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Original Article

Analysis of mitochondrial DNA mutations in Pakistani population diagnosed with cardiovascular diseases

Análise de mutações do DNA mitocondrial na população paquistanesa diagnosticada com doenças cardiovasculares

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Abstract

Heart and blood vessel disorders, such as coronary heart disease, brain vessel disease, rheumatic heart disease, and others, are together referred to as cardiovascular disease (CVD). In this study, we sought to determine how mitochondrial Leucine Transfer RNA genes and CVDs are related (MT-L1 and MT-L2). From CVD patients in Peshawar, a total of 27 saliva samples were taken. Leu-tRNA genes expressed by mitochondria were amplified using polymerase chain reaction after DNA was removed. Ten samples were sent for sequencing after PCR and gene cleaning. We obtained all of the sequenced results, which were subsequently aligned and evaluated against the mitochondrial revised Cambridge Reference Sequence (rCRS). However, in our sequenced samples, Leu-tRNA MT-L1 and MT-L2 genes were determined to be unaltered. Thus, it is suggested that a large population be taken into account while screening for mutations in the mitochondrial encoded Leu-tRNA MT-L1 and MT-L2 genes of cardiac patients in areas of Pakistan. Additionally, it is recommended that patients with cardiac problems should also have other mitochondrial encoded genes checked for potential mutations. This could result in the identification of genetic markers that could be used for early CVD screening in Pakistan.

Keywords: mitochondria, mtDNA, Leu-tRNA genes, mutation, sequencing, CVD.

Resumo

Distúrbios do coração e dos vasos sanguíneos, como doença cardíaca coronária, doença dos vasos cerebrais, doença cardíaca reumática entre outros, são referidos juntos como doença cardiovascular (DCV). Neste estudo, procuramos determinar como os genes mitocondriais do RNA de transferência de leucina e as DCVs estão relacionados (MT-L1 e MT-L2). Foi coletado um total de 27 amostras de saliva de pacientes com DCV em Peshawar. Genes de Leu-tRNA expressos por mitocôndrias foram amplificados usando reação em cadeia da polimerase (PCR) após a remoção do DNA. Dez amostras foram enviadas para sequenciamento após PCR e limpeza gênica. Obtivemos todos os resultados sequenciados, que foram posteriormente alinhados e avaliados em comparação com a Sequência de Referência de Cambridge revisada (rCRS). No entanto, em nossas amostras sequenciadas, os genes Leu-tRNA MT-L1 e MT-L2 foram determinados como inalterados. Assim, sugere-se que uma grande população seja levada em consideração durante a triagem de mutações nos genes Leu-tRNA MT-L1 e MT-L2 mitocondriais codificados de pacientes cardíacos em áreas do Paquistão. Além disso, recomenda-se que outros genes mitocondriais codificados de pacientes com problemas cardíacos também sejam verificados quanto a possíveis mutações. Isso pode resultar na identificação de marcadores genéticos que podem ser usados para triagem precoce de DCV no Paquistão.

Palavras-chave: mitocôndrias, mtDNA, genes Leu-tRNA, mutação, sequenciamento, DCV.

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1. Introduction

The sole function of mitochondria is to transform oxidative phosphorylation energy into adenosine triphosphate (ATP), a "fuel" that may be utilized to catalyze cellular functions. Mitochondria are significant organelles. Other crucial functions include calcium storage, metabolism and apoptosis control, and cell signaling (Govers et al., 2021). All eukaryotic cells, except adult red blood cells, include mitochondria, hence any organ has the potential to be impacted by mitochondrial malfunction, which can show a wide range of symptoms (Munnich et al., 1992). "Mitochondrial cytopathies" refers to a group of abnormalities affecting mitochondrial function. The fundamental causes of these cytopathies are still not fully known, despite advances in our knowledge of the mitochondria and their DNA. Their estimated prevalence is 1 in 5000, however, this number may be underestimated because many patients with mitochondrial cytopathy brought on by a mutation in the mitochondrial DNA (mtDNA) may be evading identification (Govers et al., 2021).

Though all organs can be affected, mitochondrial diseases are most commonly known to impact the muscles and neurological system. Heart and blood vessel disorders, such as coronary heart disease, brain vessel disease, rheumatic heart disease, and others, are together referred to as CVDs (Khan et al., 2022). 17.9 million people worldwide die from CVDS every year, accounting for about 31 percent of all deaths worldwide and costing the global economy about $1 trillion annually. It is noteworthy that fatty deposits on the inner walls of these blood vessels account for 85% of all CVD deaths from myocardial infarction and strokes, which primarily obstruct the blood supply to the heart and brain (Khan et al., 2022). Numerous studies found that mitochondrial DNA mutations have a significant role in the onset of CVDs in addition to the conventional risk factors.

