terminator reaction buffer, each of the primer (3.2 pmol/µL) and molecular grade water. This reaction was programmed to perform at 96°C for 1 min followed by 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min using the Peltier thermal cycler. The amplified gene in the reaction mix was precipitated after several alcohol washes at 95 and 70%, respectively dried using the vacuum centrifuge, resuspended in Hi-Di formamide and loaded onto the 3500 Genetic Analyzer, Applied Biosystems for sequencing. The resultant sequences were compared with the Cambridge sequence (GenBank Accession No. NC_012920) (Andrews et al., 1999).

Statistical analysis

Epi info version 3.5.1 (CDC Atlanta, USA) was used to assess the effect of clinical characteristics on probands based on parental transmission of T2D. A value of p<0.05 was considered significant.

RESULTS

Effect of transmission pattern on clinical characteristics

Out of the 1,110 patients screened for maternally

Table 1. Baseline characteristics of probands according to their parent's diabetic status. The Data is presented as mean ± standard deviation and frequency in percentage.

Variable	Parental diabetes		
	Paternal	**Maternal**	**Bilineal**
Number	27	73	12
Age at diagnosis	43.7 ± 10.9	44.5 ± 11.8	46.1 ± 10.1
Age at examination	51.8 ± 8.4	52.1 ± 13.0	56.1 ± 11.5
BMI (kg/m^2)	27.7 ± 4.3	26.0 ± 6.2	27.5 ± 3.9
SBP (mmHg)	136.3 ± 19.3	136.9 ± 24.6	138.8 ± 14.9
DBP (mmHg)	81.0 ± 9.7	85.4 ± 12.0	82.2 ± 8.9
Frequency of neurosensorial deafness (%)	7.4	7.0	0.0
Frequency of cardiomyopathy (%)	37.0	43.5	50.0
Frequency of stroke (%)	7.4	10.9	21.4
Frequency of retinopathy (%)	74.1	60.9	71.4
Frequency of diabetic foot disease (%)	7.4	6.5	7.1
Frequency of neuropathy (%)	88.9	78.3	85.7

inherited T2D, 85 patients could recall the diabetic status of their mothers (grandmothers inclusive) and they were subsequently recruited for the study. 27 patients with diabetic fathers were also recruited but only served as controls. The influence of the transmission patterns of T2D on the clinical outcome of patients was divided according to the probands with diabetic mother only (maternal), diabetic father only (paternal) and diabetic mother and grandmothers (bilineal). Results show no significant difference in clinical presentations between the controls, maternal and bilineal (Table 1). The ANOVA p-value of these presentations was >0.05 which is not statistically significant; no significant differences between family groups. However, maternal excess is 2.7; where it is expressed as the ratio of affected mothers to affected fathers for probands with T2D.

tRNA$^{Leu(UUR)}$ PCR- RFLP and sequence analysis

The amplified tRNA$^{Leu(UUR)}$ from all the patients was screened for the presence of A3243G mutation by PCR-RFLP. The A3243G mutation created a restriction *Apa*I site and the digestion resulted in two fragments of 234 and 294 bp (as compared with the molecular markers-100 bp DNA ladder) if the mutation is present, whereas in the absence of this mutation, a 528 bp fragment corresponding to the PCR product should be observed (Figure 1). Results were compared with the molecular markers and evidence to show that *Apa*I can create a restriction site (restricted DNA lambda) was proven. *Apa*I created a restricted site of the DNA lambda at position 10,090 bp thereby generating two bands of 10,090 and 39,000 bp, respectively (positive control), while the DNA lambda was uncut without the *Apa*I as 49,000 bp (negative control) (Figure 2). All the results from the 112 patients were compared with these controls.

More also, results were further determined by visual inspection of the size of fragments with the molecular markers as a reference and the restricted DNA lambda as a proof of the functional activity of the endonuclease enzyme. It was observed that none of the 112 patients were found to carry the A3243G mutation in the mitochondrial tRNA$^{Leu(UUR)}$ gene in the homoplasmic or in the heteroplasmic form. Therefore, we confirm the absence of the A3243G mutation in this gene. Furthermore, the tRNA$^{Leu(UUR)}$ gene of the patients with maternal history only were further screened for mutations using direct sequencing in order to identify different mutation from A3243G.

tRNA$^{Leu(UUR)}$ mutations

The tRNA$^{Leu(UUR)}$ gene of patients with maternal history were screened for mutations by comparing their sequences with the annotated human mitochondrial genome database in order to identify different mutation from A3243G. The visual inspection identified one single nucleotide polymorphism (C3254A) from 2 patients with these identity; 37/10 and 45/10. C3254A is indicated in the Figure 3. This mutation has also been classified as haplogroup marker-L2d.

