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RESEARCH

Human Mitochondrial DNA Haplogroup M5 is Associated with Type 2 Diabetes Mellitus in South Indian Population: A High-Resolution Case Control Study using Next Generation Sequencing

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ABSTRACT

Energy is required for life on Earth, and it is provided by the small organelles of cells called mitochondria, also referred to as the cell's powerhouses. Mitochondrial DNA (mtDNA), which is grouped into several human mtDNA haplogroups, is frequently employed in population genetics to identify individuals or communities based on mutation sites found by comparison with the reference sequence (rCRS). Previous studies in various populations have connected particular mtDNA haplogroups and polymorphisms to a range of human disorders, including Type 2 Diabetes Mellitus (T2DM). In addition, a number of mitochondrial DNA polymorphisms have been connected to elevated reactive oxygen species (ROS) generation and an elevated risk of a number of malignancies, including type 2 diabetes mellitus (T2DM), in the Indian patients.

As a result, we conducted a high-resolution assessment of the mtDNA hypervariable area in our study to trace distinct mtDNA haplogroup connections with type 2 Diabetes Mellitus (T2DM) in south Indian communities. We discovered that mtDNA Haplogroup M was present in 60% of type 2 Diabetes Mellitus (T2DM) patients and about 55% of the control samples examined. Haplogroup M is the most frequent mtDNA cluster observed in south Indian people. We further segmented macro haplogroup M and revealed sub haplogroups (M8, M7, M6, M5, M3, and M2) with variable frequencies. Patients with Type 2 Diabetes Mellitus (T2DM) and haplogroup M5 were significantly associated, according to our research ($p = 0.026$). Haplogroup M5 was discovered in our study in 3.3 percent of control populations and 13% of south Indian T2DM patients. These results imply that Type 2 Diabetes Mellitus is more likely to occur in haplogroup M5 individuals.

KEYWORDS

Human mitochondrial DNA (mtDNA), Hypervariable region, Haplogroup M5, Type 2 Diabetes Mellitus, D-Loop region, Ion Torrent Sequencing platform.

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a very common household disease name in India. Due to its increasing spread on radical basis, India is considered as the Global capital of Diabetes. The people affected with T2DM in India are reaching disturbing number of 69.9 Million by 2025 and almost near to 80 Million by the year 2030. According to this, the emerging country is predicted to witness a massive 266 percent boost in growth of T2DM. 28 percent of people in cities have diabetes, compared to just 5 percent of people in rural areas, according to current data.

Diabetes mellitus (T2DM) has become one of the largest public health issues of our time [Zimmet, P *et al.*]. More than a million people die each year from diabetes-related problems such as cardiovascular disease and stroke, kidney illness, and even cancer [Roglic, G. *et al.*]. Diabetic patients could rise from 366 million in 2011 to 552 million in 2030, according to the International Diabetes Federation. As the condition progresses, insulin resistance and

decreased insulin production are hallmarks. Before the onset of type 2 diabetes (T2DM), insulin resistance has already begun to develop. The mitochondrial genome has a critical role in insulin resistance and type 2 diabetes (T2DM). Reduction in mitochondrial oxidative phosphorylation capability and sub maximal ADP-stimulated oxidative phosphorylation are examples of mitochondrial dysfunctions. The insulin resistance and diabetes have been linked to mitochondrial dysfunction and mitochondrial plasticity [Szendroedi J *et al.*, Petersen KF *et al.*, Bonnard C, Gordon. J *et al.*]

