Abstract

Introduction: In India, 90% kidneys for transplantation are obtained from living donor while only 10% come from deceased donors. Since the rate of living organ donors is high, it therefore leads to the problem of organ trafficking. To minimize the chances of organ trafficking, the Transplantation of Human Organ Act (THOA) 2014 was enacted in India that makes it mandatory to prove the relationship between patient and donor by DNA testing. The present study was undertaken to evaluate the degree of matching between maternally related patients and donors, performed using mitochondrial DNA (mtDNA).

Methods: After taking an informed consent, a total of 84 subjects were recruited in the study, 42 kidney transplant recipients and 42 their corresponding donors. An attempt was made to establish and confirm the claimed relationship between recipient and donor using mtDNA analysis.

Results: Out of the total 42 cases, mtDNA analysis supported the claimed relationship in 33 (78.57%) cases, whereas in 9 (21.42%) cases, claimed relationship could not be supported.

Conclusion: mtDNA can be used as a valuable tool to support the claimed relationship of maternal lineage. It is important that more and more organ transplant physicians, surgeons, and committees are made aware of this diagnostic modality.

Keywords: Distant relationship, mtDNA analysis, relationship test, solid organ transplantation

Introduction

In India, as per the Transplantation of Human Organ and Tissue Act (THOTA) 2014, it is mandatory to establish the biological relationship between a near living donor and a recipient. If the relationship between the prospective recipient and donor is not established,[1] DNA profiling should be considered to establish a biological relationship. Initially, human leukocyte antigen (HLA) typing was applied to establish direct relationship between donors and recipients; however, one of the limitation of HLA typing is that two unrelated individuals may also have matching in HLA genes, and thus making it difficult to use HLA typing as a sole means to prove a direct relationship. HLA is inherited as haplotype in a Mendelian style from each parent. Hence the chance of two siblings being genotypically HLA identical is 25%, haplo-identical is 50%, and a 25% chance that they do not share any HLA haplotypes. In addition, as HLA is most polymorphic gene identified in human genome with an extremely clustered and patchwork assembly of sequence motifs, sequencing ‘ambiguity’ remains an issue despite the advancement in sequencing technologies.[2] Hence, nowadays, DNA profiling in the form of autosomal short tandem repeats (STR), Y-STR, and mtDNA are being used to establish the claimed relationship between prospective recipient and donor. DNA profiling by autosomal STR analysis is considered as a gold standard DNA testing, which is used to analyze different repeated DNA sequence usually ranged from 2 to 7 base pairs (bp) long regions known as microsatellites, simple sequence repeats (SSR) or short tandem repeats (STR), to prove direct relationship as it is transferred from parents to their offsprings.[3] The limitation of autosomal DNA analysis is that it may only be used to prove direct relationship as autosomal DNA is only half matched between first degree relatives and complicated to evaluate and to prove a distant relationship. In the cases of distant relationship, Y-STR and mtDNA analysis are being employed for paternal

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and maternal lineage, respectively.\textsuperscript{[4]} Y-chromosome is inherited from the biological father at the time of conception and remains unchanged for the rest of the life. The Y-STR analysis is used when two or more male individuals are related through their paternal lineages. On the other hand, mtDNA analysis is usually used to prove maternal lineage relationship as the mtDNA is passed from the cytoplasm of the ovum; therefore, it is maternally inherited from mothers to all their offsprings\textsuperscript{[6]} Mode of inheritance and subsequent lack of hetero-logous recombination make the mtDNA analysis to retrace evolutionary relationships unambiguously down the maternal lineage and without the confounding effects of recombination, an issue identified in HLA inheritance.\textsuperscript{[9]} Mitochondrial DNA can be very useful in India, where about 90% of kidneys are obtained from living donors, whereas 10% are obtained from deceased donors.\textsuperscript{[7]}

The mitochondrial genome is 16,569 base pairs (bp)\textsuperscript{[8]} long and contains the most variable segments of the human genome, the hyper-variable (HV) region. The HV region is 1200 bp long. The variability in this region is used to distinguish the maternal relations.\textsuperscript{[9]} In the present study, to evaluate the degree of matching using mtDNA analysis between donor (mother or brother or sister or maternal relative) and the recipient of kidney for transplant has been undertaken.

**Methods**

In this study, we recruited a total number of 84 subjects, comprising of 42 kidney failure subjects and 42 their prospective donors, to analyze the relationship between them by analyzing their hyper variable region I (HVI) and hyper variable region II (HVII) of mtDNA sequences. After taking informed consent for DNA profiling from all the subjects, 5 ml venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA) coated vials from all the subjects and transferred to the laboratory to store at 4°C till they were processed for DNA isolation.