Leu-tRNA (UUR) gene mutations in mtDNA most typically display an A-to-G transition at position 3243, which has been found to affect the mitochondrial structure, methylation, amino-acylation, and Leu-tRNA codon recognition (Goto et al., 1990). About 80% of MELAS patients have this mutation, which can cause a variety of symptoms such as cardiomyopathy, deafness, intolerance to exercise, and diabetes (Uusimaa et al., 2007). Maternally inherited myopathy and cardiomyopathy (MIMyCa, recently described as a maternally inherited adult-onset syndrome) is clinically characterized by variable combinations of skeletal and heart muscle failure, and molecularly by the presence of a heteroplasmic point mutation in the mitochondrial DNA (mtDNA) Leu-tRNA (UUR) gene (Zeviani et al., 1991). An A-G transition at mtDNA nucleotide position 3260 makes the mutation potentially pathogen (Anderson et al., 1981). However, there are several ways in which the mitochondrial genome and its genetics are distinct from the nuclear genome and Mendelian genetics. Interesting biological and clinical ramifications stem from these disparities.

Although mtDNA mutations are frequently transmitted from the mother, they can sometimes happen spontaneously. Furthermore, several nuclear genes are necessary for the correct upkeep of mtDNA; mutations in these genes can consequently result in both quantitative (mtDNA depletion) and qualitative (mtDNA deletions) problems in mtDNA. Based on earlier research, we carried out the current investigation to examine the Leu-tRNA gene for mutation in patients with heart disease and to contrast our amplified sequences with previously published sequences.

2. Materials and Methods

2.1. Ethical approval

The ethical form was approved from the Ethics Committee of Hazara University Mansehra Pakistan. All Volunteers be informed from the research objectives and will be signed informed consent following the Declaration of Helsinki.

2.2. Collection of samples

Doctors at the hospital confirmed that all patients had CVD. After brushing their teeth, each person received a five percent sugar solution. Patients were instructed to wash their mouth for three to five minutes with the solution in it, move their tongue over all of their teeth and jaws, and then spit the fluid into sterile cups. Samples were spontaneously transported to the lab while being kept on ice and frozen at -20°C. Saliva was used to extract DNA, which was then stored in the molecular genetics lab at -20°C at the Hazara University in Mansehra.

2.3. DNA extraction

For extraction of mtDNA, 1ml saliva was used following Aider and Line., 2007 protocol with some modifications (Ait-Aider et al., 2007). Saliva was centrifuged for 2 minutes at 8000rpm to get a pallet from the saliva cells, the supernatant was a washout, and for extraction of DNA following steps were used. Lyses solution was added to each tube containing (3µL of BME, 0.5% SDS, 10 µL proteinase K) and vortexed with pellet until it dissolved. For one and half hours the tubes were kept incubated at 60°C. Every 30 minutes during the incubation period, the tubes were turned over.

On completion of incubation, 200 µL phenol plus chloroform was added, shacked well, and left for five minutes at room temperature. For 15 minutes again centrifugation was done at 8000rpms. We took the upper layer from the tube and put it in a new tube and wasted the old one. We added 300 µL of isopropanol and left it overnight for purification. To get the pellet centrifugation was carried out at 8000rpm for 20 minutes, and then discarded supernatant. For washing ethanol was added in an amount of 300µl-500µl and was shaken until clods were removed. Again centrifugation was done at 8000rpms and keeps it air-dried. After removal of full ethanol, we added double distal water to the pellet in the amount of 30-50µl.

2.4. Agarose gel electrophoresis of DNA samples

Agarose gel electrophoresis was used to find out the quality of obtained DNA.

2.5. Procedure for preparation of agarose

To analyze the obtained DNA, 0.5g of agarose was dissolved in 29.4ml double distal water. With 600µL of 50X Tris-acetate elution Buffers having 10ul ethidium bromide (EB) were taken,5 µl of extracted DNA and 2 µl of loading dye were loaded and run on the gel for thirty-minute at 60 volts, gel was photographed using ultraviolet rays gel documentation system.

