ANALYSIS OF mt-DNA ENCODED tRNA^{Cys}GENE MUTATIONS IN POLYCYSTIC OVARIAN SYNDROME PATIENTS OF DISTRICT ABBOTABAD

Uzma Rashid[1], Kehkashan Akbar[2], Hania Akbar[3], Bibi Hajira[4], Sadaf Bibi[5], Sahib Gul Afridi[6], Hamid Ali[5], Sara Rashid[7]

[1]Department of Zoology, Hazara University, Mansehra Pakistan
[2]Department of Biochemistry, Abbottabad International Medical College, Abbottabad
[3]Senior Registrar Gastroenterology, District Hospital Abbottabad Pakistan
[4]Department Of Biochemistry, Women Medical College, Abbottabad Pakistan
[5]Department Of Biotechnology and Genetic Engineering, Hazara University Mansehra, Pakistan
[6]Department of Biochemistry, Abdul Wali Khan University Mardan, Pakistan
[7]Ayub Medical Complex Abbottabad, Pakistan

Corresponding Author:
Kehkashan Akbar
Department of Biochemistry, Abbottabad International Medical College,Abbottabad

ABSTRACT

Polycystic ovarian syndrome is a hormonal disorder which is very usual in women of reproductive age. The etiology of this disorder is due to environmental and genetic factors. The genetic factors which cause PCOS are mutations in Nuclear DNA, mt-mRNAs and mt-tRNAs. The highly conserved sequences of mt-tRNA genes are more vulnerable to such mutations. Current research was conducted to evaluate the mtDNA encoded tRNA^{Cys} gene in women suffering from PCOS. The information about patient's weight, age, on set of disease etc was also recorded after getting informed consent. From 40 patients of District Abbottabad, about 10ml of saliva was collected in sterilized cups. The genomic DNA was extracted from collected saliva samples using phenol chloroform method. The PCR amplification of mt-tRNA^{Cys}gene was carried out and among them, 11 samples were redirected to TSINGKE Biological Technology (Cheng Du, China) for sequencing. The sequence results were compared with revised Cambridge Reference Sequence (rCRS) Accession Number (NC_012920.1) available at NCBI gene bank. No mutation was detected in either of the sequenced samples. It could be concluded that PCOS in subjects studied might not be due to mtDNA encoded tRNA^{Cys} gene mutation. However, it is therefore recommended that whole mitogenome should be sequenced in large sample set to evaluate the association of mtDNA mutations with PCOS.

INTRODUCTION

Polycystic ovarian syndrome is a heterogeneous condition with unknown etiology characterized by anovulatory infertility and hyperandrogenism and occurs in association with insulin

resistance causing compensatory hyperinsulinemia, which stimulates production of ovarian androgen (Ehrmanne*et al*., 2005).

PCOS is sometimes called Stein–Laventhal Syndrome. As stated by European Society for Human Reproduction and Embryology (ESHRE) and American Society for Reproductive Medicine (ASRM) (ESHRE/ASRM), existence of any two of the underlying 3 criteria can be used for diagnosis (also called Rotterdam method):

- Oligo and/or an ovulation
- Clinical or biochemical proof of androgen excess
- On ultra sound scan multiple cysts are found in ovaries

The clinical presentation of PCOS varies greatly but menstrual disturbance often gained much importance. Where menstrual disturbance includes oligomenorrhea, amenorrheaand sustained irregular menstrual bleeding (Farquhar, 2007). About 90-95% of anovulatory females visiting to infertility health centres are suffered with polycystic ovarian syndrome. The number of primordial follicles in PCOS women is usual but the amount of primary and secondary follicles notably grow. On reaching diameter of 4-8mm, disturbing factors stop normal follicular development. The main hindrance in ovulation is the absence of dominant follicle (Teede*et al*., 2010; Brassard *et al*., 2008). Moreover, the incidence of miscarriages is 42-73% in PCOS patients (Glueck*et al*., 2001). It is anticipated that more than 80% of PCOS women show signs of high level of androgens (Aziz *et al*., 2006). The most general clinical appearance of elevated androgen is hirsutism (Fauser*et al*., 2012). Acne is also regarded as another important clue of hyperandrogenism but it is not as much precise as hirsutism and is not very common in this disorder. (Wijevaratne, 2002). Acne, hirsutism, androgenic alopecia and 66% females with male characteristics are the clinical signs and symptoms of elevated androgens (Naderi*et al*., 2011).

Morphology of Polycystic ovaries is defined as the occurrence of 12 or more follicles in each ovary measuring 2-9mm in diameter and/or increased ovarian volume (>10ml) (Balen*et al*., 2003).

