Detection of 3243 A/G and 3316 G/A mitochondrial DNA mutations in Nagpur population

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ABSTRACT

The present study aims to detect 3243 A/G and 3316 G/A mitochondrial DNA (mtDNA) mutations in Nagpur population. Total of 142 patients of type 2 diabetes mellitus and 142 healthy control individuals were selected for the study from Nagpur city. Selected mutations studied using restriction fragment length polymorphism method and confirmed by DNA sequencing. Results showed that 3316 G/A mt DNA mutation found in seven patients of type 2 diabetes mellitus with a 4.92% prevalence, however, found absent in healthy control individuals. Chi-Square and Fisher's exact test showed a significant association between healthy control individuals and type 2 diabetes mellitus patients detected with 3316 G/A mutation ($p \leq 0.01$). ODDS ratio found significant (for 95% CI; $p = 0.05$) for 3316 G/A mutation. Furthermore, we did not find 3243 A/G mtDNA mutation in the studied population. Among studied mutations, 3316 G/A mutation in the ND1 gene is a pathogenic mutation that may responsible for type 2 diabetes mellitus in the Nagpur population.

1. INTRODUCTION

Worldwide, diabetes mellitus is a major health problem, often accompanied by polyuria, polydipsia, and glycosuria [1]. In the year 2000, the prevalence of diabetes mellitus was 171 million, which may increase to 366 million by the year 2030 [2] and about 70% of this burden will be shared by the developing countries. In India, epidemiological studies revealed a 1-4% prevalence of diabetes mellitus in the urban population, while 1%–2% prevalence in the rural population. This critical situation may be due to the change in life style, consumption of non-traditional food, and a genetic predisposition to various diseases [3,4].

Mitochondria are the main sites for respiration and inherit maternally [5,6]. Hitherto, prodigious work on mitochondria revealed that mtDNA mutations may cause type 2 diabetes mellitus. mtDNA is double stranded, measuring 16,569 bps in length, code 22 genes for transfer RNA (tRNA), 2 genes for ribosomal RNA (rRNA), and 13 polypeptides of the electron transport chain (ETC). Furthermore, the paucity of histone proteins and DNA repair enzymes make

mtDNA more susceptible to oxidative damages, which may result in homoplasmic (either mutated DNA or wild DNA) or heteroplasmic (both mutant and wild DNA) type of mutations [7]. However, maternal inheritance [8] and lack of recombination are considered the unique properties of mitochondria often use to identify maternal ancestors in a population [9].

For the present study, Authors selected 3243 A/G and 3316 G/A mtDNA mutations, the reason being their high prevalence rate worldwide. The 3316 G/A mtDNA mutation is responsible for the replacement of tyrosine by histidine in ND 1 gene [codes complex 1 (Nicotinamide adenine dinucleotide Dehydrogenase) of ETC] of the mitochondrial genome [10,11]. Also, 3243 A/G mutation results in an abnormal tRNA, which incorporates leucine amino acid in the growing polypeptide chain of the ETC [8].

Nagpur is a big city of central India and the vice-capital of Maharashtra state. This city is located near the boundaries of three states namely Madhya Pradesh, Chhattisgarh, and Andhra Pradesh and provides a varied range of populations with different dietary habits and living standards. These features make the Nagpur population a better model to study mtDNA polymorphism. Therefore, we have undertaken this study to know the status of mtDNA mutations and type 2 diabetes mellitus in the Nagpur population.

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2. MATERIALS AND METHODS

2.1. Family History and Sample Collection

For the present study, a total of 142 patients of type 2 diabetes mellitus was selected, who had a history of maternal inheritance in their family. To compare, 142 healthy control individuals were also selected (ages ranged from 18 to 75 years). All of them were selected from the Nagpur population. Blood samples were collected after taking signed consent. Family history was recorded meticulously.

2.2. Inclusion Criteria

Patients having a history of maternally inherited type 2 diabetes mellitus.

2.3. Exclusion Criteria

Any kind of history of paternal inheritance of type 2 diabetes mellitus, type 1 diabetes mellitus, juvenile diabetes mellitus, alcoholic individuals, smokers and surgery.