2.6. Polymerase Chain Reaction (PCR)

Obtained DNA from the heart patient was processed with PCR to amplify the desired mtDNA Leu-tRNA genes. A total of 20 valume PCR reagents were used for one reaction to amplify MT_L1 Leucine (UUR) such as: Buffer D 2.5 µl, DNTPs Mixture2 mM 2.0 µl, MgCl2 25Mm 2.0 µl, Forward Primer 10 pM/ul 1.0 µl, Reverse Primer 10 pM/ul 1.0 µl, Taq Polymerase 5U\Pl 0.2 µl, DNA Template 2.0 µl, and Double distal water 9.3 µl.For amplification of Leu-tRNA *MT-L1, MT-L2* following primers were used; F 5'CAAATTCCTCCCTGTACGAAAGG 3' ; R 5' AATGAGGAGTAGGAGGTTGGCC 3'.

A total of 16 valume PCR reagents were used for one reaction to amplify *MT-L2 Leucine (CUN)* such as H_20 6.8 µl, DNTPs 2 µl, MgCl2 1.5 µl, Buffers 1.5 µl, Forward primer 1 µl, Reverse primer 1 µl, DNA polymerase 0.2 µl, and DNA templets 2 µl. A set of primers used for Leu-tRNA *(CUN)* F 5'TTTACCACAACACAATGGGG3' and 5' GCTCAGTTGCAGTTCGAGATA 3'.

2.7. PCR amplification condition for MT-L1

A total of 40 cycles of PCR started with a pre-PCR step at 95°C for 5 minutes. The 40 cycles of the first cyclic step were denaturation at 95°C for 30 seconds. The second cycling step was annealing at 50°C for 45 seconds and 3rd cyclic step was elongation at 72°Cfor 45 seconds and finally post PCR step at 72°Cfor five minutes as shown in Figure 1.

2.8. PCR amplification condition for MT-L2

A total of 40 cycles of PCR started with a pre-PCR step at 95°C for 5 minutes. The first cyclic step was denaturation at 94°C for 30 seconds. Following the cycling, a step was annealing at 52°C for 1 minute and 3rd cyclic step was elongation at 72°C for 40 seconds, and finally post PCR step at 72°C for 5 to 7 minutes as shown in Figure 2.

2.9. Gene cleanup of amplified fragment DNA from the gel

For elution of amplified PCR products TIAN gel, Midi purification Kit having Cat # DP20902 was used for purification. The PCR band including Leu-tRNA *(UUR) MT-L1* and Leu-tRNA (*CUN)MT-L2* genes was cut out with a blade and kept in labeled Eppendorf tubes. To keep the balance equal, we added 300ul buffer BL in each spin column and centrifuge at 8000 RPMs for 2 minutes. The amplified product was taken and added PN buffers of 150 µl and was heated up at 60°Cuntil the gel completely dissolved. Then add Isopropanol to the half of PN buffers and kept at 25°C for some time. Put dissolved solution

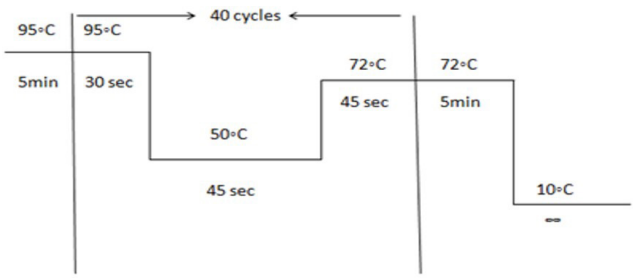

Figure 1. Condition of PCR amplification for Leu-tRNA *MT-L1 (UUR)*.

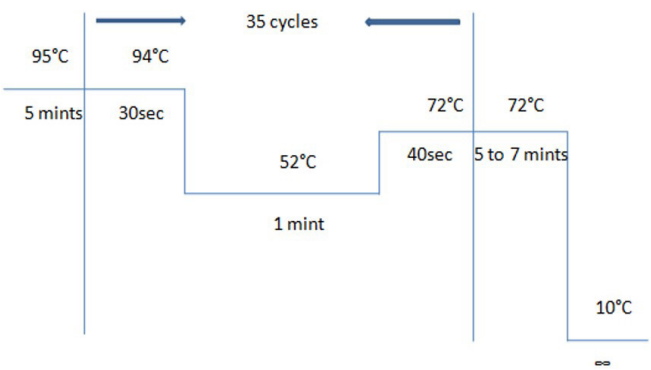

Figure 2. Condition of PCR amplification for Leu-tRNA *MT-L2 (CUN)*.

into column CA2. For 1 to 2 minutes' centrifuge again at 8000 revolutions per minute. Discarded the flow-through and added about 400ul buffer PW to the spin column. At 8000 rpm centrifuged again for 2 mints. And discarded flow through. In the 5th step, the 4th step was done again. After the repetition of the 4th step the spin column CA2 silicon membrane was centrifuged empty at 8000rpm for 5 minutes and kept the cap opened to air-dried. We put the column CA2 into a new clean Eppendorf tube of 1.5ml. 30 µl elution buffers were added to the column and centrifugation was processed at 8000rpm for two minutes. The flow-through from Eppendorf was taken again and centrifuged at 8000rpm for two minutes. The resulted buffers contained clean amplified DNA.

