Mitochondrial Genome Variations in Advanced Stage Endometriosis: A Study in South Indian Population

Suresh Govatati1, Nageswara Rao Tipirisetti2, Shyam Perugu1, Vijaya Lakshmi Kodati3, Mamata Deenadayal4, Vishnupriya Satti2, Manjula Bhanoori1*, S. Shivaji5*

1 Department of Biochemistry, Osmania University, Hyderabad, Andhra Pradesh, India, 2 Department of Genetics, Osmania University, Hyderabad, Andhra Pradesh, India, 3 Vasavi Medical and Research Centre, Hyderabad, Andhra Pradesh, India, 4 Infertility Institute and Research Centre, Secundrabad, Andhra Pradesh, India, 5 Centre for Cellular and Molecular Biology, Hyderabad, Andhra Pradesh, India

Abstract

**Background:** Endometriosis is a chronic gynecological benign disease that shares several features similar to malignancy. Mitochondrial DNA (mtDNA) mutations have been reported in all most all types of tumors. However, it is not known as to whether mtDNA mutations are associated with endometriosis.

**Methodology:** We sequenced the entire mitochondrial genome of analogous ectopic and eutopic endometrial tissues along with blood samples from 32 advanced stage endometriosis patients to analyze the role of somatic and germ-line mtDNA variations in pathogenesis of endometriosis. All ectopic tissues were screened for tumor-specific mtDNA deletions and microsatellite instability (MSI). We also performed mtDNA haplgrouping in 128 patients and 90 controls to identify its possible association with endometriosis risk.

**Principal Findings:** We identified 51 somatic (novel: 31; reported: 20) and 583 germ-line mtDNA variations (novel: 53; reported: 530) in endometriosis patients. The A13603G, a novel missense mutation which leads to a substitution from serine to glycine at the codon 423 of NDS gene showed 100% incidence in ectopic tissues. Interestingly, eutopic endometrium and peripheral leukocytes of all the patients showed heteroplasmy (A/G; 40–80%) at this locus, while their ectopic endometrium showed homoplasmic mutant allele (G/G). Superimposition of native and mutant structures of NDS generated by homology modeling revealed no structural differences. Tumor-specific deletions and MSI were not observed in any of the ectopic tissues. Haplogrouping analysis showed a significant association between haplogroup MS and endometriosis risk (P: 0.00069) after bonferroni correction.

**Conclusions:** Our findings substantiate the rationale for exploring the mitochondrial genome as a biomarker for the diagnosis of endometriosis.

Introduction

Endometriosis is a chronic gynecological disorder that occurs in 10% of women of reproductive age and in up to 50% of women with infertility [1]. It is characterized by the presence of endometrium like tissue outside the uterine cavity. Endometriosis is a benign disease but shares some features with cancer [2]. Several mechanisms have been proposed to explain the development of endometriosis [3], but the etiology and pathogenesis remain unclear. Previously we demonstrated the correlation of endometriosis [3], but the etiology and pathogenesis remain unclear. Previously we demonstrated the correlation of endometriosis with infertility [1]. It is characterized by the presence of endometrium like tissue outside the uterine cavity. Endometriosis is a benign disease but shares some features with cancer [2]. Several mechanisms have been proposed to explain the development of endometriosis [3], but the etiology and pathogenesis remain unclear. Previously we demonstrated the correlation of endometriosis with infertility [1]. It is characterized by the presence of endometrium like tissue outside the uterine cavity. Endometriosis is a benign disease but shares some features with cancer [2]. Several mechanisms have been proposed to explain the development of endometriosis [3], but the etiology and pathogenesis remain unclear. Previously we demonstrated the correlation of endometriosis with infertility [1]. It is characterized by the presence of endometrium like tissue outside the uterine cavity. Endometriosis is a benign disease but shares some features with cancer [2]. Several mechanisms have been proposed to explain the development of endometriosis [3], but the etiology and pathogenesis remain unclear. Previously we demonstrated the correlation of endometriosis with infertility [1].

Our findings substantiate the rationale for exploring the mitochondrial genome as a biomarker for the diagnosis of endometriosis.
Mitochondria are semiautonomous organelles and have a variety of important roles to play, including energy metabolism, generation of ROS, aging, and regulation of apoptosis [17]. Hence mitochondria may serve as the switching point between cell death and abnormal cell growth. Human mtDNA (16,569 kb) encodes 13 subunits of the electron transport chain (ETC), as well as a distinct set of rRNAs and tRNAs [18]. It exhibits higher mutation rate than nuclear DNA and is more susceptible to oxidative damage due to a lack of protective histone proteins, limited DNA repair mechanisms and a high rate of ROS generation [19]. During the course of evolution, several mutations have accumulated in the mtDNA that allows human populations to be categorized in to various discrete, region specific, mitochondrial clades or haplogroups. Recent investigations showed an association of mtDNA haplogroups with various diseases including cancer [20–21]. However, studies investigating the role of mitochondrial haplogroups in pathogenesis of endometriosis are not well explored and studied.

The mtDNA mutations could arise either in the germ-line and predispose cancer or in somatic tissues and participate in the tumor progression process. Both types of mutations have been reported in various types of tumors [22]. Although the molecular genetics of endometriosis has been in the focus of many research laboratories for a long period of time, relevant prognostic and diagnostic markers are still missing. At the same time mtDNA mutations have been reported in various types of tumors during the last two decades [22]. It is therefore very likely that the mitochondrial genotype is one of the tumor susceptibility factors. We propose that mtDNA mutations and/or haplogroups might be anticipated in the initiation or progression of endometriosis. To the best of our knowledge, no screening of the whole mtDNA has been performed to date in endometriosis. In the present study, we attempted comprehensive scanning of somatic & germ-line mtDNA mutations in matched ectopic (endometriotic) and eutopic (normal) endometrial tissues from 32 endometriosis patients. For high stringent data quality control, we compared the entire mtDNA sequences of matched ectopic and eutopic endometrial tissues along with blood samples from the same patients. Mitochondrial haplogrouping was performed in 128 cases and 90 controls to identify its possible association with the risk of endometriosis. Moreover, also we analyzed tumor specific large-scale mtDNA deletions and mitochondrial microsatellite instability (MSI) in ectopic tissues to verify their role in the pathogenesis of endometriosis.

