



A Case-control Study on the Association of Mitochondrial Transcription Factor a Gene +35G/C Polymorphism and Mitochondrial DNA Copy Number with the Risk of Endometriosis in Indian Women

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Authors' contributions

This work was carried out in collaboration among all authors. Author HB Execution of experiments, analysis and interpretation of data, statistical analysis and drafting of manuscript. Author TVR Acquisition of data. Author SG Acquisition of data. Author SS Data analysis. Author MD Acquisition of data. Author SS Analysis and interpretation of data, drafting of manuscript. Author MB Conception & design of study, analysis and interpretation of data, statistical analyses, drafting of manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The Mitochondrial transcription factor A (TFAM) and mitochondrial (mt) DNA copy number variations are known to contribute in disease development. Genetic factors play an important role in the development of endometriosis. Therefore, this case-control study aimed to analyze the association of TFAM+35G/C polymorphism and mitochondrial copy number with the risk of endometriosis in Indian women.

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Study Design: This study was carried out on 418 subjects including 200 endometriosis cases and 218 controls.

Methodology: Genotyping of TFAM +35G/C polymorphism (rs1937) was carried out by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Quantification of mtDNA copy number was carried out using a real time quantitative polymerase chain reaction (qRT-PCR).

Place and Duration of Study: Department of Biochemistry, Osmania University, 2014 to 2020.

Results: TFAM genotype as well as allele distributions were all in Hardy-Weinberg equilibrium. The results indicated a significant reduction of GG genotype frequency ($P=0.009$), high 'C' allele frequency ($P=0.017$) and significantly decreased mtDNA copy number in endometriosis cases compared to controls ($P= 0.0001$).

Conclusion: Present study revealed a statistically significant association of decreased GG genotype of TFAM +35G/C polymorphism and mtDNA copy number with the risk of developing endometriosis in Indian women.

Keywords: Endometriosis; TFAM; mitochondrial copy number; Indian women.

1. INTRODUCTION

Endometriosis is a chronic gynecological disease characterized by the growth of ectopic endometrial tissue outside the uterine cavity, ovaries, fallopian tubes, intestine and even on the bladder, with clinical presentations of dysmenorrhea, dyspareunia, dyschezia, subfertility and sometimes diarrhea [1,2]. It is an estrogen dependent disease and has been associated with chronic inflammation and infertility, which negatively affect the quality of life [3]. It affects 5 to 10% of reproductive aged women globally [4]. Several epidemiological studies have been identified various risk factors for endometriosis, but the etiology and pathogenesis remains unclear [5,6].

Mitochondrion is majorly involved in energy production via the electron-transport chain, oxidative phosphorylation system (OXPHOS) and required for normal cellular physiology [7]. Mitochondrial transcription factor A (TFAM) plays a key role in replication and transcription of mtDNA. It is also important for the maintenance of mtDNA integrity and stability, thus mirrors the changing levels of mtDNA in the cell [8,9,10]. Mitochondrial DNA copy number is a promising biomarker of mitochondrial function and altered levels are associated with the development of human pathologies like cancer including endometrial cancer [11]. The regulation of mtDNA copy number is complex and depends on several factors, including mtDNA mutations and genetic variations within the genes involved in mtDNA replication and transcription. Consistent with its key role in packaging mtDNA into nucleoids, TFAM serves as a key regulator of mtDNA copy number [12].

Endometriosis is a benign but invasive gynecological disease with increased levels of reactive oxygen species (ROS) and oxidative stress. Mitochondria as a major source of ROS generation may be implicated in the pathogenesis of endometriosis. TFAM +35G/C is a missense mutation leading to an amino acid substitution from serine to threonine at 12th position (Ser12Thr) [13]. This mutation may interfere in the architectural binding capability of TFAM affecting overall mitochondrial functioning leading to disease susceptibility. Several studies showed an association of TFAM polymorphism with the progression of various diseases like Alzheimer's, Huntington's, diabetic neuropathy and ovarian cancer [14,15,16,17], however no reports are documented in endometriosis. Ekstrand et al. has elucidated the novel role of TFAM in direct regulation of mtDNA copy number in mammals [8] and a significant increase in elevated mtDNA copy number was demonstrated in endometrial adenocarcinoma [18].

To contribute to the analysis of the genetic background of endometriosis, we evaluated for the first time the frequency of TFAM +35G/C polymorphism and mitochondrial copy number in Indian women with and without endometriosis.