There have been numerous studies looking into the link between type 2 Diabetes and the mitochondrial DNA. Several disorders, including type 2 Diabetes Mellitus, have been linked to mutations in mitochondrial DNA's reactive oxygen species (ROS) (T2DM). Numerous researches have examined the relationship between human nuclear DNA variations and type 2 Diabetes Mellitus (T2DM), but only a few have examined the relationship between human mitochondrial DNA variations and T2DM in the south India. A haplogroup is a group of individuals having a unique set of mutations and polymorphisms in the mitochondrial DNA region according to the Cambridge revised reference sequence (rCRS). It's impossible to recombinate mitochondrial DNA, which explains why it's so unique and follows maternal inheritance. As a result, it has a high mutation rate and a high copy number. The mtDNA has no introns or spacers, and there is no repetitive DNA in the mtDNA. All 37 coding genes in the human mtDNA are involved in the synthesis and storage of ATP, which is a form of energy. A 16,536bp in length, the human mitochondrial genome is composed of two rRNAs, thirteen mRNAs, and twenty-two tRNAs. Human mtDNA has been shown to have a higher rate of substitution than the nuclear genome (Brown *et al*. 1979).

However, there is a lack of evidence for the link between human mtDNA haplogroups and type 2 Diabetes Mellitus in the south Indian population [Tipirisetti NR and others, Govatati S and Wallace DC *et al.*, Wallace DC *et al.*]. The molecular clock is made up of mitochondrial DNA. The regulatory region of the mtDNA that contains 16536bp is known to carry the genetic signals required for transcription and replication. The base alterations and DNA substitutions detected in the control region of human mitochondrial DNA can be used to evaluate the relatedness between individuals. There are approximately 10 times more point mutations per nucleotide in the regulatory region of DNA than there are in nuclear DNA.

On average, the replacement rates in the human mtDNA regulatory region were found to be as high as 5 times (Aquadro and Greenberg, 1983) the replacement rates in the rest of the genome (Cann *et al*., 1984). Studies using mtDNA control region sequences tend to focus on intraspecific variation and phylogenetic links between closely related species, such as the study of human population evolution (see Cavalli-Sforza *et al.* 1994). There are two "Hypervariable segments" HVRII position and HVRI position within this loop that contain polymorphic nucleotide sites (Wilkinson-Herbots *et al*. 1996). As a result, HVSI and HVSII data can be used to provide insight into population variance between and within species. This study aims to identify haplogroups associated with type 2 Diabetes Mellitus (T2DM) in the south Indian population by sequencing the two hypervariable regions (HVR1: np 16024–16383 and HVR2: np 57–333) of mitochondrial DNA in 100 case and 90 control samples from the south Indian population, respectively.

MATERIALS AND METHODS

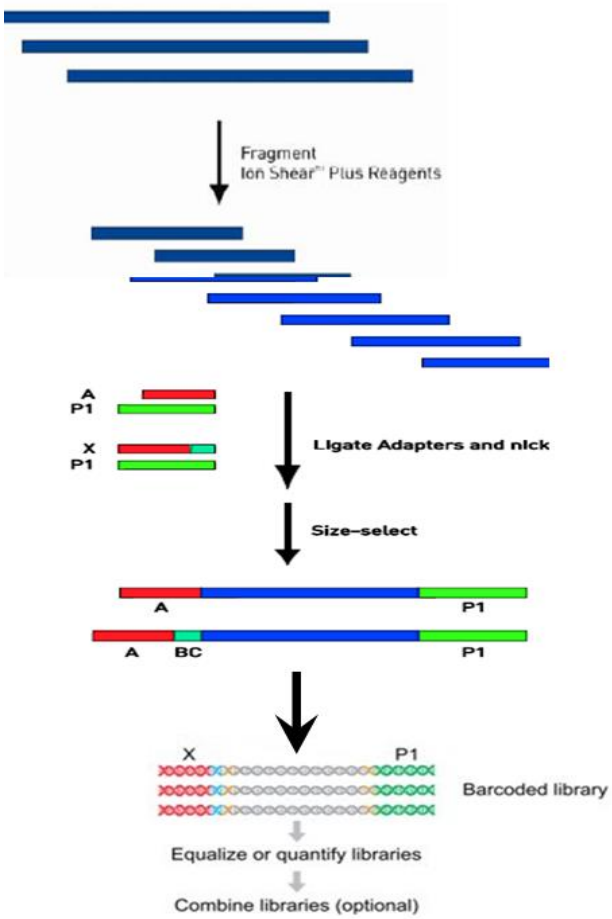
The samples for this study were collected from the medical department of the Nizam's Institute of Medical Sciences (NIMS), Hyderabad, India, for the purpose of assessing the mtDNA haplogroup connection with Type 2 Diabetes Mellitus (T2DM). All verified cases of Type 2 Diabetes Mellitus (T2DM) were collected in EDTA vacutainers by providing thorough informed consent to the patients. The phenol chloroform technique mentioned before was used to isolate genomic DNA (Gill *et al*., 1987). The purity and integrity of the genomic DNA was evaluated using agarose gel electrophoresis after the genomic DNA isolation from all blood samples. The UV Vis nanodrop spectrophotometer was used to quantify the concentration of genomic DNA in all of the case and control samples. Each sample has a working supply of 10ng/ul of genomic DNA kept at -20 degrees Celsius.