2.4. Isolation of mtDNA

mtDNA was isolated using a commercially available Abcam kit (ab65321) as per the manufacturer's instruction.

2.5. Detection of 3243 A /G Mutation

Total 422 nucleotides containing DNA was amplified by polymerase chain reaction (PCR), using ready to use master mix (Promega M7122). The forward primer was taken from nucleotide sequence 3035 to 3054 as 5′ -CGTTTGTTCAACGATTAAAG-3′ and the reverse primer was taken from nucleotide sequence 3437 to 3456 as 5′-AGCGAAGGGTTGTAGTAGCC -3′ [12]. The specificity of the primers was confirmed by primer BLAST using the national centre for biotechnology information (NCBI) database. The amplified PCR products were digested by Apa I restriction endonuclease (Promega: R6361). After electrophoresis, the presence of the mutation generates two bands (212 and 210 bp) and the absence of the mutation generates a single band (422 bp).

2.6. Detection of 3316 G/A Mutation

To identify 3316 G/A mutation authors used a new set of primers from the revised Cambridge reference sequence of human mtDNA (MITOMAP). A total 261 nucleotides containing DNA was amplified by PCR, using ready to use master mix (Promega M7122). The forward primer was taken from 3150 to 3175 as 5′TACTTCACAAAGCGCCTTCCCCCGTA 3′ and the reverse primer was taken from 3388 to 3410 as 5′ TTGCGTAGTTGTATATAGCCTAG 3′. The specificity of the primers was confirmed by primer BLAST using the NCBI database. The amplified PCR products were digested by Hae III restriction endonuclease (Promega: R6041). After electrophoresis, the presence of the mutation generates a single band (261 bp), while the absence of the mutation generates two bands (167 and 94 bp).

2.7. PCR Reaction Conditions and the Detection of Amplified DNA Bands

Primers were synthesized by integrated DNA technologies. A 12 µl PCR reaction cocktail consisted of 1 µl DNA as a template (100 ng), 2 µl of forward primer (100 ng), 2 µl of reverse primer (100 ng), and 7µl of the Promega master mix. PCR condition consisted: incubation for 3 minutes at 94ºC, forwarded by 30 seconds at 94ºC, 30 seconds at 55ºC, 45 seconds for 72ºC, and a final incubation for 5 minutes at 72ºC, in Bio Rad thermal cyclar. The resultant amplicons of both mutations were identified using 2% agarose gel containing ethidium bromide, in the Gel Doc system (Bio Rad) and compared with 100 to 1,000 bp DNA ladder. The identified mutation was further confirmed by DNA sequencing.

2.8. Statistical Analysis

All statistical analyses were done using Med Calc statistical software (version 10.2.1.0). The Chi-Square test and Fisher's exact test were used to show an association between type 2 diabetes patients and healthy control individuals. The prevalence of the mutation was observed by the number of mutated samples over the total number of samples analyzed. ODDS ratio was used to define exposure of mutation and its probable outcome. $p \leq 0.05$ was considered as a level of significance.

3. RESULTS

Authors screened 142 patients of type 2 diabetes mellitus and 142 healthy control individuals to detect 3243 A/G and 3316 G/A mtDNA mutations. The restriction fragment length polymorphism analysis showed that seven patients of type 2 diabetes mellitus found positive for 3316 G/A mutation with 4.92 % of prevalence, while this mutation found absent in the healthy control individuals (Fig. 1). Furthermore, the presence of this mutation was confirmed by DNA sequencing (Fig. 2).