2. METHODOLOGY

2.1 Subjects

A total of four hundred eighteen women of 20–40 years of age were involved in this case–control study. All the subjects were randomly selected pre-menopausal unrelated Indian women, recruited at the Infertility Institute and Research Center (IIRC), Hyderabad, India, and Institute of Reproductive Medicine (IRM), Kolkata, India. Written informed consent was given by all

participants in the study. The Institutional Review Board of the Center for Cellular and Molecular Biology (CCMB), Hyderabad, approved the study (IEC/CCMB26/2008/6th February, 2008).

All cases (n=200) were with moderate-severe (III–IV) endometriosis staged using the revised American Fertility Society classification system (rAFS). All women had a trans-vaginal ultrasound scan (TVS) performed at screening and followed by a laparoscopy to confirm the diagnosis (rAFS III = 86; IV = 114). Their mean age \pm SD was 26.5 \pm 5.5 (range: 20–40) years. All the patients complained of dysmenorrhoea (mild, 45%; moderate, 31% and severe, 24%) and 75% had dyspareunia. Most of women (98.1%) were infertile (primary, 82% and secondary, 18%). All cases exhibited various forms of endometriosis such as adhesions, peritoneal lesions and endometriomas. Women with fibroids, ovarian cysts, adenomyosis, stage I and II endometriosis, and ovarian cancer were excluded from the study. The main aim was to focus on patients with more severe forms of endometriosis (Stage III and IV), because the more severe forms comprise ovarian cystic disease, which has almost certainly a different etiology to peritoneal forms, and the diagnosis can be usually unequivocal, which isn't the case for Stage I and II.

The control group was composed of two hundred and eighteen (n=218) healthy women with no evidence of endometriosis on TVS and had an equal opportunity to be identified as cases, thereby meeting the criteria for appropriate controls set by Zondervan et. al. [19]. Their mean age \pm SD was 27.9 \pm 4.95 (range: 22–40).

Extraction of DNA

Five milliliters of Peripheral blood samples were obtained from all subjects in EDTA vacutainers and preserved at -20° C for further use. Extraction of genomic DNA was carried out from 1 ml of EDTA anti-coagulated whole blood as per protocols established earlier [20]. The results were analyzed in a blinded fashion.

2.2 Determination of TFAM Genotype

Genotyping of TFAM +35G/C polymorphism (rs1937) was carried out by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis as described earlier [21,13]. PCRs were carried out in a total volume of 25 μ l, containing, 2–6 pmole of each primer, 50 ng genomic DNA, 0.25 U of Amplitaq

DNA polymerase (Perkin Elmer, Foster City, USA) and 1X Taq polymerase buffer (1.5 mM MgCl₂). The primers used were 5'-CCCCGCCCCCATCTACCGA-3' (forward), and 5'-GACGTCCTGGGCCCTGCTG -3' (reverse). PCR amplification was carried out in a programmable thermal cycler gradient PCR machine (Eppendorf AG, Hamburg, Germany). The PCR amplification run was performed with initial denaturation, at 96°C (5 min) followed by 35 cycles of denaturation at 94°C (1min), annealing at 59°C (50 sec), extension at 72°C (1 min) and final extension at 72°C (10 min). 326 bp PCR product of was analysed by 1.5 % agarose gel stained with ethidium bromide and then subjected to RFLP analysis. For the RFLP analysis, 20 μ L of PCR product was digested with 1 unit of restriction enzyme *DdeI* (New England Biolabs) at 37°C for 3hours. DNA fragments were electrophoresed through a 3% agarose gel and stained with ethidium bromide. The results were confirmed by PCR–DNA sequencing with a Taq-Dye deoxy-terminator cycle sequencing kit (Applied BioSystems, Foster City, CA) using an automated ABI 3770 DNA sequencer (Applied BioSystems). Genotype calling was performed using the software Chromas V0.2 (Technelysium Pty Ltd, South Brisbane, Australia).