Human mtDNA hypervariable region I and II primers were used in the polymerase chain reaction. It was determined that PCR products had been amplified by electrophoresis of the gels with specified 1kb ladders. Following Exo-SAP treatment, the Big Dye cycle was performed on the PCR products following the manufacturer's instructions for the PCR technique sequencing. As a result, both the forward and reverse sequencing reactions were carried out separately. The products were denatured with Formamide after post-sequencing cleaning using Ethanol Precipitation. Applied Biosystems 3730 DNA analyzer was used to sequence the sequencing products, and bidirectional sequencing was performed for both HVR I and II to provide the wholesome amplicon required read length. Following the base calling, mutations and polymorphism were examined using Auto Assembler software by comparing the sequences to the revised Cambridge reference sequence (rCRS).

Selected samples of case samples are sequenced using next generation sequencing (NGS) using Ion torrent platform. Mitochondrial DNA is amplified using long range PCR method. One PCR amplifies product of around 8.5 kilo bases (KB) and another PCR amplified product of 8 kilo bases. Primers for long range PCR are selected basing on the previously published literature [Mark Stoneking *et al.*]. Amplified long range PCR products (8KB and 8.5 KB) are purified using Ampure XP beads protocol as per the recommendation from the Agencourt Beckman Coulter manufacture. After purification both the long-range PCR products (8 and 8.5 KB) are pooled in equimolar ratio and

followed the Ion fragment library kit protocol as recommended by the Manufacturer (Ion torrent, Thermo Fisher scientific, USA) to shear the amplicon to a region of 200-250 base pairs. Amplicon library preparation involves fragmentation of the pooled PCR products, followed by purification using Ampure XP beads. After purification of the fragmented products, it is further carried out for adapter ligation, size selection using method and finally for the library amplification. Amplified libraries are purified and checked the size of library using Agilent Bioanalyzer DNA High sensitivity chip kit as an indicator of the libraries range from 180-230 basepairs. Equimolar concentration of libraries are prepared and pooled together in one reaction and carried out emulsion PCR using Ion ONE Touch 2 system. The enriched products are then sequenced using Ion torrent systems for a 200 base pair chemistry sequence. (**Fig. (a)**).

Fig. (a). Amplicon sequencing for sequencing whole mitochondria DNA using Fragment library approach is depicted below.



Statistical analysis

SPSS package (V11.0) is used to perform the required analysis. Fisher's exact test is employed to test the Hardy–Weinberg equilibrium genotype distributions among the studied case and control samples. The P Value of 0.05 is determined as statistically significant. The 95% confidence interval is used for calculation of ODDs ratio. For the NGS data analysis, we used Ion Reporter software and Torrent suite software server for the variant calling.

Phylogenetic analysis

Human mtDNA sequence of HVR I and II is used to build maximum parsimonious phylogenetic tree. Human mtDNA haplogroup was denoted to all the case and control samples in the present study based on the available literature (Palanichamy *et al*. 2004; Thangaraj *et al*. 2005).