Materials and Methods

Sample Collection and Diagnosis

Matched ectopic and eutopic endometrial tissue samples were collected from thirty two pre-menopausal unrelated women of South Indian origin (Dravidian linguistic group) with moderate-severe (III–IV; rAFS [23]) endometriosis. The samples were collected from Infertility Institute and Research Centre (IIRC), Secundrabad, India (n = 7) and Vasavi Medical and Research Centre, Hyderabad, India (n = 25). Each pair of ectopic and eutopic endometrial tissues was collected from the same patient to confirm the heteroplasmic mutations in tumor tissues because it is possible that the apparent heteroplasmic mutation is attributable to the contamination of the surrounding non-tumor cells. All women had a trans-vaginal ultrasound scan (TVS) at screening followed by a laparoscopy to confirm the endometriosis stage (rAFS III = 10; IV = 22). All the patients had different manifestations of endometriosis such as peritoneal lesions, adhesions and endometrioma. Women with other ovarian cysts, adenomyosis, ovarian cancer, fibroids, and stage I and II endometriosis were excluded from the study. The aim was to focus on patients with more severe endometriosis (stage III and IV) because the more severe forms include ovarian cystic disease, which almost certainly has a different etiology to peritoneal forms, and the diagnosis is usually unequivocal, which is not the case for stage I and II. Tissue samples were frozen immediately in the operation room and stored at –80 °C until isolation of genomic DNA was performed. For haplogrouping analysis peripheral blood samples were collected from 128 patients and 90 controls. The control group was selected from same clinical population as per the criteria of appropriate controls set [24]. Written informed consent was obtained from all participants. The Institutional Review Board of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, approved the study.

Sequencing of Entire Mitochondrial Genome

DNA was isolated from all the samples by salting out method [25]. The entire mitochondrial genome mutations were analyzed by PCR-sequencing analysis as per the protocols described by Bhanoori et al. [7]. Twenty-four pairs of overlapping primers (Table S1) were used to amplify the entire 16,569 kb mitochondrial genome. The generated DNA fragments vary in size from 801 bp to 1161 bp with an average of 895.96 bp. The amplified fragments totaled 21503 bp, 29.8% more than the mt-genome because of the overlapping regions.

Mutational Analysis & Haplogrouping

The individual mtDNA sequences were compared against the Revised Cambridge Reference Sequence (rCRS) [26] using Auto Assembler-Ver 2.1 (Applied Biosystems, Foster City USA). Sequences were aligned by using CLUSTAL X and mutations were noted by using MEGA software ver 3.1. Independent sequencing readings were performed by three different individuals (SG, NRT & SP). Sequence variations found in both ectopic and matched eutopic mtDNA were scored as germ-line variations. Each was then checked against the Mitomap database. Those not recorded in the database were categorized as novel mtDNA variations, and those that appeared in the database were reported as polymorphisms or mutations. Any DNA sequence differences between ectopic and matched eutopic mtDNA were scored as somatic mtDNA mutations. All the somatic heteroplasmic mutations were crosschecked and confirmed by comparing the ectopic mtDNA sequences with the analogous eutopic as well as blood mtDNA sequences form the same patients. The level of heteroplasmy was calculated based on the ratio of the height of the mutant peak relative to the wild type plus mutant peak at the same nucleotide position in the sequencing results. Pathogenic potential of missense mutations was predicted by PolyPhen software (www.tux.embl-heidelberg.de/ramesnys/polyphen.cgi). It works based on sequence comparison with homologous proteins. Profile scores (PSIC) were generated for the allelic variants which represents the logarithmic ratio of the likelihood of a given amino acid occurrence at a particular site relative to the likelihood of this amino acid occurrence at any site (background frequency). Generally, PSIC score differences >2 indicate a damaging effect, scores between 1.5 and 2 suggest that the variant is possibly damaging and scores <1.5 indicate that the variant is benign. Mitochondrial haplogroups were assigned to all samples based on updated comprehensive phylogenetic tree of global human mitochondrial DNA variation [27].
Microsatellite Instability (MSI) Analysis
To study mtDNA MSI, 9 mononucleotide (nt 303, 311, 3566, 6692, 9478, 12385, 12418, 13231, and 16184), a dinucleotide (nt 514), and a trinucleotide (nt 12981) repeats at various nucleotide positions throughout mt-genome were analyzed by using sequencing data.

mtDNA Deletion Analysis
The common 5 kb deletion (nt 8469 to nt 13447) in tumor tissue was analyzed by PCR method using forward primer mtFL8150 (5’-CCGGGGTTATACCGGTCA-3’) and reverse primer mtRH14020 (5’-ATAGCTTTTCTAGTCAAGTT-3’). The PCR will produce either 894 bp fragment (4977 bp mtDNA deletion) and/or 536 bp fragment (5335 bp mtDNA deletion) or 5871 bp fragment (wild type mtDNA) based on the type of deletion. Both ectopic and matched eutopic tissues were included in this study.

Statistical Analysis
Statistical analysis was performed using SPSS statistical package (ver 11.0). The frequency of each haplogroup among cases and controls were compared with Pearson $\chi^2$ or Fisher’s exact test. A $P$-value less than 0.05 was considered significant. Bonferroni correction was used to adjust the significance level of a statistical test to protect against Type I errors when multiple comparisons were being made. Since we have 39 mitochondrial haplogroups, the Bonferroni correction should be 0.05/39 = 0.00128. Therefore, a $P$-value less than 0.00128 was considered significant.

Results
Endometriosis Harbored Tumor Specific Somatic mtDNA Mutations
The first objective of this study was to evaluate whether endometriosis presented with the incidence of tumor specific somatic mtDNA mutations like many other tumors types. We identified 51 somatic mutations (Table 1) in which 45.1% (23/51) were located in the D-loop region, 17.6% (9/51) were in rRNA genes, 3.9% (2/51) were in tRNA genes and 33.3% (17/51) were present in protein coding region of mt-genome. The relative somatic mutation frequency in the D-loop region is 11.4-fold higher than the remaining region of mt-genome. Interestingly all the identified somatic mutations in protein coding region (n = 17) are novel. Overall, among the identified 51 somatic mutations 38 were point mutations, 2 were nucleotide deletions, 5 were nucleotide insertions and 6 were heteroplasmic mutations. Among six heteroplasmic mutations 5 (3 in 12S rRNA genes; 1 in 16S rRNA genes and 1 in ND4 gene) showed heteroplasmcy in ectopic endometrium while in analogous eutopic endometrium they were homoplasmic wild type. Interestingly the remaining 1 heteroplasmic mutation, located in ND5 gene at nt 13603 showed heteroplasmcy (A/G) in eutopic endometrium while homoplasmic mutant type (G/G) in analogous ectopic endometrium. To confirm the heteroplasmic mutations, we sequenced and compared the mtDNA of peripheral leukocytes along with the eutopic and ectopic tissues from the same patients, because heteroplasmcy could be attributed to the contamination with the surrounding tumor or the morphologically normal tissue that have already undergone molecular changes. The blood mtDNA sequence pattern is fully consistent with eutopic tissue, indicating the purity of ectopic tissue.