For the prevalence of 3316 G/A mutation, Chi-Square and Fisher's exact test showed a significant association between type 2 diabetic patients and healthy control individuals ($p \leq 0.01$). Moreover, for this mutation ODDS ratio demonstrated a significant association

Figure 1: Identification of 3316 G/A mitochondrial DNA mutation. Well 1 shows DNA ladder. Well 2 to 8 represents mutation (diabetic patients); however well 9 and 10 show the absence of the mutation (healthy individuals).
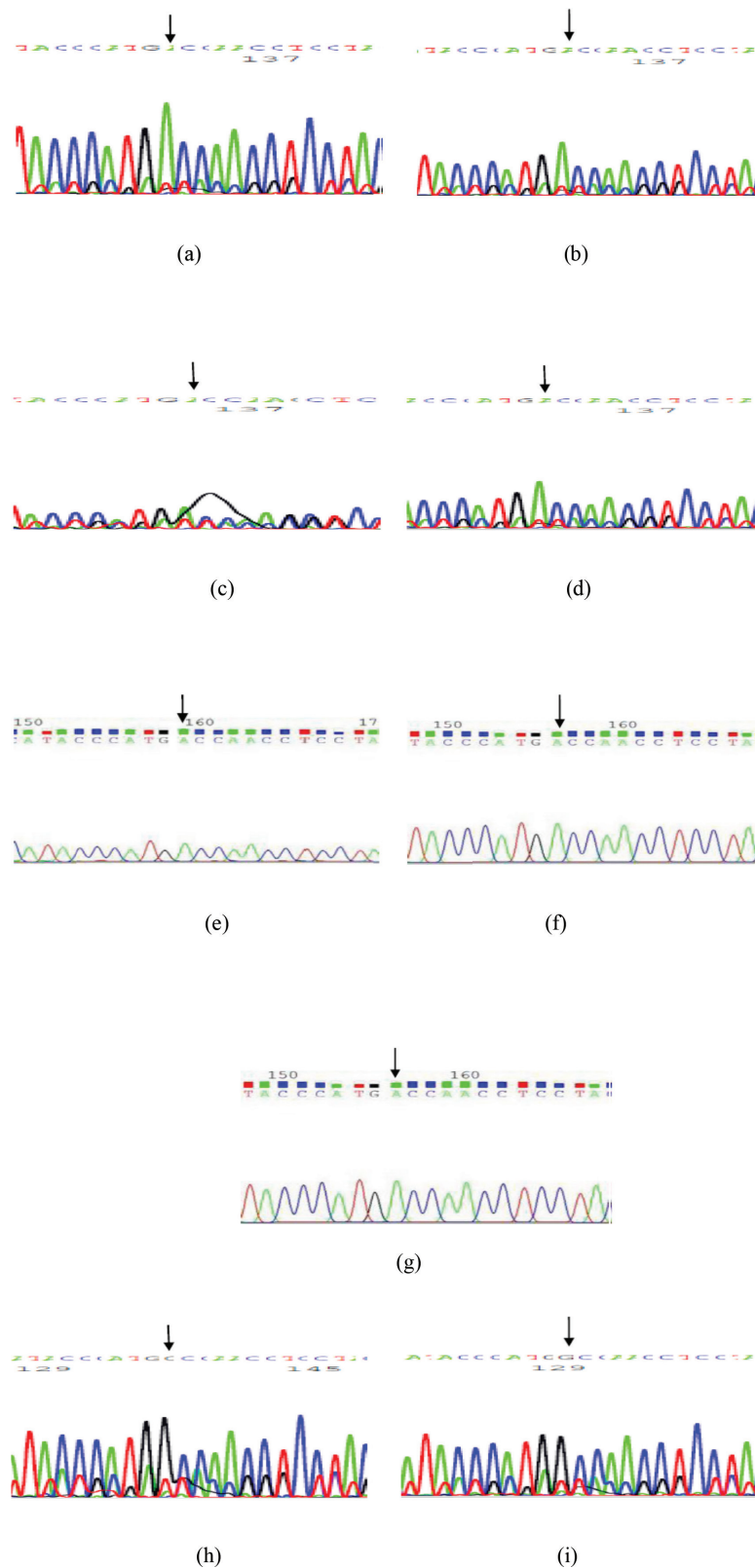
Figure 2: DNA sequencing result shows the presence of 3316 G/A mutation in seven patients of type 2 diabetes mellitus (a–g) and absence of the mutation in healthy control individuals (h and i).

between diabetic patients and healthy control individuals (p = 0.05). 3243 A/G mutation found absent in type 2 diabetes patients and healthy control individuals.