RESULTS AND DISCUSSION

Type 2 Diabetes Mellitus (T2DM) and mtDNA Control (D-loop) Region

The control area, or D-Loop region, is the only non-coding section of the human mitochondrial DNA. It is located between base pairs 16024 and 16576. Since point mutations accumulate 10 times faster than in nuclear DNA, the non-coding regions of human mtDNA are referred to as hypervariable. Since DNA and RNA polymerase binding sites are distinct only by short nucleotide sequences, a high mutation rate can be tolerated in the hypervariable region of mtDNA. As a possible explanation for why mtDNA has a high mutation rate in the hypervariable area, oxygen free radicals, which are part of the cell's respiratory apparatus, may be to blame. This genetic variant found in Hypervariable region, which is responsible for the respiratory chain's increased amounts of reactive oxygen species, could have a significant impact on the respiratory chain's role in nuclear genome damage and cancer instigation and progression (Lievreand Laurent-Puig, 2005). It is also clear that mitochondrial apoptosis can lead to mtDNA damage if respiratory chain alterations are made [Zamzami and Kroemer, 2001]. Somatic D-Loop mutations have been linked to a variety of malignancies, including type 2 Diabetes Mellitus (T2DM) (Richard *et al.*, 2000; Lievre and Laurent-puig, 2005; Gille and Joenje, 1992). Carcinogenesis may be linked to the non-coding part of the human mtDNA D-loop, according to these studies.

There were 179 differences in the D-loop region compared to the reference sequence from Cambridge in this investigation. **Table 1** lists the unique mutations, of which there were nine (**Table 1**), as well as 170 previously described mutations. A total of five of the identified mutations have been linked to mitochondrial disorders. Mitochondrial DNA translation and transcription are controlled by the D-loop which includes promoters, transcription factor binding sites, and other critical regulatory regions Variations in this region have been linked to a variety of ailments. In this region, nine new mutations were discovered & on the whole, D-loop has a 5.33-fold mutation rate (179 mutations/1121 NP = 0.16) than the rest of the mitochondrial genome. According to the majority of prior studies, the D-loop area is a hot site for mutations.

Table 1

HAPLOGROUP	CASE	CONTROL
H2a2a	3	2
J1	1	4
M	1	3
M18	1	5
M2	7	4
M3	6	2
M30	8	9
M31	0	1
M33	1	0
M34	0	2
M35	4	2
M36	1	0
M37	0	2
M39	4	1
M4	6	5
M40	0	1
M41	0	1
M42	1	0
M49	2	0
M5	12	4
M52	1	0
M58	0	1
M6	5	7
M64	0	1
R	7	2
R2	0	1
R7	0	3
R30	2	0
R31	1	0
R5	1	4
R6	3	6
R8	1	1
T1	1	0
T2	1	0
U1	0	1
U2	10	12
U5	1	0
U7	7	3
W	1	0
TOTAL	100	90

Mutations linked to different disorders have been reported

Human mitochondrial DNA from type 2 Diabetes Mellitus patients was also examined to see if the mutations detected in the HVRI and HVRII of mitochondrial DNA were associated with other human diseases. A total of five of the identified mutations have been linked to mitochondrial disorders. One patient had a C insertion at the 12th position, and another patient had a G insertion at the 16520th position in 9 unique variations. Eight patients had mutations in the transition/transversion gene. 5 patients and 2 controls were found to have the C150T variant, which has been linked to longevity disease, in this investigation. In total, 14 patients and 13 controls were found to have the T16189C variation, which has been linked to dilated cardiomyopathy. A189G, a mutation previously linked to leukemia, was found in three patients and three healthy controls. Patients with "cyclic vomiting syndrome with migraine" were found to have the T16519C mutation in 76 cases and 65 controls.

For both patients and controls, the D-loop area was determined to have a model class of 10-15 with comparable numbers of mutations (**Fig. 1**). For the patients and the control group, the area under the curves did not change considerably. Furthermore, there was no correlation between the clinical factors and the results. The type 2 Diabetes Mellitus (T2DM) risk is not increased by mutations in the D-loop region alone, since the ratio of mutation frequency in patients and controls was determined to be 1 (**Fig. 1**).