Effect of Somatic Mutations on Mitochondrial Function
Among the 17 somatic mutations identified in mtDNA protein coding region, 7 (41.2%) were missense mutations that may have the potential to cause defects in the oxidative phosphorylation system (OXPHOS).

The C5444A in ND2 gene (Figure 1A) and the T7372C in COI gene (Figure 1B) result in substitution from phenylalanine to leucine and methionine to threonine respectively. These two point mutations are missense mutations, located in poorly conserved region of proteins. Thus presumably they have no impact on protein structure and/or function. The Profile score (PSIC) difference was 0.031 for C5444A and 0.184 for T7372C, thus they were predicted to be benign by PolyPhen analysis. The A8698G mutation results in substitution from methionine to valine at codon 58 of the ATPase 6 (Figure 1C). This may be harmful missense mutation because the substitution position is highly conserved among vertebrates (PSIC = 1.893). The T11544T/A heteroplasmcy (48–51% heteroplasmy) was identified in ectopic endometrium of 7 patients. It causes a substitution from leucine to histidine at codon 262 of the ND4 gene (Figure 1D). The location of this mutation is evolutionally highly conserved (L or I) among most of the euukaryote, thus it may be a potentially harmful mutation (PSIC = 2.050).

The T11792G in ND4 gene and the C12398T in ND5 gene result in substitution from serine to alanine and threonine to isoleucine respectively (Figure 1E–F). These two point mutations may not be harmful missense mutations as they occur at poorly conserved region of proteins. PolyPhen analysis predicted both of these variants as benign (PSIC = 0.450 for T11792G & not applicable for C12398T).

Association of ‘A13603G’ Mutation of ND5 with Endometriosis
We identified a novel somatic missense ‘A→G’ transition mutation at the nt 13603 (Figure 2A) in ectopic endometrium of all the patients (n = 32) which causes a substitution from serine to glycine at the codon 423 of ND5 gene. It may be a potentially harmful mutation as it occurs in highly conserved functional domain of the peptide (PSIC = 2.193). Interestingly, eutopic endometrium & peripheral leukocytes of all the patients showed different levels heteroplasmcy (A/G; 40–80%) at this locus, while their ectopic endometrium showed homoplasmic mutant allele (G/G). The level of heteroplasmcy (mutation load) was inconsistent between different tissues of the same patients. Overall, eutopic endometrium showed high mutationload than the analogous peripheral leukocytes. Furthermore, we did not observe this mutation among age matched controls from the same ethnic group. These results emphasize the correlation between ‘A13603G’ mtDNA mutation and risk of endometriosis.