2.10. Sequencing and data analysis

Eluted Ten samples were sent to Microgen Inc. Korea for sequencing analysis. The delivered results from Microgen were aligned with the reference genome of mtDNA (NC-012920.1). To find out any mutation in mitochondrial encoded Leu-tRNA genes.

3. Results

3.1. Sample collection

Samples collection of heart patients were done in different hospitals in Peshawar. Total of 27 samples were collected, out of 27 patients, 16 were females and 11 were males. The age of the patients was 20 to 90 years. Besides heart disease, the patients were suffering from a disease like diabetes, Hearing problem, eye problems skin problem, and kidney problems. Out of 27 samples 10 samples were sent to Microgen Inc. Korea for nucleotide sequence analysis Table 1.

3.2. DNA extraction and amplification of Leu-tRNA MT-L1 and MT-L2 genes

After collection, the samples were spontaneously brought into the molecular lab of Hazara University Mansehra. DNA was extracted from the saliva of the subjects and was kept at -20°C in Molecular Genetics Lab at Hazara University. The total DNA extracted from 16 samples is shown in the following Figure 3. In addition, the Condition for PCR amplification was optimized by changing the annealing temperature and time. To amplify the desired region two sets of primers were used. Primer set 1 for *MT-L1* is 279bp, while the second set of primers amplifies the *MT-L2* gene with a fragment size of 553bp as shown in Figure 4.

3.3. Sample F-1

In this case, a female patient was diagnosed with Heart Disease Mitral valve replacement (MVR) having the age of 46 years. Family history showed that parents were not suffered from heart disease. But her sister was suffering from heart problems as shown in Figure 5. The weight of the subject was 52kg and others reported diseases were; skin

Table 1. List of patients with heart diseases whose samples were sent for sequencing.

S. No	Samples No	Gender	Age	Heart diseases	Others disease
1	F1	Female	46	MVR defect	Eye diseases, Hypertension problems
2	F2	Male	50	Valve defect	Kidney, Diabetes Mellitus DM
3	F3	Male	66	CCF/CAD	Hearing problems, DM
4	F6	Male	67	Myocardial infarction	Eye problems
5	F10	Male	28	CAD/ Palpitation	Skin problems,
6	F1	Male	46	RHD	Eye, stomach problem
7	F2	Male	50	Valve defect	Skin, diabetes, Kidney
8	F9	Female	46	MVR defect	Hearing problems
9	F14	Male	50	Valve defect	Diabetes mellitus
10	F22	Male	66	CCF/CAD	Skin

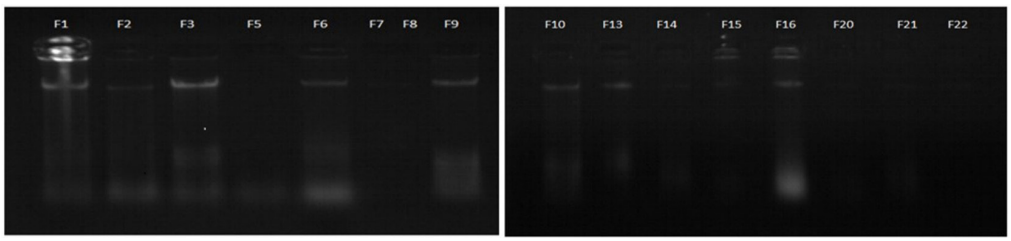

Figure 3. The figure shows extracted DNA results of 16 samples.