Psychological gradients of PCOS are depression and anxiety (Pastore*et al.*, 2011). PCOS has become the major reason of infertility in as it the most common gynecological endocrine disorder of reproductive age which affects about 6-10% of women during their reproductive years (Spritzer, 2002).Studies have shown that hot spots for mutations correlated with PCOS are mitochondrial OXPHOS complexes and also various mitochondrial transfer RNA mutations. Such tRNA mutations included $tRNA^{Gln}$T4395C, $tRNA^{Asp}$ A7543G, $tRNA^{Lys}$ A8343G, $tRNA^{Cys}$ G5821A, $tRNA^{Arg}$ T10454C and $tRNA^{Glu}$A14693G. Such mutations in mt-tRNA were found at highly conserved nucleotides which are said to be responsible to cause structural and functional changes and causes malfunctioning of mt-tRNA metabolism (Ding *et al*., 2016).

In the mt-$tRNA^{Cys}$gene, mutation G5821A occurred in the highly conserved nucleotide sequence of anticodon stem. This mutation occurred at position 6 in the anticodon of gene that encodes mt-$tRNA^{Cys.}$ As a result of lack of mt-$tRNA^{Cys}$ problems occur in the production of proteins of mitochondria. My research was carried out for the investigation of mt-$tRNA^{Cys}$ mutations in polycystic ovarian syndrome positive subjects from District Abbottabad.

MATERIAL AND METHODS

Our study area was Abbottabad District.Various health centers and clinics of District Abbottabad were visited where PCOS patients were diagnosed using Rotterdam diagnostic method.To assure the non-existence of other syndromes like thyroid disease, hyperprolactinemia,adrenal and deficiency of 21 hydroxylase, an in-depth and detailed examination was done.For the validity of examination further medical procedures such as laboratory tests of metabolic syndrome and routine ultrasonography were performed. Informed consent was also taken from the patients before sample collection.In order to collect saliva from our selected subjects, brushing of teeth was done of those patients and for this they were provided with tooth brushes

and tooth paste. Because of brushing the rate of contamination reduces. Then 1 ml of 5 % sucrose solution were given to respective subjects and were directed to place this sample in their mouth for 3-5 minutes and at the same time they were asked for rubbing their tongue against their cheeks for sufficient production of saliva. Subjects were then asked to spit out spittle in sterilized labeled cups after 3-5 minutes. Afterwards, for extraction of DNA sterilized labeled cups were placed in freezer at -20^0 C in the laboratory. Using Aider and Line method standard DNA extraction procedure was carried out from subject's epithelial cells found in spittle:By using pipette, 1.5 ml eppendorf tube was filled with 1ml of buccal wash and spun at high speed for 1 min. Supernatant was disposed to get the pellet of epithelial cells. Procedure was repeated twice to get enough cells for DNA isolation.Then in each eppendorf tube, 150ul of lysis solution carrying Beta-mercaptoethanol and proteinase K, was added and was inverted 5-10 times for complete mixing. Tubes were then incubated at 56 ^{0}C for 50 minutes in incubator. In incubated samples, about 200ul of iso-amyl alcohol or also known as PCL was added after incubation. Then for 10 minutes they were placed at room temperature. The tubes were then overturned for 10 times. For the formation of three layers (in which DNA was in upper layer while lower layers hold proteins and phenol contents) the tubes were spun at 8000rpm for 10 minutes. In the next step the upper DNA layer was transferred into the new labeled tubes and afterwards 500ul of ice-cold isopropanol was added.After that, for whole night these samples were placed in freezer at -20^0C.Then for 20 minutes tubes were centrifuged at 8000rpm and supernatant was disposed off with much attention and DNA pallet was isolated and washed with 500ul ethanol (70%ethanol).The tubes were overturned 10 times or vortexed and again centrifugation was carried out at 8000rpm for 5 minutes. After that supernatant was thrown away and pallet was completely dried by placing it in incubator or air dried.Then addition of 30-40ul of molecular grade

water for complete dissolution of pallet was done.

Agarose gel electrophoresis was performed after DNA isolation to verify the excellence of DNA.