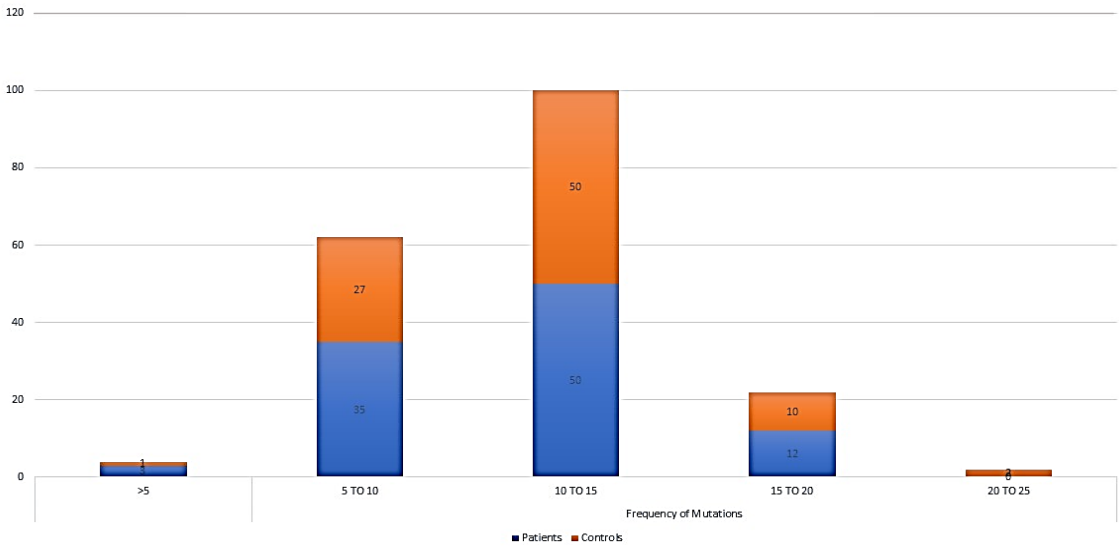


Fig. 1. Distribution of mutations per individual in disease and control group of D-loop region.

We aim to understand the association between the excess weight and the time to first diagnosis of Type 2 DM and its complications among elderly individuals, Distribution of mutations in HVRI and HVR II of mtDNA in different stages of body mass index (25 to 27.49, 27.50-29.99, 30 to 39.99 and greater than or equal to 40). Out of the 100 samples in the patient group (33 females and 67 males with a mean age of 54 ± 7 years) were categorized into different stages as per BMI.

Distribution of mutations in HVRI and HVR II of mtDNA in different stages of body mass index 25 to 27.49, 27.50-29.99, 30 to 39.99 and greater than or equal to 40) are represented in (**Fig. 2**).