Structure Prediction and In Silico Functional Analysis of ‘A13603G’ of ND5
We compared all 164 sequence depositions of human ND5 gene available from the NCBI database using Clustal X software for similarities. Using protein prediction servers we found 1EHK as template structural sequence for the ND5 target sequence. There is no full pledge crystalized structure available for the ND5. Template sequence has 30% identity to the ND5. We performed the homology modeling and generated 40 structures for ND5 protein using modeller9V9 software [28]. Of these we considered one structure (Figure 2B) with low energy value (geometrically favorable). The structural accuracy of the selected structure was evaluated by Procheck software [29] (Figure 2C). It indicates that...
| Gene/region | Nucleotide position | Ref | Base change | Eut → Ect pattern | F | Codon & AA change | Reported/Novel |
|-------------|---------------------|-----|-------------|-------------------|---|------------------|----------------|
| D-loop      | A56 del             | A   | A A A –     | HM → HM           | 1 | NPCR            | Novel          |
| D-loop      | A73G                | A   | A A A G     | HM → HM           | 2 | NPCR            | Reported       |
| D-loop      | C105 del            | C   | C C C –     | HM → HM           | 2 | NPCR            | Novel          |
| D-loop      | 149 ins T           | –   | – – T       | HM → HM           | 1 | NPCR            | Novel          |
| D-loop      | T195C               | T   | T T C       | HM → HM           | 1 | NPCR            | Reported       |
| D-loop      | T204C               | T   | T T T C     | HM → HM           | 1 | NPCR            | Reported       |
| D-loop      | G207A               | G   | G G G A     | HM → HM           | 3 | NPCR            | Reported       |
| D-loop      | 303–9 ins C         | –   | – – C       | HM → HM           | 7 | NPCR            | Reported       |
| D-loop      | 303–9 ins CC        | –   | – – CC      | HM → HM           | 5 | NPCR            | Reported       |
| D-loop      | T310C               | T   | T T C       | HM → HM           | 1 | NPCR            | Reported       |
| D-loop      | 311-15 ins C        | –   | – – C       | HM → HM           | 2 | NPCR            | Reported       |
| D-loop      | G316C               | G   | G G G C     | HM → HM           | 2 | NPCR            | Reported       |
| D-loop      | C394T               | C   | C C T       | HM → HM           | 1 | NPCR            | Novel          |
| D-loop      | A438G               | A   | A A A G     | HM → HM           | 1 | NPCR            | Novel          |
| D-loop      | G709A               | G   | G G G A     | HM → HM           | 1 | NPCR            | Reported       |
| 12S rRNA    | C775C/G             | C   | C C C G/G   | HM → HT           | 1 | NPCR            | Novel          |
| 12S rRNA    | G811G/A             | G   | G G G G/A   | HM → HT           | 3 | NPCR            | Novel          |
| 12S rRNA    | A1171A/G            | A   | A A A A/G   | HM → HT           | 10| NPCR           | Novel          |
| 12S rRNA    | A1383G              | A   | A A A G     | HM → HM           | 1 | NPCR            | Novel          |
| 16S rRNA    | A1811G              | A   | A A A G     | HM → HM           | 1 | NPCR            | Reported       |
| 16S rRNA    | C2005T              | C   | C C C T     | HM → HM           | 1 | NPCR            | Novel          |
| 16S rRNA    | G2536G/A            | G   | G G G G/A   | HM → HT           | 30| NPCR           | Novel          |
| ND1         | C3573T              | C   | C C C T     | HM → HM           | 1 | NPCR            | Reported       |
| ND2         | C4799T              | C   | C C C T     | HM → HM           | 1 | P110P          | Novel          |
| ND1         | C5444A              | C   | C C C A     | HM → HM           | 1 | F325L          | Novel          |
| 18S rRNA    | C5821A              | G   | G G G A     | HM → HM           | 3 | NPCR            | Reported       |
| COI         | C7019T              | C   | C C T       | HM → HM           | 1 | Y372Y          | Novel          |
| COI         | C7372C              | T   | T T C       | HM → HM           | 1 | M490T          | Novel          |
| COII        | A8116G              | A   | A A A G     | HM → HM           | 1 | G177G          | Novel          |
| ATPase6     | A8698G              | A   | A A A G     | HM → HM           | 1 | M58V           | Novel          |
| ATPase6     | C9061T              | C   | C C C T     | HM → HM           | 1 | L179L          | Novel          |
| ND2         | C9650T              | C   | C C C A     | HM → HM           | 1 | H148H          | Novel          |
| ND2         | C11399C             | T   | T T C       | HM → HM           | 1 | L204L          | Novel          |
| ND2         | C11542T             | C   | C C T       | HM → HM           | 1 | F261F          | Novel          |
| ND2         | C11544T/A           | T   | T T T A/A   | HM → HT           | 7 | L262H          | Novel          |
| ND2         | A11707G             | A   | A A A G     | HM → HM           | 1 | M316M          | Novel          |
| ND2         | T11792G             | T   | T T G       | HM → HM           | 1 | S345A          | Novel          |
| ND4         | C12398T             | C   | C C C T     | HM → HM           | 1 | T211           | Novel          |
| ND5         | C12867T             | C   | C C C T     | HM → HM           | 1 | I177I          | Novel          |
| ND5         | A/G13603G           | A   | A/G A/G     | HT → HM           | 32| S423G          | Novel          |
| tRNA        | C15926T             | C   | C C T       | HM → HM           | 2 | NPCR          | Novel          |
| D-loop      | G16145A             | G   | G G G A     | HM → HM           | 3 | NPCR          | Reported       |
| D-loop      | C16172T             | C   | C C C T     | HM → HM           | 2 | NPCR          | Reported       |
| D-loop      | A16182C             | A   | A A A C     | HM → HM           | 1 | NPCR          | Reported       |
| D-loop      | T16189C             | T   | T T C       | HM → HM           | 5 | NPCR          | Reported       |
| D-loop      | C16325T             | C   | C C C T     | HM → HM           | 1 | NPCR          | Reported       |
the generated structure was 98% geometrically favorable and can be reliable for further studies. Then we have mutated the native structure at amino acid position 423 by glycine (Figure 2D) and superimposed the native and mutated structures (Figure 2E) to find out the structural variations. Our results showed no structural difference between native and mutated forms. Serine is a polar amino acid, while glycine is hydrophilic, both of them, however belongs to the class of the smallest amino acids, with short side chain. It seems that the presence of small amino acids in this position is crucial for the maintenance of the protein structure without causing destabilization. The exact role of this mutation in pathogenesis of endometriosis is currently unknown. Further extensive biochemical and molecular functional studies are necessary to confirm the role of this mutation in endometriosis.

In addition, we identified two somatic mutations in tRNA coding genes. The G5821A mutation (Figure S1A) in the tRNA^Cys^ gene interrupts conserved Watson- Crick base pairing in the anticodon loop. Thus it may alter the overall tertiary structure of the cloverleaf. The C15926T mutation (Figure S1B) in the tRNA^Thr^ gene newly establishes Watson- Crick base pairing in the anticodon loop. Thus it may alter the overall structural stability of tRNA^Thr^.

Germ-line mtDNA Mutations are Common in Endometriosis

We observed 53 novel and 530 reported germ-line mtDNA variations among the patients. Among 53 novel mutations, 11 are missense mutations (Table 2), 22 are synonymous mutations (Table S2) and remaining (n = 20) are present in non protein coding region of mt-genome (Table S3). The overall frequency of novel germ-line mutations was 16.90% (9/53) in the D-loop and 83.02% (44/53) in the coding regions. Forty one of the 53 (77.35%) novel variations occurred only once, whereas the remaining variations showed differential frequency. All the above 53 novel variations are completely absent in controls.

Among 530 reported germ-line mutations, 73 (13.8%) are missense mutations, 236 (44.5%) are synonymous mutations and remaining are located in non protein coding region of mt-genome (data not shown). In overall, 151 (28.3%) are located in the D-loop region. The relative germ-line mutation frequency in the D-loop region is 6.5-fold higher than the remaining region of mt-genome.

Association of Haplogroup M5 with Endometriosis Risk

We identified 39 mtDNA haplogroups among the patients and controls (Table 3). We found a significant association between haplogroup M5 and endometriosis risk (P = 0.00069). Further we tested the correlation between identified macro-haplogroups and endometriosis incidence. We found no statistical significant difference between cases and controls.

Microsatellite Instability (MSI) is Less Frequent in Endometriosis

Somatic homoplasmic insertions were found in nt 303–309 and 311–315 poly C tracts only. In addition we found a T to C substitution at nt 16189 which resulted in a stretch of 10 C’s in the region. None of the remaining MS loci showed obvious insertion or deletion of C’s.

Tumor Specific Deletions are Absent in Endometriosis

The common 5 kb tumor-specific deletion (nt 8469 to nt 13447) was analyzed in ectopic endometrial tissues by PCR method. This method is sensitive enough to detect 0.01% of deleted mtDNA. Deletions were not detected in any of the ectopic tissues.

Discussion

A new role of ROS as second messenger of cellular proliferation has been reported. According to this, normal cell proliferation correlated with production of endogenous ROS through the activation of growth-related signaling pathways [30]. Modulation of ROS production may control tumor cell proliferation [31]. The mitochondrial ETC is a major source for ROS generation. Mutations in mitochondrial DNA have been found to increase endogenous ROS production and enhance metastatic potential of tumor cells [16]. It has been suggested that ROS or free radicals may increase growth and adhesion of endometrial cells in the peritoneal cavity, promoting endometriosis and infertility [32–33]. However, the published studies on the association between oxidative stress and endometriosis have been inconsistent. Some of the studies have found a positive association between oxidative stress and endometriosis [14,34] whereas others have not found an association [35–36]. In addition, increased oxidative stress and mtDNA deletions have been observed in endometriotic tissues from patients [37]. All these observations strongly correlate the mitochondrial malfunction with the pathogenesis of endometriosis.