DISCUSSION

It has been well documented that maternal inheritance has been implicated with the diagnosis of T2D. Therefore, the maternal excess reported in this study is not different from most published studies on maternally inherited T2D (Alcolado and Alcolado, 1991; Lin et al., 1994; Young et al., 1995; Karter et al., 1999; Bjornholt et al., 2000). The recent publication in Tunisia reported that
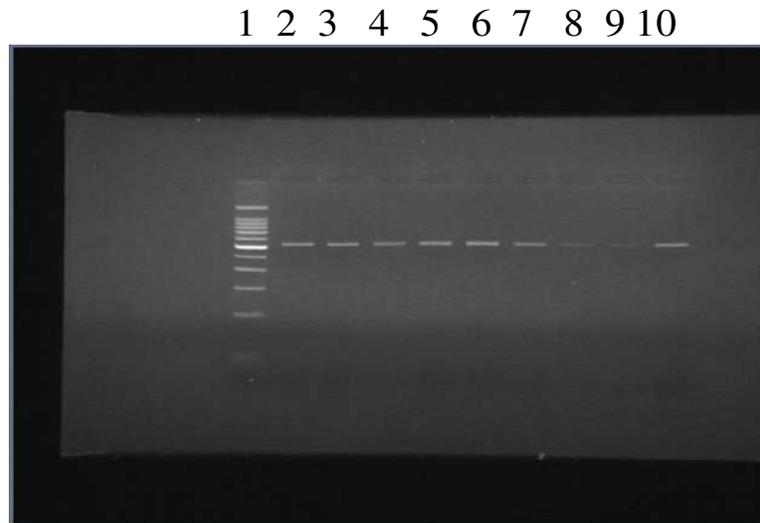
Figure 1. Screening of the A3243G mutation by PCR-RFLP. PCR fragment of 528 bp was digested with *Apa* I. The wild type PCR product does not contain the *Apa*I restriction site but PCR product with A3243G is expected to cleave into two fragments of 234 and 294 bp. Hence, Lanes 2 to 10 is a representation of restriction assay in patient's samples that resulted in an uncut fragment of 528 bp while Lane 1 is the 100 bp DNA ladder (molecular marker).
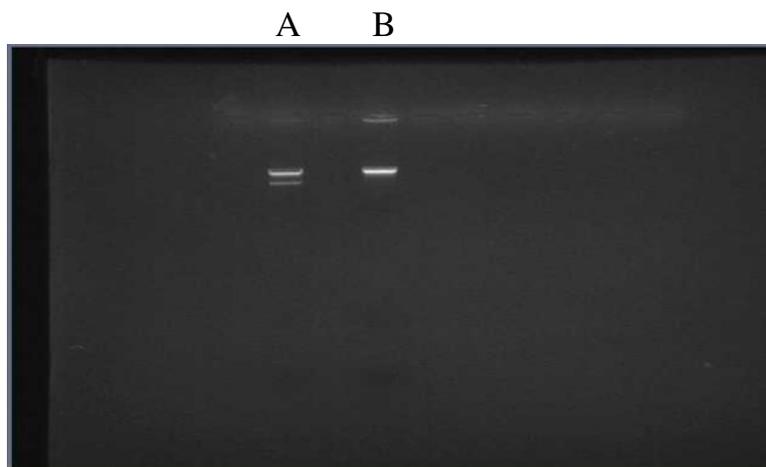

Figure 2. Restriction digestion of unmethylated lambda DNA. Lane A represents the cut DNA; *Apa*I digested the lambda DNA into two fragments (10,090 and 39,000 bp, respectively), but remained uncut in Lane B in the absence of the endonuclease enzyme.

patients with T2D are more likely to have an affected mother than their father (Imen et al., 2007) which is the same with the South Africa study (Erasmus et al., 2001).The two studies from Africa did not however evaluate the contribution of mitochondrial tRNA[Leu(UUR)] in the transmission of maternally inherited T2D which has been well reported from Europe, America and Asia. To our knowledge, our documented data is likely to be the first in this region to further document the association of tRNA[Leu(UUR)] mutations (particularly A3243G) with T2D as reported in studies not from Africa.