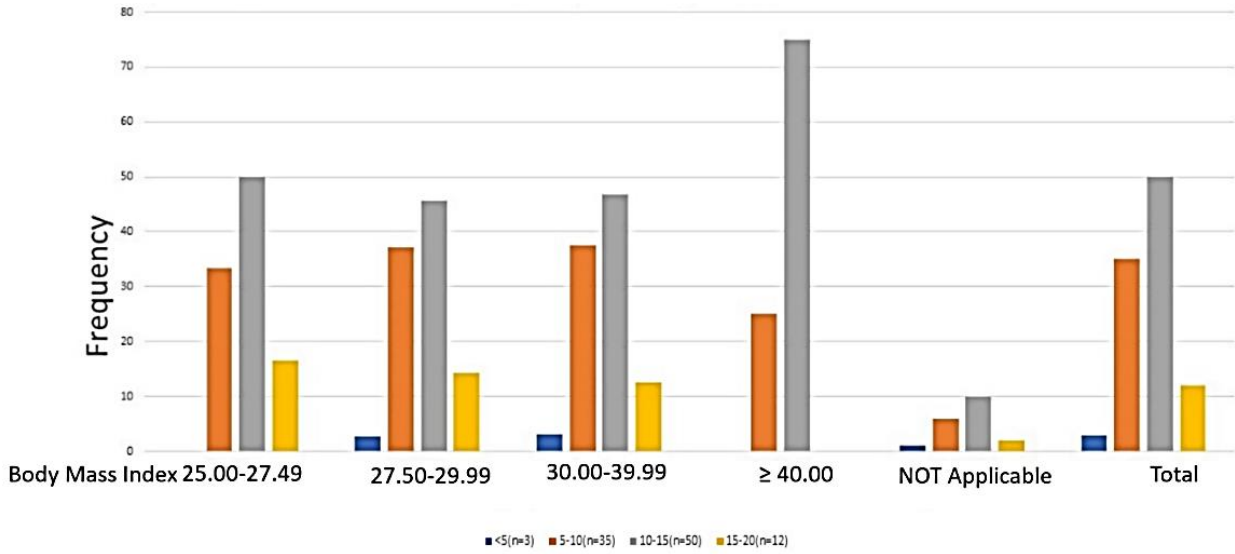


Fig. 2. Distribution of mutations with respect to Body mass index association and Type 2 Diabetes.

Type 2 Diabetes Mellitus (T2DM) Status in relation to Macro Haplogroup M and N

The major human mtDNA macro haplogroups are L, M, and N, and their distributions vary geographically. L is the oldest of the three major human mtDNA macro haplogroups, and it is primarily restricted to the African continent, with a focus on African Sub-Saharan populations. L7, L6, L5, L4, L3, L2, L1, L0 are sub haplogroups of macro haplogroup L. Haplogroups M and N are the most common mtDNA haplogroups in south Asian populations. It is widely accepted that around 60,000 years ago [Quintana-Murci *et al.*, 1999; Mishmar *et al.*, 2003], the L3 haplogroup radiated out of Africa in the form of M and N Macro haplogroups, paving the way for modern-day south Asia. Numerous population genetics studies on Indian populations using uniparentally transmitted genetic tools like the Y chromosome and mtDNA clearly demonstrated that India played a pivotal role in the early dispersal of humankind. This is demonstrated by the widespread distribution of M and N haplogroups among many Indian populations (Kivisild *et al.*, 1999; Metspalu *et al.*, 2004; Palanichamy *et al.*, 2004; Thangaraj *et al.*, 2006). M50, M49, M48, M41, M40, M39, M38, M37, M36, M35, M34, M33, M32, M31, M30, M25, M18, M6, M5, M4, M3 and M2 are distinct haplogroups of Indian origin. Both eastern and western Eurasian mtDNA haplogroups are represented in Indian populations (Bamshad *et al.*, 1997; Passarino *et al.*, 1996). M and U make up the vast majority (75 percent) of the Indian maternal pool, which is distributed evenly across the country (Kivisild *et al.*, 1999).

The absence of most Indian maternal mtDNA clades in adjacent European and East Asian populations, as well as elsewhere in the world, demonstrates the autochthonous development of these haplogroups and further indicates a limited gene flow out from the Indian subcontinent over time.

We investigated if there is any significant association between major macro-haplogroups M and incidence of type 2 Diabetes Mellitus (T2DM) in south Indian population by sequencing D -Loop region of mtDNA in our current case control study on type 2 Diabetes Mellitus (T2DM) patients in south Indian population. According to our findings, mtDNA Haplogroup M is found in 60% of Type 2 Diabetes Mellitus (T2DM) patients and around 55% of control samples. The most common mtDNA cluster found in Indian populations is Haplogroup M. [**23, 24**]. We further subdivided macro haplogroup M and discovered sub haplogroups – M8, M7, M6, M5, M4, M3, M2 (**Fig. 4, Table 3**). In our current study, we found a significant association of haplogroup M5 in Type 2 Diabetes Mellitus (T2DM) patients compared to control samples ($p = 0.026$). Haplogroup M5 is found in 12% of studied Indian type 2 Diabetes Mellitus (T2DM) patients and 5% of studied control populations (**Table 3**).