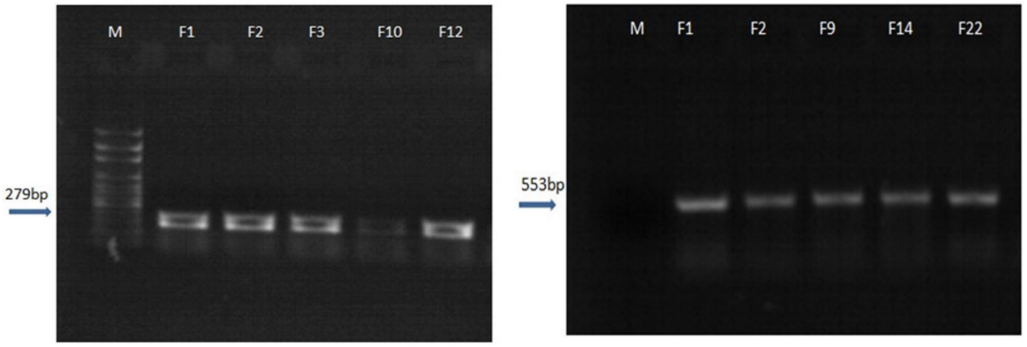

Figure 4. Indicates PCR results of Leu (UUR) and Leu (CUN) genes.

problems, eye problems, and blood pressure. The patient used the following tablets canita-D, jouit. Mutation in Leu-tRNA *MT-L1* encoded by mitochondria was not found in this subject as shown in Figure 6. Moreover, the sequence alignment result is shown in Figure 7.

3.4. Sample F-2

The patient was suffering from heart disease CCF (Congestive cardiac failure) having the age of 50 years. Family history showed that the patient father suffered from a heart problem, same as her sister as shown in Supplementary Material Fig S1. The recorded weight of the patient was 70Kg and others reported diseases were Diabetes, skin, hearing, and eye problems. The patient used the following tablets accord- 75, Probest (10 mg), and Cardinal. Mutation in Leu-tRNA *MT-L1* encoded by mitochondria was not found in this subject as shown in Supplementary Material Fig S2.

3.5. Sample F-3

In sample 3 the patient was suffering from heart disease Coronary artery disease (CAD) having the age of 66 years. Family history showed that his mother was suffering from heart problems and also he younger brother. His wife also died because of a heart problem and there is no heart disease found in his offspring as shown in Supplementary Material Fig S3. The recorded weight of the patient was 65 Kg and other reported diseases were, hearing problems and diabetes. The medicines the patient used were spirimoda (40mg), rorista lon (10mg), and lopren. Mutation in Leu-tRNA *MT-L1* encoded by mitochondria was not found in this subject as shown in Supplementary Material Fig S4.

3.6. Sample F-10

Heart disease (arteriosclerosis and palpitation) was found in subject F10 at age of 28 years. The history of the family showed that his grandfather suffered from a heart problem. The patient father and mother were not suffering from heart disease but his younger uncle was suffering from heart problems as shown in Supplementary Material Fig S5. The recorded weight of the patient was 90Kg and reported pain problems. Tablets used by the patient were pro base, zopent, and estar. Mutation in Leu-tRNA *MT-L1* encoded by mitochondria was not found in this subject as shown in Supplementary Material Fig S6.

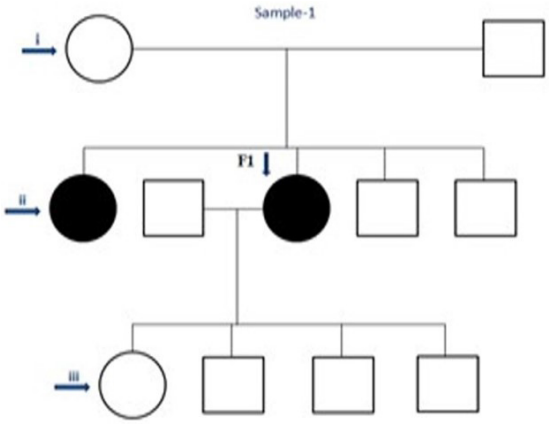

Figure 5. Shows the family history of the F-1 sample. Circles represent females while squares represent male members. Circles/squares are filled with black color show patients while the white color shows healthy individuals.

3.7. Sample F-12

In this case patient with heart disease CCF/TVD (congestive cardiac failure and triple vessels disease) having aged 65 years. Family history showed that his father suffered from heart disease and died because of stenosis. His uncle also suffering from heart disease. Of his children, the second younger son has also a problem with his heart as shown in Supplementary Material Fig S7. The recorded weight of the patients was 75Kg and the other reported disease was eye problems. Medicines used by the patient were, Maxflow (75mg) and leopard. Mutation in Leu-tRNA *MT-L1* encoded by mitochondria was not found in this subject as shown in Supplementary Material Fig S8.