Previously investigators have screened the whole mt-genome in a variety of human diseases including cancer [22]. However none of the investigators have reported whole mtDNA mutations in endometriosis. The current sequencing based analysis of the entire mt-genome demonstrates that endometriotic tissues harbor a plethora of tumor-specific somatic mtDNA mutations much like other tumors. Our study provides, for the first time, an insight into the prevalence and distribution of entire mtDNA mutations in endometriosis. Association between 3 reported germ-line mtDNA mutations (A10398G, G13708A and T16189C) and endometriosis was analyzed in ectopic endometrial tissues by PCR method. This method is sensitive enough to detect 0.01% of deleted mtDNA. Deletions were not detected in any of the ectopic tissues.

Table 1. Cont.

| Gene/region | Nucleotide position | Ref | Base change | Eut → Ect pattern | F | Codon & AA change | Reported/Novel |
|-------------|---------------------|-----|-------------|-------------------|---|------------------|---------------|
| D-loop      | 16351ins C          |     |             |                   |   |                   |               |
| D-loop      | T16362C             | T   | T           | C                 |   | 3 NPCR           | Novel         |
| D-loop      | T16519C             | T   | T           | C                 |   | 1 NPCR           | Reported      |

1Total number of mutations: 51; 2Novel mutations: 31; Reported mutations: 20;
Ref, Cambridge reference sequence; Bld, Blood; Eut, Eutopic endometrium; Ect, Ectopic endometrium; F, Frequency of mutations; HM, Homoplasmic mutation; HT, Heteroplasmic mutation; NPCR, Non Protein Coding Region; doi:10.1371/journal.pone.0040668.t001
Mitochondrial Mutations in Endometriosis

A

EUT

ECT

Blood 320 TKTF P LPTLI 330
Eutopic 320 TKTF P LPTLI 330
Ectopic 320 TKTF P LPTLI 330

485 VEEPS M NLEWL 495
53 TKQNM M AHMT 63

Human 320 TKTF P LPTLI 330
Gorilla 320 TKTF P LPTLI 330
Mouse 320 TKTF P LPTLI 330

485 VEEPS M NLEWL 495
53 TKQNM M AHMT 63

280 TKTF P LPTLI 330
Cow 320 TKTF P LPTLI 330
Blue Whale 320 TKTF P LPTLI 330

485 VELTA T NIEVI 495
53 TKQNM M AHMT 63

Chicken 320 QLIS P LSLNM 330
Zebra fish 320 QLIS P LSLNM 330
Fruit fly 320 QLIS P LSLNM 330
Mosquito 320 QLIS P LSLNM 330

485 QLIS P LSLNM 330
53 TKQNM M AHMT 63

D

EUT

ECT

Blood 257 MAYFF L VLSLW 267
Eutopic 257 MAYFF L VLSLW 267
Ectopic 257 MAYFF L VLSLW 267

340 RIMIL S QGLQT 350
16 IPPIL T TLVNF 26

Human 257 MAYFF L VLSLW 267
Gorilla 257 MAYFF L VLSLW 267
Mouse 257 MAYFF L VLSLW 267

340 RIMIL S QGLQT 350
16 IPPIL T TLVNF 26

257 MAYFF L VLSLW 267
Horse 257 MAYFF L VLSLW 267
Blue Whale 257 MAYFF L VLSLW 267

340 RIMIL A RGLQM 350
16 IPPIL T TLVNF 26

Chicken 257 MAYFF L VLSLW 267
Zebra fish 257 MAYFF L VLSLW 267
Fruit fly 257 MAYFF L VLSLW 267
Mosquito 257 MAYFF L VLSLW 267

340 RIMIL A RGLQM 350
16 IPPIL T TLVNF 26
have recently been reported [38]. Although interpretation of linking germ-line mtDNA mutations to tumor can be confounded by the high background frequency of functional mtDNA polymorphisms, studies of somatic mtDNA mutations can be more definitive since the tumor cell should have the neoplastic mtDNA mutation while the normal tissue should not. Moreover, if germ-line mtDNA mutations induce oncogenic transformation, all the offspring of a mother carrying such mutations should develop tumors due to the maternal inheritance of mtDNA, but no bias toward maternal inheritance of tumor development has been reported. In the present study we reported a comprehensive study of somatic and germ-line mtDNA mutations in endometriosis.

Previously several reporters have shown the prevalence of somatic mtDNA mutations in various types of tumors [22]. The high frequency of somatic mutations was attributed to artifacts instead of real somatic events [39]. A critical reassessment of the reported somatic mutations in tumors was carried out using a phylogenetic method and found that many of these investigations were affected by flawed data which were mainly caused by sample crossover and contamination [39]. In the present study we maintained high stringent data quality control to avoid these problems. Of the 51 somatic mutations that we found, 20 (17 in the D-loop and 3 in non-D loop areas) have been previously reported in other cancer types (Table 1) suggesting that these sites are susceptible to mutation in a variety of human malignancies. Our observations underpin the previous findings that somatic mtDNA mutations in tumors are concentrated in the D-loop and that the D-310 region is a mutation hot spot. The sequence between nt 303–315 is highly conserved and the length variation or mutations in this region affects regulation of mtDNA replication. The 5 mutations that we and others observed in this conserved region in tumor specimens may therefore reflect neoplastic initiation.