Table

Haplogroup	Number of patients	number of controls	%	χ^2	P-value
H2a	4	3	2.2	0.123	0.7258
J1	1	4	4.4	2.141	0.14341
M	1	3	3.3	1.23	0.26741
M18	1	5	5.5	3.115	0.07757
M2	7	4	4.4	0.593	0.44126
M3	5	2	2.2	1.761	0.1845
M30	8	9	10	0.222	0.63752
M31	0	1	1.1	1.1	0.29427
M33	1	0	0	1	0.31731
M34	0	2	2.2	2.2	0.13801
M35	4	2	2.2	0.523	0.46956
M36	1	0	0	1	0.31731
M37	0	2	2.2	2.2	0.13801
M39	4	1	1.1	1.649	0.1991
M4	6	5	5.5	0.022	0.88209
M40	0	1	1.1	1.1	0.29427
M41	0	1	1.1	1.1	0.29427
M42	1	0	0	1	0.31731
M49	2	0	0	2	0.1573
M5	12	3	3.3	4.947	0.02614*
M52	1	0	0	1	0.31731
M58	0	1	1	1	0.29427
M6	5	7	7.8	0	0.43404
M64	0	1	1	1	0.29427
R 7	2	2	0.2	2	0.11356
R2	0	1	1.1	1	0.29427
R7	0	3	3.3	3	0.06928
R30	2	0	0	2	0.1573
R31	1	0	0	1	0.31731
R5	1	4	4.4	2	0.14341
R6	3	6	6.7	1	0.23489
R8	1	1	1.1	0	0.94363
T1	1	0	0 1	0	0.31731
T2	1	0	0 1	0	0.31731
U1	0	1	1.1	1	0.29427
U2	10	1	2 1	3	0.49437
U5	1	0	0 1	0	0.31731
U7	7	3	3.3	1	0.24898
W 1	0	0	1	0	0.31731

Because India served as a conduit for early human dispersal throughout the world, its population is expected to have a high level of diversity in mitochondrial sequences. It is estimated that approximately 50 million people are affected by genetic diseases. However, only genetic diseases of nuclear origin have been studied so far, and there is no comprehensive study on mitochondrial diseases in India.

Despite technological advancements and a massive focus on oncology research, there is still a scarcity of studies on the molecular mechanisms underlying the genetic causes of cancer and mtDNA polymorphisms. It is

widely accepted that mitochondria are the primary source of cellular energy synthesis and production, i.e., ATP, and that any genetic changes in mitochondrial DNA may have a significant impact on mitochondrial dysfunction.

As a result, mitochondrial deficiency is likely to result in nuclear genome instability as well. Furthermore, the significance of specific mtDNA polymorphisms should be investigated in conjunction with other nuclear polymorphisms in Type 2 Diabetes mellitus epidemiology and genetic basis of complex disease like Type 2 Diabetes Mellitus.

CONCLUSIONS

Type 2 Diabetes Mellitus (T2DM) patients in the south Indian population were shown to have mtDNA haplogroup M5 as a risk factor for developing the disease. Understanding mtDNA mutations offers us with several advantages over nuclear DNA changes in the identification of mtDNA Genetic markers involved in the T2DM. Diseased cells have a larger mitochondrial DNA concentration than normal cells, and homoplasmic mutations in human mtDNA are more common. Therefore, homoplasmic mtDNA mutations are the most common sign of clonality. Developing new indicators that provide useful evidence for disease prevention would be made possible by efforts made to better understand the role of mtDNA polymorphisms in "mitochondrial genetic studies." As a result, it is necessary to conduct high-resolution research. To have a better understanding of mitochondrial mutations' significance in type 2 Diabetes Mellitus (T2DM), the full human mitochondrial DNA should be sequenced.

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AUTHOR CONTRIBUTIONS

RD and KM conceived, designed and wrote the manuscript. KM analyzed the data and wrote the manuscript with inputs from RD.

CONFLICT OF INTEREST

All authors read, approve the manuscript and there is no conflict of interest.

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