**DNA extraction and quantification**

Genomic DNA isolation from the peripheral blood leucocytes was performed by using phenol chloroform isoamyl alcohol (PCI) extraction method. The concentration of DNA samples were estimated by checking on 2% agarose gel prepared in 1X TAE buffer containing ethidium bromide (0.5 μg/ml) while purity and quality was measured by measuring its concentration using Qubit\textsuperscript{\textregistered} 2.0 Flurometer (Invitrogen, Life Technology) and the observed DNA concentration ranged between 25-50 ng/ml, which depicted that good quality of DNA has been extracted.

**mtDNA analysis**

The targeted regions of HV were amplified by polymerase chain reaction (PCR). Primers used for amplification of HVI were; forward: 5′-CACATTAGCACCCAAAGCT-3′ and reverse: 5′-GAGGATGGTGTAAGGAC -3′ with a product size of 446 bp. Amplification of HVII was performed using a set of primer forward: 5′-CTCACGGGAGCTCTCCATGC-3′ and reverse: 5′-CTGTTAAAAAGTGCACTACCACCCA-3′ with the product size of 407 bp. PCR cycling was carried out by using ABI Veriti Thermal Cycler (Applied Biosystems, USA). PCR amplification conditions consisted of denaturation at 95°C for 30 seconds; annealing at 58°C for 1 minute; and extension was at 72°C for 1 minute and a final stage of polymerization for 5 minutes. For PCR sequencing, the reaction was set up with 1 μl of amplified mtDNA, 1 μl of forward or reverse primer of HVI or HVII, 0.2 μl of Taq Polymerase, 2.5 μl PCR buffer 10X (500 mM KCl, 100 mMTris pH 8.4, 15 mM MgCl2, and 0.01% gelatin) and 2 mM of each dNTPs (8 mM total dNTP). The amplified products were subjected to electrophoresis in 2% agarose gel for assessment of PCR products. The PCR products were used for fluorescence-based mtDNA sequencing analysis by using Sanger sequencer. The observed mtDNA sequencing results were obtained in the form of chromatogram (ABI file format). This obtained chromatogram in ABI format was analyzed using Finch TV 1.4.0 [Figures 1-4]. The alignment of both the individual nucleotide sequence was done using online bio informatics BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). mtDNA sequences in text file (FASTA format) of two individuals (relatives) were uploaded and analyzed by using online BLAST tool to find the regions of similarities and differences between them.

**Results**

Out of total cases, 10 cases were of mother and her offspring and the mtDNA matching analysis between them showed 100% match [Table 1]. Seven cases were siblings, and among them, 5 were 100% matched, whereas 2 cases were mismatched [Table 2]. Table 3 representing the cases of 8 maternal cousins (brothers and sisters), and

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**Table 1: Analyses of mitochondrial DNA matching between mother and her offspring**

| Case no. | Recipient | Donor | Mito Results |
|---------|-----------|-------|--------------|
| 1       | Mother    | Son   | Match        |
| 2       | Mother    | Son   | Match        |
| 3       | Mother    | Son   | Match        |
| 4       | Mother    | Son   | Match        |
| 5       | Brother   | Sister| Match        |
| 6       | Mother    | Daughter| Match     |
| 7       | Son       | Mother| Match        |
| 8       | Son       | Mother| Match        |
| 9       | Mother    | Daughter| Match     |
| 10      | Son       | Mother| Match        |
among them, only 4 cases were matched [Table 3]. Among other cases, 17 cases among maternal aunt, uncle, nephew and niece (distant maternal relationship) showed 100% matching in 14 cases [Table 4]. Thus, of all the cases, mtDNA analysis supported the claimed relationship in 33 (78.57%) cases whereas in 9 (21.42%) cases claimed relationship could not support.

**Discussion**

mtDNA has been extensively used in the field of forensic investigations and population genetic study. Multiple copies of mtDNA genomes are present outside the cell nucleus, which contains only 2 copies of genomic DNA. Therefore, the hundred copies of mtDNA increase the probability of recovering DNA from the damaged and degraded as well as from the very small biological samples. The non-coding regions in the mtDNA have two hyper variable (HV) regions: hyper-variable regions I (HVI) and II (HVII), which are highly polymorphic and the polymorphisms in these regions exhibit a high degree of diversity\[10\]. Therefore, HV regions of mtDNA have also been used widely in population genetics study. Additionally mtDNA haplo-group is also being studied. It is unique set of markers that define those haplogroup. Each individual of a single haplogroup, bears a unique set of mtDNA markers that differentiate them from other haplogroup. At present, 26 mitochondrial DNA haplo-groups are known\[11\]. But in this paper, we only analysed sequencing data of HVI and HVII of all the tested participants.

After the implementation of Transplantation of Human Organ Act (THOA) in India in 1994 to streamline organ donation and transplantation, a potential living donor needs to be a biological relative of the recipient or has proven ties...
of love to the recipient. After the USA, the living kidney transplantation programs in India, the 2nd largest program, has evolved in the past 45 years.\textsuperscript{[7]} Instead of this major advancement, currently, approximately 5 lakh people die due to lack of organ availability. There are approximately 200,000 kidneys, 50,000 hearts and 50,000 livers that are required for transplantation each year.\textsuperscript{[12]} Living donors are source of 90% of kidney transplant at present in India, whereas, as mentioned earlier, only 10% depend on deceased donors. This clearly shows the importance of establishment of relationship between prospective recipient and donor as mentioned in THOTA 2014.

