Mitochondrial DNA variations associated with recurrent pregnancy loss among Indian women

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Abstract

Several genetic factors have been found to be associated with recurrent pregnancy loss (RPL). However, not many attempts have been made to associate the mitochondrial DNA (mtDNA) variations with RPL. Therefore, we have analyzed the complete mtDNA of 100 women with RPL and 12 aborted fetal tissues. Our analysis revealed a total of 681 variations, most of which were in NADH Dehydrogenase (ND) genes that encode mitochondrial enzyme Complex I. Presence of T4216C variation (ND1 gene) in 9% of the RPL women and several pathogenic, and novel mutations suggest the role of mtDNA variations in RPL.

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1. Introduction

Recurrent pregnancy loss (RPL) is the spontaneous loss of 2 or more consecutive pregnancies before 20 weeks of gestation. RPL is one of the most common obstetrical complications that affect about 5% of the women trying to conceive (Sierra and Stephenson, 2006). Approximately 30% to 50% of all conceptions and 15% of clinically recognized pregnancies fail to result in a live birth (Wilcox et al., 1998). RPL is attributed to problems in the implantation of the fetus and any impediment in its growth in the uterus. Uterine anomalies, infections in the uterine lining, impaired folliculogenesis and immunological influence of the paternal antigens are some of the known factors involved in RPL (Rock and Murphy, 1986; Homer et al., 2000; DiZerega and Hodgen, 1981; Summers, 1994; Hill, 1992).

Chromosomal abnormalities account for more than half of the recurrent pregnancy loss (Simpson, 2000). Genetic variations in the regulatory enzymes of the crucial metabolic pathways, clotting factors, hormones and hormone receptors had been shown as possible causes of RPL (Sierra and Stephenson, 2006). Although various genetic, hormonal and anatomical factors were implicated in recurrent miscarriage, etiology of 50–60% of cases is idiopathic (Gupta et al., 2007).

The two important mitochondrial functions such as; oxidative phosphorylation and apoptosis were found to be involved in the RPL. Oxidative phosphorylation has been found to be essential for the early embryo, before its implantation (Jansen and deBoer, 1998) and defective oxidative phosphorylation might lead to the early loss of the pregnancy. For the normal post implantation development, a crucial threshold of cells in the inner cell mass is required (Brison and Schultz, 1996; Tam, 1998). Loss of fewer cells can result in fetal resorption or malformation and conversely if more cells undergo cell death, it would lead to RPL (Tam, 1998). Thus, the mitochondrial functions are very critical in the early pregnancy. The crucial subunits of the mitochondrial respiratory chain are encoded by the mitochondrial genes. Hence, it is very much appropriate to speculate the role of mitochondrial DNA (mtDNA) mutations as a possible cause of the RPL, which is known to cause multisystem disorders. We have made an attempt, for the first time in Indian RPL subjects, to look for mtDNA mutations.

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2. Materials and methods

2.1. Subjects

Women attending the obstetrics and gynecological department at the Institute of Reproductive Medicine, Kolkata; Infertility Institute and Research Centre, Hyderabad; and Fertility Research Centre, GG Hospital, Chennai were evaluated for the known causes of recurrent pregnancy loss such as uterine malformations, abnormal placental insertions, abnormal karyotypes and thrombophilic abnormalities. About 80% of the women had a natural menarche at the age of 12 to 16 years. The average age at the time of conception was 31 years, and 16 out of 100 women were above 35 years. After careful evaluation, 100 women with unexplained cause of recurrent pregnancy loss were selected for the complete mtDNA analysis. For further confirmation of mutations detected in the initial screening, we have selected additional 55 patients randomly from the Lakshmi Fertility Clinic and Research Centre, Nellore, Andhra Pradesh, using the similar selection criteria. About 10 ml of intravenous blood sample was collected from each woman.

In addition to the blood samples, tissue samples from 12 aborted fetuses belonging to independent RPL women were also included in this study. A total of 300 aged and ethnically matched women who never had any history of abortion and have children were selected as controls. An informed written consent was obtained from all the donors of the blood samples, and from mothers of the aborted fetuses.

DNA was isolated from the blood and the tissue samples by standard methods (Thangaraj et al., 2002). This study was approved by the institutional ethical committee of the respective institutes.

2.2. Analysis of complete mitochondrial DNA

Complete mtDNA of 112 samples (100 blood samples and 12 aborted tissues) was amplified using 24 sets of overlapping primers as described earlier (Rieder et al., 1998; Vanniarajan et al., 2006). Amplicons were electrophoresed using 2% agarose gel and the cycle sequencing reaction was carried out in a GeneAmp 9600 thermal cycler using BigDye Terminater ready reaction kit (Applied Biosystems, Foster City, USA) (Thangaraj et al., 2003a,b). Extended products were precipitated with ethanol and dissolved in Hi-Di formamide, followed by analysis in an ABI 3730 automated DNA analyzer (Applied Biosystems, Foster City, USA). The sequences were carefully edited and aligned with revised Cambridge reference sequence (rCRS) (Anderson et al., 1981; Andrews et al., 1999) using sequence analysis and AutoAssembler tools (Vanniarajan et al., 2006; Thangaraj et al., 2003a,b). The variations detected in the women with RPL were checked in the mitochondrial databases such as Mitomap (http://www.mitomap.org), and mtDB (http://www.genepat.uue.se/mtDB) for their significance. Mutations obtained in women with RPL were also compared with the mtDNA database of our centre (Centre for Cellular and Molecular Biology).