The role of somatic mtDNA mutations in tumor progression has not been investigated. Mutations in the conserved regions, origins of replication, promoters or transcription factor binding sites, may affect the total amount of mitochondrial transcripts and mature proteins. Eventually, the overall OXPHOS activity of the mitochondria may be affected. We observed 7 somatic missense mutations that led to changes in amino acids (Table 1). All these mutations are novel and most of them are located in highly conserved regions of different mitochondrial genes. Each of these mutations may have functional significance, but more extensive biochemical and molecular studies are necessary to determine their effects on energy metabolism in the tumor cells. Interestingly, the A13603G (S423G) of ND5 gene was observed in ectopic endometrium of all patients (n = 32) while completely absent in matched eutopic endometrium and peripheral leukocytes of the same patients. More specifically, mtDNA of blood and eutopic endometrium of all patients showed different levels of heteroplasm [12; 40 - 80%] at this locus while the analogous ectopic endometrium carried exclusively mutant mtDNA (homo-plasm) without any co-existing normal mtDNA (heteroplasm), as is found in mitochondrial genomic degenerative diseases [18,22]. This may be consequence of ‘replicative segregation’ – a process in which with repeated cell divisions (clonal expansion) the percentage of mutant and normal mtDNAs can drift until it reaches either pure mutant or normal homoplasm (Figure S2). These findings support the idea that pathological mtDNA mutations are particularly deleterious in specific cell types, which can explain some of the tissue-specific aspects of mitochondrial DNA diseases. Interestingly, all control group individuals carried homoplasmic wild allele (A/A) at this locus. These observations emphasize the role of A13603G in pathogenesis of endometriosis. We predicted mitochondrial ND5 protein structure for the first time and observed no structural difference between native and mutated forms (Figure 2). Further studies are required to elucidate the exact pathogenic role of this mutation in endometriosis.

Several mitochondrial mutations have been reported in association with various human diseases. However, very few of them have been confirmed as markers. For LHON disease, three markers have been reported in approximately 95% of all patients while their frequency is 0% in controls (www.mitomap.org). Interestingly, in our study we identified 100% incidence of A13603G novel missense somatic mutation in ectopic endometrium and 0% incidence in eutopic endometrium and peripheral leukocytes of patients. However, further studies are required considering different ethnic groups to elucidate the A13603G mutation as marker of endometriosis.

Most of the ectopic tissues had more than one somatic mutation. Specifically, of the 32 ectopic tissues with somatic mutations, 30 (93.75%) had two or more, 16 had three or more (50%) and 5 had six or more (15.6%) mutations (Table 1). Multiple mtDNA mutations are common in most of the human tumors [40]. Transcription of mitochondrial genome produces two polycistrionic primary transcripts that are processed by endonuclease to yield the mature rRNA, tRNA, and mRNA molecules. Thus, mutations anywhere in the genome affecting the folding and secondary structure of the RNA precursors are potentially detrimental to RNA processing. However, most of the somatic mtDNA mutations in tumor may represent passenger mutations that do not play any primary role in tumorigenesis.

We identified a huge number of novel (n = 53; reported: 530) of germ-line mtDNA mutations in endometriosis patients. Of 530 reported germ-line mutations, 151 (28.4%) are present in the D-loop, an area that acts as a promoter for both the heavy and light strands of mtDNA which contains essential transcription and replication elements. The frequency of novel germ-line variations in coding regions [83.01% (44/53)] is much higher than in the D-loop [16.98% (9/53)]. Although we cannot be sure, this may simply reflect the fact that the D-loop has been more intensively investigated than the coding regions.

Mutations in different mitochondrial gene complexes have been shown to contribute to tumorigenicity through ROS generation in several tumor types [41]. The high incidence of missense mutations in complex I has been reported in several tumors [41–42]. Our results are consistent with this finding. We observed 3/7 somatic and 8/11 novel germ-line missense mutations in complex I genes (Table 1 & 2). Mitochondrial OXPHOS produces most of the cellular ROS at complexes I and III, by the transfer of an unpaired electron to O2,i t exhibits distinct geographic distribution. L is the oldest macro-
haplogroup, restricted to Africans and consists of haplogroups L0, L1, L2, L3, L4, L5, L6 and L7, of which L3 radiated out of Africa in the form of macro-haplogroups M and N around ~60,000 ybp [43]. Among the two macro-haplogroups M and N, M is more prevalent in Indian subcontinent. The haplogroups are associated with region-specific mtDNA sequence variations as a result of genetic drift and/or adaptive selection for an environment-favored mitochondrial function [44]. Several investigators have showed association between different mtDNA haplogroups and various diseases including cancer [20–21]. Difference in redox signaling as a consequence of haplogroup-associatedOXPHOS capacity has been suggested as the molecular mechanism involved in the haplogroups-associated phenotypes [45]. In the present study we report for the first time an association between haplogroup M5 and endometriosis in South Indian population.

Although not a primary aim of our investigation, also we analyzed MSI of mtDNA in ectopic endometrium of patients. Contradictory observations have been reported regarding mtDNA MSI in different tumors [46–47]. The methods used to detect the MSI were either fragment size analysis or RFLP analysis, which may not be the most accurate means because of the interference of shadow bands and the possibility of incomplete digestion, respectively. Differences in the frequency of MSI may be related to differences in methodology used, as well as the loci evaluated.

We investigated the mtDNA MSI by direct sequencing method, and found insertions only in the D loop regions of nt 303–309 and nt 311–315. MSI was not detected in any of the other 10 selected STR regions except at nt T16189C. These observations indicate that the regions of nt 303–309 and nt 311–315 are probably mutation hot spots rather than the reflection of true MSI.

Tumor-specific large scale mtDNA deletions (4977 bp) have been detected in various diseases including cancer [48–49]. mtDNA deletions occur at a region that is flanked by short direct repeat sequences (class I deletions) or by imperfect repeats containing a few mismatches (class II deletions) which is usually lost during the deletion process [50]. Defects in mtDNA replication caused by inappropriate alignment of direct repeats [51] and/or altered repair of mtDNA damage [52] may be responsible for mtDNA deletions. Tumor-specific mtDNA deletions have also been reported in endometriotic tissues [37]. However in the present study we did not found any large-scale mtDNA deletions both in ectopic and eutopic endometrium of the patients.