Table 1 represents the clinical characterization of seven patients of type 2 diabetes mellitus found positive for 3316 G/A mutation. The ages of the patients were ranged from 36 to 64 years, however,

the ages of the onset for type 2 diabetes mellitus varied from 34 to 52 years. The levels of fasting and postprandial glucose indicate severe hyperglycemia, resulted in high glycosylation of hemoglobin (HbA1C). Patient 2 (represented by I3 in the pedigree; Figure 3) was mentally retarded and the level of urea (48 mg/dl) and creatinine (1.9 mg/dl) were found elevated, which showed kidney dysfunction, however, rest of the family members exhibited these values in the normal range.

The mtDNA sequences were deposited in the NCBI gene bank with following accession numbers: LC064880, LC064881, LC064882, LC064883, LC064884, LC064300, LC064711, LC064712, and LC064713.

4. DISCUSSION

Due to its non Mendelian inheritance, mtDNA polymorphism can be used as a marker for the genetic elucidation of the world population; hence the detection of single nucleotide polymorphism in mtDNA has attended much prominence. In the last few decades, the extensive body of literature has reported the role of mtDNA

Table 1. Clinical Characterization of patients identified with 3316 G/A mutation.

Parameters	P1	P2	P3	P4	P5	P6	P7	Normal range
Age (years)	64	62	59	57	41	36	37	–
M/F	F	M	F	F	F	M	M	–
Onset (years)	48	52	45	39	38	34	34	–
Fasting Glucose	180	213	185	199	156	162	192	60–105 mg/dl
PP Glucose	298	345	276	299	289	302	280	<126 mg/dl
HbA1C	10.3	11.4	10.2	9.4	8.8	9.8	10.1	4–6 %
Urea	21	48	20	19	18	19	21	7–22 mg/dl
Creatinine	0.8	1.9	1.2	1.4	0.9	1.1	1.1	0.8–1.5 mg/dl

P = Patient; PP = Post Prandial; HbA1C = Glycosylated Hemoglobin. M/F = Male/ Female Position of patients in the pedigree analysis was represented as: P1 (I2), P2 (I3), P3 (I6), P4 (I7), P5 (II2), P6 (II9), P7 (II12).
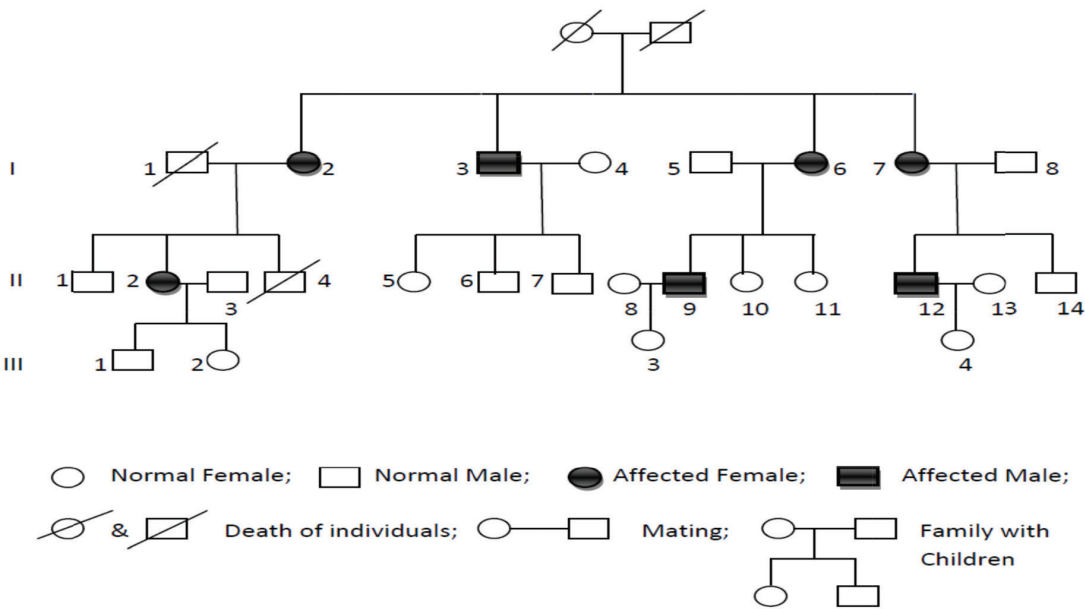
in type 2 diabetes mellitus [13–15]. Approximately, 40 different mtDNA mutations have been reported yet for the pathogenesis of type 2 diabetes mellitus [7].