2.3. Quantification of heteroplasmy

The level of heteroplasmy at the nucleotide position 4216 was evaluated by PCR-RFLP, followed by densitometric analysis (Santos et al., 2004). The samples that were showing the heteroplasmy (electropherogram) were amplified with a pair primers (4216F: GATTCCGCTACGCCAATCT and 4216R: GGATCTCGAGGATATGTTCC). The amplicons were digested with NulII restriction enzyme and size fractionated using 2% agarose gel. The levels of the mutant and wildtype alleles were quantified by the densitometric analysis.

2.4. Phylogenetic analysis

The maximum parsimonious phylogenetic tree was constructed based on the mutations obtained from the complete mtDNA sequences of individuals with RPL. All the essential quality control measures and the haplogroup nomenclature were followed based on our published information (Thangaraj et al., 2005a).

3. Results

We have analyzed a total of 100 women with RPL, of which more than 70% of them had ≥3 losses. All the RPL women have been evaluated for the known causes of the RPL, but none of them had uterine malformations, abnormal placental insertions, abnormal karyotypes and thrombophilic abnormalities. The complete mtDNA analysis of 112 samples (100 blood samples and 12 fetal tissues) revealed a total of 681 variations compared to rCRS. Of the total 112 samples analyzed, 61 possessed at least 1 novel or disease associated mutation (Table 1).

The mutations were more prevalent in NADH Dehydrogenase (ND) genes (256 variations) that encode mitochondrial enzyme Complex I. Out of 256 variations in the ND genes, 29 were novel and 10 were reported to be associated with other diseases. The novel mutations include 6 missense mutations: C4139T (ND1); A5484G (ND2); T13154C, A12724G, G13204A (ND5); and C14433T (ND6), two frame shift mutations; 11084insA, and 11683insG (ND4) and the rest were silent mutations. Among the disease-associated mutations, we have also observed 8 were reported to be associated with Leber Hereditary Optic Neuropathy (LHON): G3316A, T3394C, A3397G, T4216C (ND1); C4640A, A4917G (ND2); T11253C (ND4) and A13708G (ND5) (Mitomap).

The LHON associated mutation T4216C (Fig. 1A–B) was found in 9 samples with RPL, of which 3 showed heteroplasmy (Fig. 1C). This mutation was also detected in 5 out of the 55 RPL patients, who were exclusively checked for this mutation. Hence, the frequency of this mutation was 9% of the total (155) RPL cases studied. The G3316A mutation reported in diabetes and Progressive External Ophthalmoplegia (PEO) was found in 7 women with RPL. The G5460A (Fig. 1D–E) mutation, which was reported earlier in Alzheimer’s and Parkinson’s diseases was detected in 8 women with RPL. A diabetes-specific pathogenic mutation A12026G was found in one woman with RPL.

In Cytochrome b (Cytb) gene, 9 out of 54 variations observed were novel that includes; two missense mutations (T15292C and C15828T), altering the highly conserved amino acids (Isoleucine to threonine and Threonine to methionine, respectively). Fourteen novel mutations including 2 missense (T6631A in COI and T7710C in COII) mutations were observed in Cytochrome Oxidase (CO) genes. Two missense mutations (G6267A and G9804A) detected in CO genes were reported earlier in prostate cancer and LHON, respectively. Seven novel mutations were detected in ATP synthase genes that comprise of 2 frame shift mutations [8840insG (ATPase 6) and 8411insC (ATPase 8)].

In addition to the mutations observed in the protein coding genes, 33 variations were also observed in the tRNA genes, of which C12187G (tRNA His) and A5529G (tRNA Trp) were novel. The mutation A12308G in tRNA Leu was observed in 16 patients. This mutation was previously described in CPEO, stroke, and cardiomyopathy and has been found to be associated with increased risk of renal and prostate cancers (Booker et al., 2006). The disease associated tRNA Thr mutations, A15924G (Fig. 1F–G) and G15928A, were observed in 7 and 5 patients, respectively. In rRNA genes, 43 variations were observed, that includes two novel mutations in 16S rRNA. In 12S rRNA gene, 2 mutations T961C and T1243C were detected in 2 RPL each, which were reported with deafness. In addition to the above mutations, we have also observed five novel variations in the hypervariable regions (HVRs).