In conclusion, our study confirms the strong association between mtDNA variations and endometriosis risk. In addition, we found that haplogroup M5 exhibited an increased risk of endometriosis incidence. To our knowledge, this is the first report of complete mt-genome sequencing and haplogrouping in endometriosis. Present study gives a cumulative evidence that

Table 2. Novel missense mtDNA mutations observed in endometriosis patients.

| Gene/ region | Nucleotide position | Ref | Base change | Germline/Somatic | Codon & AA change | Con |
|--------------|---------------------|-----|-------------|------------------|-------------------|-----|
|             |                     |     |             | F                |                   |     |
| Bld Eut Ect |                     |     |             |                  |                   |     |
| ND2         | T4509C              | T   | C           | C                | germ-line         | 1   |
|             | A4701G              | A   | G           | G                | germ-line         | 2   |
|             | C5444A              | C   | C           | A                | somatic           | 1   |
|             | C5445T              | C   | T           | T                | germ-line         | 1   |
| COI         | T7372C              | T   | T           | C                | somatic           | 1   |
| COII        | G7775A              | G   | A           | A                | germ-line         | 2   |
| COII        | T7836C              | T   | C           | C                | germ-line         | 1   |
| ATPase6     | A8698G              | A   | A           | G                | somatic           | 1   |
| ATPase6     | A8704G              | A   | G           | G                | germ-line         | 1   |
| ND4         | T11544T/A           | T   | T           | T                | somatic           | 7   |
| ND4         | T11792G             | T   | T           | T                | somatic           | 1   |
| ND5         | C12398T             | C   | C           | T                | somatic           | 1   |
| ND5         | C12498T             | C   | T           | T                | germ-line         | 4   |
| ND5         | T13154C             | T   | C           | C                | germ-line         | 1   |
| ND5         | T13543C             | T   | C           | C                | germ-line         | 1   |
| ND5         | A/G13603G           | A   | A/G         | A/G              | somatic           | 32  |
| ND5         | T13820C             | T   | C           | C                | germ-line         | 1   |

1Total number of mutations: 18; 2Germ-line mutations: 11, Somatic mutations: 7; 3Conservation; Ref, Cambridge reference sequence.

Bld, Blood; Eut, Eutopic endometrium; Ect, Ectopic endometrium; F, Frequency of mutations; PC, Poorly conserved; HC, Highly conserved; CN, Conserved.

doi:10.1371/journal.pone.0040668.t002
mtDNA is a rational biomarker for the detection of endometriosis. Further investigation is warranted as to the functional implications of identified mutations in endometriosis.

Supporting Information

Table S1 Primers used in this study for whole mitochondrial genome sequencing.

Table 3. Mitochondrial haplogroup distribution in endometriosis patients and controls.

| Haplogroup | Cases | Controls | \(P\)-value\(^2\) | \(\chi^2\)-value | Odds ratio | 95% CI |
|------------|-------|----------|-----------------|-----------------|------------|--------|
| H2a2a      | 4 (3.1) | 2 (2.2) | 0.69568 | 0.153 | 0.7045 | 0.1262 to 3.9314 |
| J1         | 1 (0.8) | 4 (4.4) | 0.11443 | 2.492 | 5.907 | 0.6491 to 53.7591 |
| M          | 1 (0.8) | 3 (3.3) | 0.21702 | 1.524 | 4.3793 | 0.4481 to 42.7975 |
| M18        | 9 (7.0) | 4 (4.4) | 0.05778 | 3.6 | 7.4706 | 0.8576 to 65.0753 |
| M2         | 7 (5.4) | 3 (3.3) | 0.47644 | 0.507 | 0.5961 | 0.1499 to 2.3702 |
| M30        | 10 (7.8) | 9 (10) | 0.60199 | 0.272 | 1.3111 | 0.5101 to 3.3696 |
| M31        | 0 (0) | 1 (1.1) | 0.29427 | 1.1 | – | – |
| M33        | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |
| M34        | 0 (0) | 2 (2.2) | 0.13801 | 2.2 | – | – |
| M35        | 5 (3.9) | 2 (2.2) | 0.49115 | 0.474 | 0.5591 | 0.106 to 2.9479 |
| M36        | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |
| M37        | 0 (0) | 2 (2.2) | 0.13801 | 2.2 | – | – |
| M39        | 5 (3.9) | 1 (1.1) | 0.2105 | 1.568 | 0.2764 | 0.0317 to 2.4071 |
| M4         | 7 (5.4) | 5 (5.6) | 0.94957 | 0.004 | 1.0168 | 0.3122 to 3.3114 |
| M40        | 0 (0) | 1 (1.1) | 0.29427 | 1.1 | – | – |
| M41        | 0 (0) | 1 (1.1) | 0.29427 | 1.1 | – | – |
| M42        | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |
| M49        | 3 (2.3) | 0 (0) | 0.12937 | 2.3 | – | – |
| M5         | 25 (19.5) | 3 (3.3) | **0.00069** | 11.511 | 0.1421 | 0.0415 to 0.4867 |
| M52        | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |
| M58        | 0 (0) | 1 (1.1) | 0.29427 | 1.1 | – | – |
| M6         | 6 (4.7) | 7 (7.8) | 0.38053 | 0.769 | 1.7149 | 0.5564 to 5.2851 |
| M64        | 0 (0) | 1 (1.1) | 0.29427 | 1.1 | – | – |
| R          | 8 (6.2) | 2 (2.2) | 0.16752 | 1.905 | 0.3409 | 0.0707 to 1.6447 |
| R2         | 0 (0) | 1 (1.1) | 0.29427 | 1.1 | – | – |
| R7         | 0 (0) | 3 (3.3) | 0.06928 | 3.3 | – | – |
| R30        | 2 (1.6) | 0 (0) | 0.2059 | 1.6 | – | – |
| R31        | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |
| R5         | 1 (0.8) | 4 (4.4) | 0.11443 | 2.492 | 5.907 | 0.6491 to 53.7591 |
| R6         | 4 (3.1) | 6 (6.7) | 0.25023 | 1.322 | 2.2143 | 0.6064 to 8.0855 |
| R8         | 1 (0.8) | 1 (1.1) | 0.82837 | 0.047 | 1.427 | 0.0881 to 23.1187 |
| T1         | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |
| T2         | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |
| U1         | 0 (0) | 1 (1.1) | 0.29427 | 1.1 | – | – |
| U2         | 11 (8.6) | 12 (13.3) | 0.31514 | 1.009 | 1.6364 | 0.6877 to 3.8937 |
| U5         | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |
| U7         | 8 (6.2) | 3 (3.3) | 0.4684 | 0.885 | 0.5172 | 0.1334 to 2.0057 |
| W          | 1 (0.8) | 0 (0) | 0.37109 | 0.8 | – | – |

\(^1\)Haplogroups observed = 39; \(^2\)Bonferroni correction for \(P\)-value = 0.05/39 = 0.00128.

doi:10.1371/journal.pone.0040668.t003

Figure S1 Somatic mtDNA mutations observed in tRNA genes of endometriosis patients. (A) The G5821A mutation in tRNA^Cys\(^\gamma\); (B) The C15926T mutation in tRNA^Thr\(^\alpha\). (TIF)

Figure S2 Differential mutation load shown by ‘A13603G’ mutation of ND5 gene. (A) Homoplasmic wild allele: 13603A; (B) 48% heteroplasm: 13603A/G; (C) 54% heteroplasm: 13603A/G; (D) 57.1% heteroplasm: 13603A/G; (E) 62.5% heteroplasm: 13603A/G; (F) Homoplasmic mutant allele: 13603G. (TIF)
Table S2 mtDNA novel synonymous mutations observed in endometriosis patients.