Our observation showed the absence of this mutation among the patients studied. This finding was also observed among the studied polish population (Maclej et al., 2001).This study and the polish studies examined probands with positive family history, maternal and paternal inclusive. Also, among the 128 patients screened for A3243G in the Tunisian study, none was
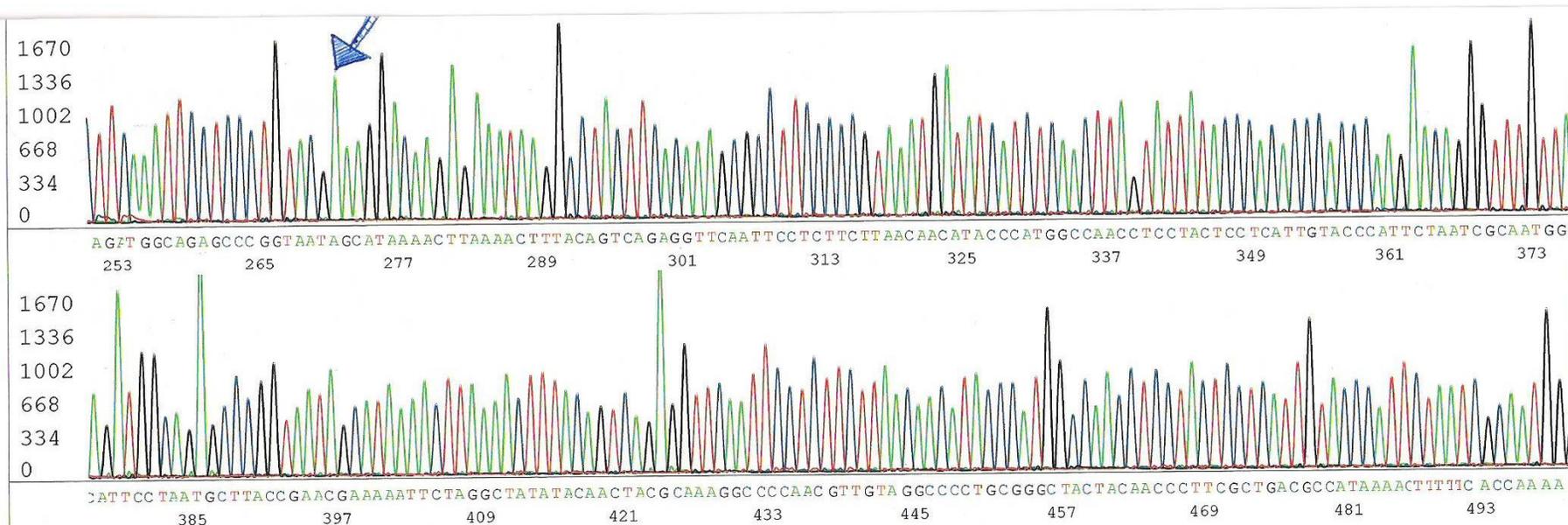
Figure 3. tRNA[Leu(UUR)] electropherogram showing the transition of C to A at position 3254 in the gene of interest of patient 45/10 as indicated by the arrow.

found with the mutation, although, only 30 diabetics contributed to the study (Emna et al., 2007). It was however, not mentioned if these diabetics were type 1 or type 2 but there were classical features of MIDD or MELAS in some of the patients evaluated. The finding of this study is in contrast to majority of studies conducted among the Caucasians with varied frequencies. The prevalence of less than 1% was documented among the studied Brazilian, Australian, Korean and British (Daisy et al., 2008; Carolyn et al., 2007; Hyun et al., 1997; Newkirk et al., 1997), while a prevalence of 4.1% was documented among the Japanese, 2.6, 2.9 and 1% were reported among the Scandinavian, Finish and Hong Kong, respectively (Suzuki et al., 2003; Odawara et al., 1995; Uchigata et al., 1996; Lehto et al., 1999; Kari et al., 1998; Ng et al., 2000). The

studied Brazilian population was made up of 344 Caucasoid and 101 African-Brazilians. This population was classified into classical type 2 diabetes mellitus (without MIDD) and type 2 diabetes mellitus (with MIDD). The identified patients with A3243G belonged to the second group. In addition, these patients presented with age at diagnosis, before 30 years, BMI <25kg/m^2, insulin treatment, and presence of proliferative retinopathy and ischemic cardiomyopathy (Daisy et al., 2008).