2.3 Quantification of Mitochondrial DNA Copy Number by Real Time PCR

Quantification of mtDNA copy number was carried out using a real time quantitative polymerase chain reaction (qRT-PCR) using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) as described earlier [22]. To analyze the nuclear DNA, forward primer 5'-GCCAATCTCAGTCCCTTCCC-3' and the reverse primer 5'-TCGGTGAGGATCTTCATGAGGTA-3' (GAPDH gene) were used to amplify 177-bp product. For analysis of the mtDNA, forward primer 5'-GGGCTACTACAACCCTTCGCT-3' and the reverse primer 5'-GAGGCCTAGGTTGAGGTTGAC-3' (ND1 gene) were used to amplify 153-bp product. The DNA (10 ng) was mixed with 10 μ l SYBR Green I Master Mix (TaKaRa, USA) that contained 10 pmol of forward and reverse primer in a final volume of 20 μ l. The qRT-PCR conditions consisted of initiation at 50°C (2 min) , 95°C (10 sec) followed by 40 cycles of denaturation at 95°C (5 sec), annealing at 59°C (35 sec), and extension at 72 °C (1 min). The threshold cycle

number (Ct) value of the ND1 gene and GAPDH gene were determined for each individual quantitative PCR run, each measurement was performed at least twice and normalized in each experiment against control DNA sample. The Ct value difference used to quantify mtDNA copy number, relative to the GAPDH gene were calculated as follows: Relative copy number (Rc) = $2^{\Delta Ct}$, where ΔCt is the $Ct_{GAPDH} - Ct_{ND1}$ [22].

2.4 Statistical Analysis

Statistical analysis was executed using SPSS statistical package (V 11.0). The genotype distribution among subjects were tested for Hardy-Weinberg equilibrium (HWE). The allele frequencies and genotype distributions of cases and controls were analyzed using the Fisher's exact test. The values of odds ratio and 95% confidence interval (CI) were calculated using online Vassar Stats Calculator (<http://www.faculty.vassar.edu/lowry/VassarStats.html>). The values of $P < 0.05$ was considered as statistically significant for all the statistical tests.

3. RESULTS

3.1 TFAM +35G/C SNP

All subjects (n=418) were successfully genotyped. Amongst both cases and controls, the TFAM genotype as well as allele distributions were all in Hardy-Weinberg equilibrium. In RFLP analysis the homozygous 'G' yielded two bands (200 bp and 126bp) and the heterozygote G/C generated three bands (200, 126, and 326 bp), the sequencing analysis of +35G/C SNP is shown in Fig. 1. In the present study, no C/C homozygote was identified. The genotype and

allele distributions of the TFAM +35G/C SNP revealed significant differences between cases and controls (Table 1). There was a significant reduction of GG genotype frequency ($P=0.009$) in cases compared to controls, whereas the 'C' allele frequency ($P=0.017$) was significantly higher in cases than in the controls.

3.2 Decreased Relative mtDNA Copy Number in Endometriosis

There are 2-10 thousand copies of mtDNA in a single cell [22]. The results revealed significantly decreased mtDNA copy number in endometriosis cases compared to controls ($P= 0.0001$). The mean mtDNA copy number was 1.430 ± 0.403 in controls and 1.100 ± 0.479 in cases (Fig. 2).

4. DISCUSSION

In the present study, we evaluated, for the first time a significant association of TFAM +35G/C polymorphism and reduced mtDNA copy number with the endometriosis risk in Indian women. We observed high frequency of GG genotype, and 'G' allele in controls when compared to cases which may be protective allele against the development of endometriosis. In this study we also did not observe CC genotype in our population similar to previous study carried out by [23] in European population. This could be due to very low frequency of minor allele 'C' (<https://www.ncbi.nlm.nih.gov/snp/rs1937>, ALFA Allele Frequency, Global: G=0.90404,C=0.09596; South Asian: G=0.93, C=0.07). Our data also showed significantly decreased mtDNA copy number in endometriosis cases compared to controls.

Table 1. Genotype and allele frequencies of the TFAM+35G/C polymorphism in endometriosis cases and controls

Genotypes/ Alleles	Cases(Freq.) (n=200)	Controls(Freq.) (n = 218)	P-value	Odds ratio	95% CI
GG	129 (0.645)	166(0.7615)	0.009 ^a	Reference	Reference
GC	71(0.355)	52(0.2386)		0.5691	0.371-0.870
CC	0(0)	0(0)			
Alleles					
G	329(0.8225)	384(0.8808)	0.017 ^b	Reference	Reference
C	71(0.1775)	52(0.1193)		0.627	0.426 - 0.923