(DOC)

Table S3 mtDNA novel non-protein coding region mutations observed in endometriosis patients.

(DOC)

References

1. Eskinazi B, Warner ML (1997) Epidemiology of endometriosis. Obstet Gynecol Clin 24: 235–258.
2. Spuijbroek MD, Duselmann GA, Menheere PP, Evers JL (1992) Early endometriosis invades the extracellular matrix. Fertil Steril 58: 929–933.
3. Giudice LC, Kao LC (2004) Endometriosis. Lancet 364: 1709–1709.
4. Govatati S, Chakravarty B, Deenadayal M, Kodali VI, Latha M, et al. (2012) p53 and risk of endometriosis in Indian women. Genet Test Mol Biomarkers 16(8): 1–9.
5. Govatati S, Tangadu NK, Deenadayal M, Chakravarty B, Shivaji S, et al. (2012) Aconcavan Ercardacion single nucleotide polymorphisms with the increased risk of Endometriosis in Indian women. Mol Hum Reprod 18(5): 280–287.
6. Bhanoo M, Kameshwar DB, Zondervan KT, Deenadayal M, Kennedy S, et al. (2008) The endothelial nitric oxide synthase Gn209Aap polymorphism is not a risk factor for endometriosis in South Indian women. J Obstet Gynecol Reprod Biol 139: 53–58.
7. Bhanoo M, Deenadayal M, Kennedy S, Shivaji S (2007) The G2986A 3′-untranslated region polymorphism of the signal transducer and activator of transcription 6 gene is associated with endometriosis in South Indian women. Hum Reprod 22: 1026–1030.
8. Bhanoo M, Arvind Babu K, Pavankumar Reddy NG, Lakshmi Rao K, Zondervan KT, et al. (2005) The vascular endothelial growth factor (VIGF) +105G>C 3′-untranslated region polymorphism and increased risk of endometriosis in South Indian women: a case control study. Hum Reprod 20: 1844–1849.
9. Bhanoo M, Babu KA, Deenadayal M, Kennedy S, Shivaji S (2005) The interleukin-6 = 174G>C promoter polymorphism is not associated with endometriosis in South Indian women. J Soc Gynecol Investig 12: 96–100.
10. Tempfer CB, Simon M, Destenaves B, Fauser BCJM (2005) Functional genetic polymorphisms and female reproductive disorders: Part II—endometriosis. Hum Reprod Update 11: 97–118.
11. Koko CA, Demir-Wuisten AV, Groothuis PG, Duselmann GAJ, de Goeij APM, et al. (2006) Menstruum induces changes in mesothelial cell morphology. Gynecol Obstet Invest 52: 14–18.
12. Langendeschi AV, Canasas-Roux F, Donnez J (2002) oxidative stress and perinatal endometriosis. Fertil Steril 77: 861–870.
13. Onur-Iyyidogan Y, Kocak H, Gurdol F, Buyru F (2004) Indices of oxidative stress in endometriosis. J Reprod Med 49(10): 866–870.
14. Szczepanska M, Kozlik J, Skrzypczak J, Mikolajczyk M (2003) Oxidative stress may be a piece in the endometriosis puzzle. Fertil Steril 79: 1288–1293.
15. Ngy C, Chereau C, Nicco C, Well B, Chapron C, et al. (2009) Reactive Oxygen Species Controls Endometriosis Progression. Am J Physiol 297: 225–234.
16. Ishikawa K, Takenaga K, Akinoto M, Koshikawa N, Yamaguchi A, et al. (2008) ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. Science 320: 661–664.
17. Chan DC (2006) Mitochondria: dynamic organelles in disease, aging, and development. Cell 125: 1241–1252.
18. Anderson S, Bankier AT, Barrett BG, De Bruijn MHL, Coulson AR, et al. (1981) Sequence and organization of the human mitochondrial genome. Nature 290: 457–465.
19. Maynard S, Schurman SH, Barocca c de Souza-Pinto NC, Bohr VA (2009) Base excision repair of oxidative DNA damage and association with cancer and aging. Carcinogenesis 30: 2–10.
20. Rani DS, Dhanapalan P, Nallari P, Govindaraj P, Singh I, et al. (2010) Mitochondrial DNA haplogroup ‘R’ is associated with Noonan syndrome of South India. Mitochondrion 10: 166–173.
21. Fang H, Shen L, Chen T, He J, Ding Z, et al. (2010) Cancer type-specific modulation of mitochondrial haplogroups in breast, colorectal and thyroid cancer. BMC Cancer 10: 421.
22. Chatterjee A, Mantho E, Sidransky D (2006) Mitochondrial DNA mutations in human cancer. Oncogene 25: 4663–4674.
23. Revised American Society for Reproductive Medicine classification of endometriosis (1997) Fertil Steril 67: 817–821.
24. Zondervan KT, Cardon LR, Kennedy SH (2002) What makes a good case-control study? Design issues for complex genetic traits such as endometriosis. Hum Reprod 17: 1415–1423.
25. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16: 1215.

Acknowledgments

Suresh Govatati would like to thank Council of Scientific and Industrial Research (CSIR), India for JRF (NET) and SRF (NET).

Author Contributions

Conceived and designed the experiments: SS MB SG. Performed the experiments: SG NRT. Analyzed the data: SG SP. Contributed reagents/materials/analysis tools: VLK MD VS. Wrote the paper: SG MB SS.
50. Sadikovic B, Wang J, El-Hattab A, Landsverk M, Douglas G, et al. (2010) Sequence Homology at the Breakpoint and Clinical Phenotype of Mitochondrial DNA Deletion Syndromes. PLoS ONE 5: e15687.

51. Holt IJ, Lorimer HE, Jacobs HT (2000) Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. Cell 100: 515–524.

52. Krishnan KJ, Reeve AK, Samuels DC, Chinnery PF, Blackwood JK, et al. (2008) What causes mitochondrial DNA deletions in human cells? Nat Genet 40: 275–279.