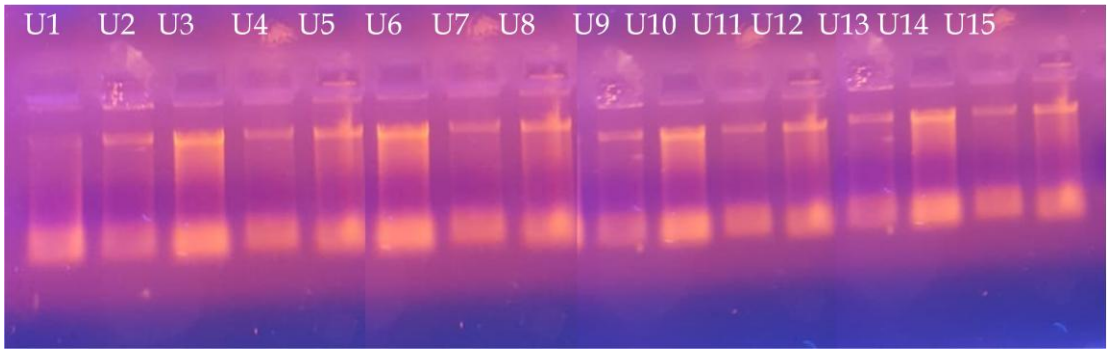

Figure: 1.Quantification of extracted DNA of 15 samples

The genomic DNA which was extracted from buccal wash of PCOS positive subjects was then subjected to the amplification of mitochondrial $tRNA^{Cys}$ gene through PCR. For the amplification of target DNA, the primers were designed using primer 3 software. Primer sequence is given below.

F- primer	5′ CTAACCGGCTTTTTGCCC 3′
R- primer	5′ ACCTAGAAGGTTGCCTGGCT3′

Table 3.1: Reagents' list for single PCR reaction

S.No	Reagents	Volume per reaction
i.	DdH2O	16.2µl
ii.	N Taq Buffer	2.5µl
iii.	dNTPs	1µl
iv.	Reverse primer RP	1µl
v.	Forward primer FP	1µl
vi.	MgCl2	1ul

vii.	DNA template	2µl
viii.	DNA polymerase	0.3µl
	Total volume	25µl

PCR amplification for mt-tRNA^{cys}was carried out in DNA thermal cycler set for pre denaturation at 95^{o}C for 5 min, then denaturation was done at 95^{0}C for 30 sec. This was followed by primer annealing at95^{o}C to 53^{o}C for 1 min. Then extension was carried out for 45 sec at 72^{o}C.Then at 72^{o}C the final elongation step was carried out for 7 minutes and in the end, temperature was dropped to 10^{o}C for infinity. Amplified products were resolved by 1% agarose gel run in 600ul50XTAE buffer. The amplified products were observed under UV light after staining with ethidium bromide.

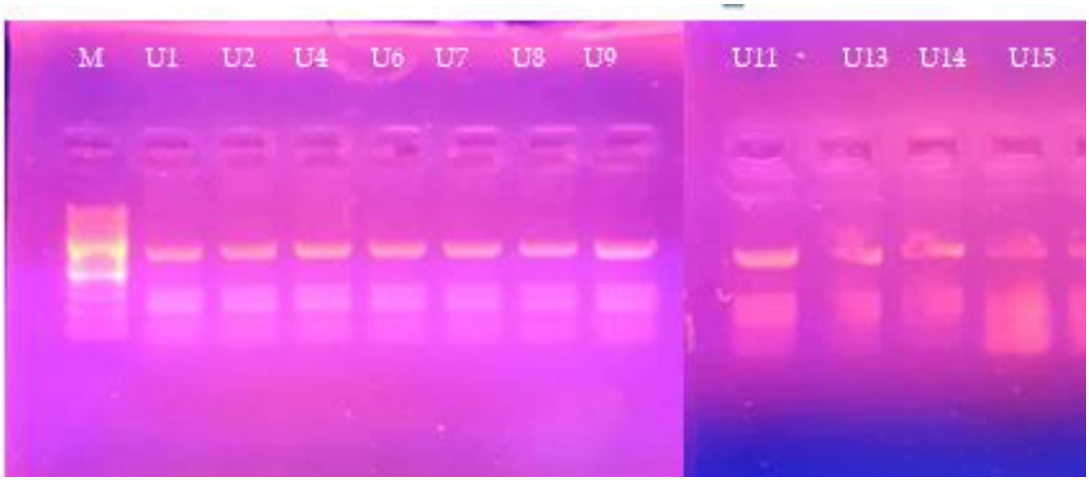

Figure: 2. The electropherogram of amplified PCR product of mt-DNA encoded tRNA^{Cys} gene of 11 samples

For sequence analysis, the amplified PCR products were sent to TSINGKE Biological Technology (Cheng Du, China). After that retrieved sequences were compared with revised Cambridge Reference Sequence accession No NC-0129920.1 for mutation detection.

Results and discussion:

Analysis of mtDNA gene was conducted for the identification of tRNA^{cys}gene. Among the 40 samples

none of the sample showed the mutation in mttRNA^{cys}gene.

Conclusion:

In the present study 40 samples which were selected for the identification of mutation in mt tRNA^{Cys} lacks any such mutation for patients of district Abbottabad . On the basis of our findings, it is strongly recommended to analyze polycystic ovary syndrome patients on large scale for tRNA^{Cys} gene mutations before reaching to the ultimate conclusion.

The mt-DNA mutations are linked to variety of different diseases. So, for the determination of exact etiology of polycystic ovary syndrome it is recommended to sequence the entire mitochondrial genome.

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