In order to find out whether the mtDNA mutations have direct role in the RPL, we have evaluated the mtDNA mutations in 12 aborted fetal tissue samples from independent RPL women. Out of the 12 samples analyzed, 5 samples possessed significant mutations, which could be of potential cause for the RPL. We found 2 novel mutations;
mtDNA mutations observed in women with RPL. Status of the mutations and its association with other diseases are also shown.

Table 1

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<td>NIDDM; LHON; PEO (A)</td>
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one missense (T8759C) and one frame shift (11084 Ains) in ATPase 6 and ND4 genes, respectively along with the other disease associated mutations.

The level of heteroplasmy was quantified by PCR-RFLP, followed by densitometric analysis (Fig. 2). This analysis confirmed the results obtained from the sequence analysis, as all the three samples with heteroplasmy showed both wildtype and mutant bands (Fig. 2). However, the densitometric analysis revealed that each one of the three heteroplasmic sample showed different levels of mutant and wildtype alleles (Fig. 2).

The phylogenetic analysis revealed haplogroup M (61.60%) to be the major haplogroup among the RPL women (Fig. 3), of which, the frequency of M2 was found to be high (11 individuals). Some of the rare haplogroups such as C4 and D4 were also found in the women with RPL. The haplogroup U was found in 21.43% of the total individuals analyzed and its sub haplogroup U2 was observed in 10 patients. The haplogroup R30 was observed in 5 and R6 in 4 women with RPL. The mutation T4216C, which had been described as a marker of JT haplogroup, was also found in many other haplogroups such as M, U as well as T.

4. Discussion

Recurrent pregnancy loss is a multifactorial disorder and elucidating the underlying cause remains the biggest challenge for the clinicians. One of the central mechanisms operating in pathophysiology of many diseases is reactive oxygen species (ROS) induced oxidative damage to the cells, which had also been proposed to be one of the causes of RPL (Gupta et al., 2007). Mitochondrial DNA mutations are known to be involved in enhancing ROS production (Ishikawa et al., 2008). Thus, mtDNA mutations in RPL patients would have profound effect in the development of the fetus. Therefore we have analyzed the mtDNA mutations in idiopathic RPL women and fetal tissue samples to check for the association of these mutations in the pathogenesis of RPL.

A high frequency of mutations was observed in the ND genes that constitute the mitochondrial enzyme Complex I. Mutations in Complex I was also found to cause neurodegenerative diseases such as Parkinson’s disease, Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke like episodes (MELAS), Leigh’s disease, etc. (Shanske et al., 2008; Malfatti et al., 2007; Smigrodzki et al., 2004).

In the ND1 gene, a missense mutation T4216C was observed in 9 (out of 100) women with RPL (3 heteroplasmic and 6 homoplasmic), accounting for overall 9% of the cause of RPL. On the other hand, control women with proven fertility show this mutation in homo-plasmic condition with extremely low frequency (1.6%). The heteroplastic nature of the mutation in the patients shows the significance of the mutation in the pathogenesis of RPL. This LHON associated mutation T4216C has been reported as a haplogroup JT-specific marker in some populations (Herrnstadt et al., 2002). However, we found this mutation to occur independently in different haplogroup backgrounds, suggesting that it might have a pathogenic role rather than a simple polymorphism.

RPL could also be due to defective mitochondrial protein translation caused by the mutations in tRNA genes. We observed

![Fig. 1. The sequence electropherogram of mtDNA mutations observed in high frequency among the women with recurrent pregnancy loss (RPL).](image-url)

The mtDNA content was decreased in newborns with abnormal reproductive age (Jansen and deBoer, 1998; Van Blerkom et al., 1998). Deletions and point mutations in mtDNA were found to be more prevalent in the oocytes extracted from women of increased age (Dhandapany et al., 2009; Thangaraj et al., 2003a,b, 2005a,b, 2008), but also in the genetic etiology of various disease (Dhandapany et al., 2009; Thangaraj et al., 2002; Singh et al., 2006; Rani et al., 2008; Rani et al., 2010).

Our previous studies on various Indian populations suggest that the variations observed in the women with RPL were not found in the Indian populations. Hence, it has been dif
cult for us to convince them and alternate methods of remediation of the RPL women, it has been dif
cult for us to convince them.

Although we could not do the functional analysis, but the in silico analysis of several mutations observed in our study revealed that these mutation affects the protein coding regions, which might lead to defective mitochondrial functions and cause serious complications if the child is born. It has now been established that engineering the mitochondrial genome can rectify the mitochondrial dysfunction. The mitochondrial gene replacement in the non-human primate by spindle transfer has been successful with normal fertilization, embryo development and birth of healthy offspring (Tachibana et al., 2009). Thus, our study becomes more relevant in the context of the reproductive biology and provides new avenues for the treatment of the mitochondrial diseases and to find alternate methods of overcoming the recurrent pregnancy loss.

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References


Fig. 3. Phylogenetic tree, constructed based on complete mtDNA sequence of 100 women with RPL and 12 aborted fetuses. Underlined nucleotide positions were recurrent mutations.