Moreover, in the year 1992, Van Den Ouweland [12] had described the role of mtDNA mutation A/G at position 3243 as a causative factor for type 2 diabetes mellitus in a large pedigree. Since then, huge work has been done globally to know about the frequency of mtDNA mutations in different populations. Among these, 3243 A/G and 3316 G/A detection were more common.

This study did not report 3243 A/G mtDNA mutation in type 2 diabetes mellitus patients and healthy control individuals, indicating this mutation is not a major cause of maternally inherited type 2 diabetes mellitus in Nagpur population. The absence of the mutation is because of the higher level of 3243 A/G mutation is detected in muscles rather than the rapidly dividing tissue like blood [16]. However, for heteroplasmic mtDNA mutation, the development of physiological and clinical consequences depends on the threshold of the mutation. Furthermore, thousands of copies of mtDNA are present in each cell; hence the level of heteroplasmy may vary from 1% to 99% among different cells and tissues [17,18]. Therefore a female carrying mtDNA mutation may transmit the different amounts of the mutated mitochondria to her offsprings, within the same family; makes prenatal and postnatal genetic testing in maternally inherited mitochondrial abnormalities more complicated and problematic [19,20].

In this study, seven patients of type 2 diabetes mellitus showed 3316 G/A mutation in the mitochondrial genome and Chi-Square and Fisher's exact test also showed a significant association between type 2 diabetes patients and healthy control individuals ($p \le 0.01$). These results represent that, mtDNA polymorphism due to 3316 G/A mutation is related to type 2 diabetes mellitus in the Nagpur population, instead of a neutral polymorphism. Furthermore, we found a significant ODDS ratio for 3316 G/A mutation and type 2 diabetes mellitus (for 95% CI; $p = 0.05$). Also, 4.6% of prevalence

Figure 3: Pedigree analysis of the individuals affected by 3316 G/A mutation. I, II, and III represent generations.

has been reported for this mutation, which is similar to the different prevalence rates observed in other populations worldwide [21].

3243 A/G mutation has been reported for muscle stiffness and mental retards [11], however, in the studied population mental retard was found associated with 3316 G/A mutation (patient 2). This is possible since the position of 3316 G/A mutation exists nearer to the 3243 A/G mutation. Hence, we could predict that 3316 G/A mutation may also be involved in the etiology of mental retardation as similar to 3243 A/G mutation.

Clinical characterization of patients detected with 3316 G/A mtDNA mutation has been recorded in Table 1. Increased level of urea and creatinine in patient 2 (Fig. 3: I3) clearly shows the diabetic nephropathy, while it is not noted in the other family members. The higher range of fasting, postprandial glucose, and HbA1C values was reported in all patients, indicating that these patients are more prone to micro and macro-vascular complications. Kidney dysfunction was recorded for a single male candidate, which is possibly due to prolonged increased exposure of hyperglycemia.

5. CONCLUSION

The 4.92% prevalence of 3316 G/A mutation and its significant association between type 2 diabetic patients and healthy individuals concluded that this mutation may be associated with maternally inherited type 2 diabetes mellitus as a pathogenic mutation in Nagpur population. However, further studies are required to know about the possible role of 3316 G/A mutation as a causative agent for mental retardation. The absence of 3243 A/G mutation concludes that this mutation is not responsible for the type 2 diabetes mellitus in the studied population. This study provides a new genetic predisposition for maternal inheritance of type 2 diabetes mellitus in the studied population. This study will be useful for a large cohort and meta-analysis studies.

CONFLICT OF INTEREST

Authors declare that they do not have any conflicts of interest.

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