On the basis of the provided evidence, if the competent authority does not find the biological relationship between the living donors and recipient, then to prove biological relatedness, several DNA analyses methods are available (STR analysis based on autosomes and Y-chromosomes). However, in case of distant relationship, other DNA profiling tests like, mtDNA and Y-STR analysis may be used to establish the biological relationship between donor and recipient.

Previously, HLA typing analysis was extensively used to test biological relationship between parents and their offspring. According to the Mendelian law, HLA is a co-dominant allele and offspring inherits a set of HLA genes from both the parents which results in the parents

| Case no. | Recipient | Donor  | Mito Results |
|---------|-----------|--------|--------------|
| 1       | Uncle     | Nephew | Match        |
| 2       | Nephew    | Uncle  | Match        |
| 3       | Uncle     | Nephew | Match        |
| 4       | Uncle     | Nephew | Match        |
| 5       | Nephew    | Uncle  | Match        |
| 6       | Aunt      | Nephew | Match        |
| 7       | Aunt      | Niece   | Match        |
| 8       | Uncle     | Nephew | Match        |
| 9       | Uncle     | Nephew | Match        |
| 10      | Nephew    | Uncle  | Match        |
| 11      | Uncle     | Nephew | Match        |
| 12      | Aunt      | Niece   | Match        |
| 13      | Uncle     | Nephew | Match        |
| 14      | Uncle     | Nephew | Match        |
| 15      | Aunt      | Nephew | Mismatch     |
| 16      | Uncle     | Niece   | Mismatch     |
| 17      | Uncle     | Niece   | Mismatch     |
being a half match to their offspring, and therefore cannot be used to prove indirect relationships such as maternal or paternal cousin relationships. In addition, HLA typing can also be match between two unrelated individuals with the matching probability 1 in 10,000[13] resulting as inappropriate tool for proofing biological relationship. At present, to establish the biological relationship Autosomal STR, Y‑STR and mtDNA, analysis in the form DNA profiling test is being used. mtDNA changes very slowly over time and it makes it more powerful tool to establish relationship over paternity that are carried out by short tandem repeat DNA loci. These microsatellite sequences mutate at a higher rate than that of bulk DNA. The mutation rate was observed between 0 and $1.5 \times 10^{-2}$ per locus per gamete per generation in paternity STR, where as in mt DNA it was observed .0043 per generation[14] Y‑STR DNA is widely used particularly in cases where autosomal DNA analysis is not useful. Y‑STRs haplotypes are used to discriminate paternal lineages of unknown male, especially informative when males and females have contributed to the same trace. Due to limited information about the Y‑STR DNA analysis in this paper we are evaluating the degree of matching using only mtDNA analysis between donor and recipient.[15] The sequencing of mtDNA nucleotide was firstly reported by Anderson et al. in 1981 (Anderson et al., 1981). In the present study sequence analysis of the mtDNA control regions, HVI and HVII were performed by PCRMethod, followed by Sanger sequencing. We observed that mtDNA matching analysis between mother and her offspring showed 100% matched because mtDNA transfers from the mother to her offspring in the form of haplotypes and hence mtDNA matching profile analysis between brother and sister also showed 100% matching on mtDNA analysis. Therefore, to prove sibling relationship mtDNA analysis can be used. The probability of mitochondrial HVI and HVII DNA sequences matching for relationship proofing is high in the maternal lineage relations like grandmother, maternal aunt, maternal uncle, nephew, niece and cousins brother and sister from the maternal aunt because they share identical mtDNA sequence.[16] In the

**Figure 3: Mitochondrial Hyper Variable region II Forward Sequence**
present study to analyze distant relationship, a comparison between mother’s offspring and maternal cousins found a matched result in mtDNA analysis. The mtDNA analysis among maternal uncle and aunt, niece and nephew also showed a significant mtDNA match among them due to sharing of same mtDNA.

In our study, there were 9 cases between them, where the mtDNA analysis was mismatched and transplantation could not proceed. The reason for these mismatches could be that they did not share the same maternal lineage, as these may be cases of professional donors. We reported these cases, as not being matched on mtDNA analysis. No further family analysis could be performed as they were lost to follow-up. These results also showed that mtDNA analysis is a vital tool for establishing the claimed distant maternal relationship.

**Conclusions**

In the field of solid organ transplantation, mtDNA can be used as tool to prove distant relationship of maternal lineage. As a result of our study, we would strongly recommend the inclusion of mtDNA analysis for routine transplantation setting when the patient and donor are maternally related.

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**Conflicts of interest**

There are no conflicts of interest.

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