CI, Confidence Interval

^aFisher's exact test (3 X 2 table at 2 df), $P < 0.05$

^bFisher's exact test (2 X 2 table at 1 df), $P < 0.05$

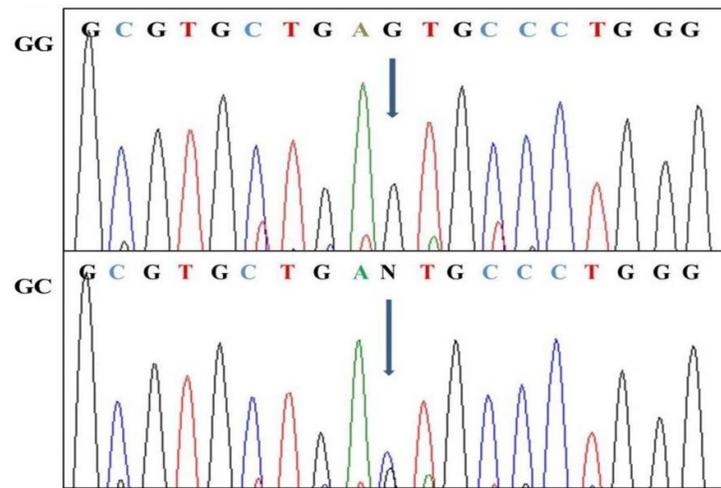


Fig. 1. Genotyping of the TFAM+35G/C polymorphism by sequence analysis of the PCR amplified product using a forward primer. The G/G homozygotes manifested as a single peak, whereas the heterozygote G/C as a double peak

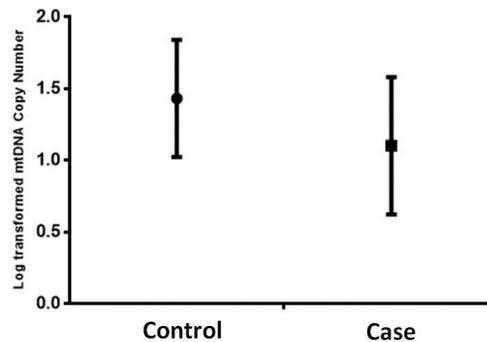


Fig. 2. Log-transformed mtDNA copy number in endometriosis cases and controls

TFAM, a nuclear coded transcription factor is a master regulator of oxidative phosphorylation and mammalian mitochondrial biogenesis. Polymorphisms of TFAM gene may lead to aberrant estrogen signaling which in turn alter its target gene BCL2L1 expression, which is an important mediator of programmed cell death [16,24] reported a high frequency of TFAM truncated mutations that led to low level of TFAM protein and reduced mtDNA copy number in cells which in turn affected apoptotic signalling in endometriotic cells. The TFAM +35G/C polymorphism results in the single amino acid change from serine to threonine in the 12th codon [25] interfering in the architectural binding capability of TFAM to mitochondrial D-loop and consequently may affect overall mitochondrial functioning [26]. TFAM binds to mtDNA in sequence-specific and non-specific manner, it is

possible that either modes of mtDNA binding contributes to the regulation of mtDNA copy number [27,28]. In the cultured cells knock down of TFAM gene has been shown to cause mtDNA depletion [29] whereas over expression of TFAM showed an increase in mtDNA copy number [30,31] showed that TFAM stabilizes and enhances mtDNA transcription in a promoter-specific fashion in the presence of mitochondrial RNA polymerase indicating its key role in maintenance of mtDNA.

Altered estrogen signaling is implicated in the development of several diseases, including cancer, cardiovascular, metabolic, and endometriosis [32,33,34]. TFAM is a structural and functional homolog of HMGB1 and has been implicated as a key contributory factor for the initiation of inflammatory responses [35,36] and

inflammation is a key feature of endometriosis. It has been reported that TFAM can induce inflammation either alone or in combination with other proinflammatory molecules such as TNF α , NF κ B through the activation of PI3K pathway [37]. Any alteration in TFAM gene may influence inflammatory signaling cascade in endometriotic lesions. Therefore, with intricate association of TFAM with factors of endometriosis it is possible that TFAM+35G/C polymorphism is involved in reduction of mtDNA copy number leading to the outgrowth of ectopic endometrial tissues.

5. CONCLUSION

In conclusion, this study represents the first evidence that the TFAM +35G/C polymorphism and reduced mtDNA copy number is associated with the risk of developing endometriosis in Indian women. However further study with large sample size is necessary to understand the potential link between TFAM, mtDNA copy number and endometriosis. Our results can be a guide for more detailed statistical evaluations, functional studies and diagnostic and therapeutic interventions.

CONSENT

Informed written consent was obtained from all subjects participating in the study.

ETHICAL APPROVAL

The Institutional Review Board of the Center for Cellular and Molecular Biology (CCMB), Hyderabad, approved the study (IEC/CCMB26/2008/6th February, 2008).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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