This is however different from the Australian study, that studied a large cohort of individuals without screening for diabetes mellitus within a defined urban area. The identified 7 patients with A3243G were previously undiagnosed, oligo-symptomatic and were thought to have age-related hearing loss (Carolyn et al., 2007). Also,

out of the 1,440 patients diagnosed as type1 and type 2 diabetes in the British study, 445 had one or more features of MIDD and/or MELAS. Only 2 of this group of patients had this mutation, A3243G (Newkirk et al., 1997). While the Korean study randomly screened patients irrespective of their diabetic types. The proband identified with A3243G had history of myoclonic epilepsy, mild sensorineural hearing loss, one year duration of diabetes mellitus and a low level C peptide response to oral glucose (Hyun et al., 1997). In addition, the Japanese study, attributed poly-neuropathy, earlier onset of diabetes and a higher frequency of retinopathy, nephropathy and insulin therapy as mostly found among type 2 diabetic patients with A3243G mtDNA mutation with the 11 identified patients. This mutation A3243G has been well documented in the Japanese population

(Suzuki et al., 2003; Odawara et al., 1995; Uchigata et al., 1996) and appears to be more prevalent than in any other population.

Unlike the Japanese study, however, Scandinavian study focused on maturity onset diabetes of the young and mitochondrial genes to early onset diabetes with a strong family history of diabetes in a cohort with a high prevalence of type 1 diabetes mellitus. However, the 3 identified MIDD patients with A3243G resembled more the phenotype of the insulin resistant type 2 diabetic patients (Lehto et al., 1999). While the Hong Kong Chinese classified the patients into early (133) and late onset (348) and all were diagnosed as having type 2 diabetes mellitus, 4 of the early onset patients carried the mutation with maternal family history, while 1 of the late onset patients carried the mutation but without the history (Ng et al., 2000). Unlike the other studies, the Finish study examined a large population but 480 samples were analyzed for A3243G based on clinical presentations of the patients. The 14 identified patients with the mutation presented with diabetes, hearing loss, hypertrophic cardiomyopathy, occipital stroke, ophthalmoplegia and epilepsy (Kari et al., 1998). In all the identified studies, study approaches in the identification of the mutation varied from one group to another, although, there were similarities in some studies. One common factor with all the mentioned studies is the high number of subjects recruited unlike our study. This may be one of the reasons responsible for the absence of the mutation in our study. Hence, this may require a further screening of an enlarged selected probands. However, our study documented some of the common co-morbidities associated with diabetes and one or two of classical symptoms of MIDD like in the Finish, Japanese, Korean and Brazillian studies.

It is also possible that the blood sample used as the source of the mtDNA could have contributed to non-detection of the mutation. This is because different samples like blood, hair follicles, buccal epithelial cells and muscle biopsy have been used to determine the presence of A3243G mutation (Kari et al., 1998; Chia-Wei et al., 2001) but it has been shown that the proportions of mutant mtDNA A3243G in diabetic subjects were noted to be the highest in muscle tissue followed by hair follicles and lowest in blood cells (Chia-Wei et al., 2001). Interestingly, the proportions of this mutation in all the tissues were found to decline with increase in age (Chia-Wei et al., 2001) suggesting that our study must have missed the mutation of interest since the average age of studied subjects at examination was approximately 52 years.

This study detected C3254A mutation from two of the patients investigated in this work, a mutation that has been reported in the MITOMAP and associated with gestational diabetes mellitus (Chen et al., 1999) but this mutation need to be further investigated for its functional meaning according to the authors. One of the patients

that carried this mutation in this study presented with diabetic retinopathy and neuropathy which are symptoms usually associated with T2D. Interestingly, this mutation has also been recognized to be associated with the Sub-Saharan Africans (non-Khoisan) (Behar et al., 2008b) confirming where the sample collection was done. It can be argued that the mutation associated with gestational diabetes mellitus as reported by Chen et al may actually be "accidental" since the parental history was not documented unlike this study. Though the geographical place where the samples were collected was not mentioned by Chen et al., (2000) it may be suggested that this mutation is associated with people from Sub-Saharan Africa based on the human mtDNA phylogeny (PhyloTree.org-Global human mtDNA phylogenetic tree), or it is also possible that this mutation identified by Chen et al may have come from a patient from Sub-Saharan Africa as a result of migration. Since the authors considered this mutation novel and the functionality is yet to be established, it can then be suggested that this mutation is associated with the population in Sub-Saharan Africa.

Conclusion

Further screening is required to fully determine whether A3243G mutation contributes to the existence of T2D among the black race.

This, along with the search of other pathogenic mitochondrial DNA mutations, will determine whether the gene, tRNA$^{Leu(UUR)}$ is a hot spot as reported in the black community. Also, the further search will allow an understanding of the molecular background of diabetes in the Nigerian population.

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