3.8. Sample F-1

In the case of subject F1, the female patient suffering from heart diseases like MVR (Mitral valve replacement) having aged 46 years. Family history showed that their parents were not suffered from heart disease, but her younger sister also suffered from heart disease as shown in

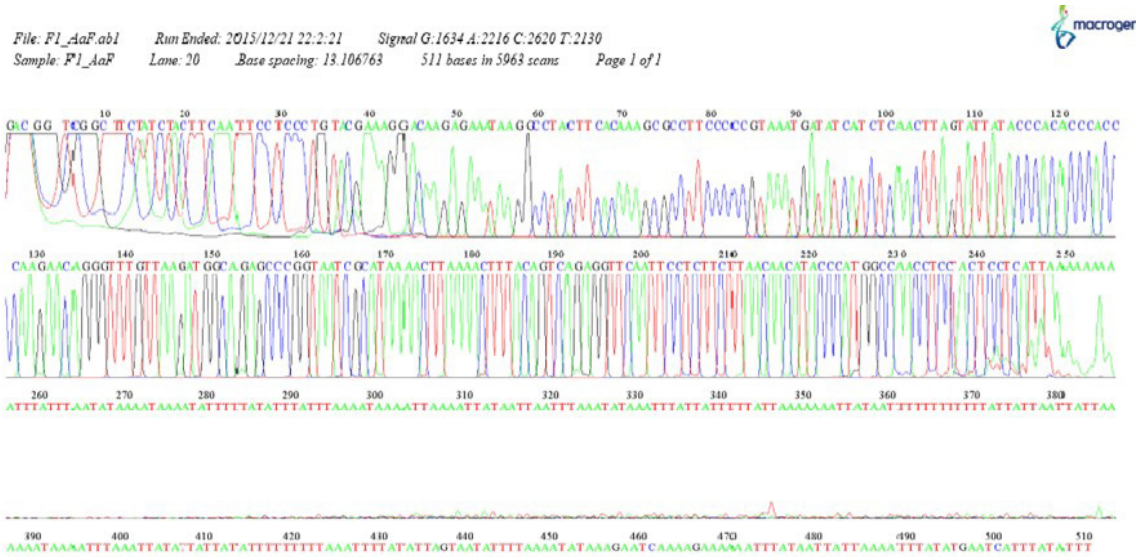

Figure 6. Chromatogram of sample F-1 as a sequencing result provided by Macrogen Inc. South Korea.

Supplementary Material Fig S9. The recorded weight was 50kg and other reported diseases were skin, eye, and blood pressure problems. The medicines the patient used were Canita-D and Jouit. Mutation in Leu-tRNA MT-L2 encoded by mitochondria was not found in this subject as shown in Supplementary Material Fig S10.

3.9. Sample F-9

The patient was suffering from heart disease RHD (Rheumatic heart disease) having age 66 years. Their family history showed that her mother was suffering from cardiac problems same as his sister as shown in Supplementary Material Fig S11. The recorded weight of the subjects was 65Kg and was also suffering from different other reported diseases, including skin allergy, kidney, eye, and mouth allergy problems. Tablets underused were a mother (2.5mg). Mutation in tRNA *MT-L2* encoded by mitochondria was not found in this subject as shown in Supplementary Material Fig S12.

3.10. Sample F-14

This patient was suffering from heart disease CCF (Congestive cardiac failure) having the age of 56 years. Family history showed that their parents were not suffered from heart problems but her brother suffered from heart problems and died because of mitral valve blockage as shown in Supplementary Material Fig S13. The recorded weight of the subjects was 60Kg and other reported diseases were kidney and stomach problems. The Medicine patient used was to start a (10mg) and NO. CLOT. Mutation in tRNA-*MT-L2* encoded by mitochondria was not found in this subject as shown in Supplementary Material Fig S14.

3.11. Sample F-22

This patient was suffering from heart disease RHD (Rheumatic heart disease) at age of 55years. Family

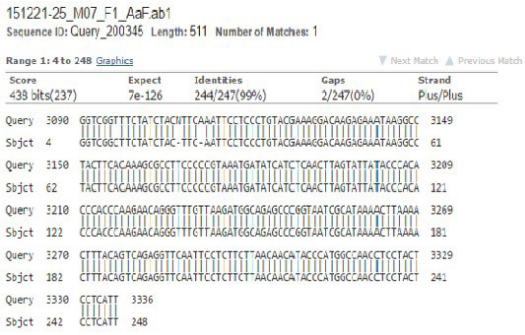

Figure 7. Alignment of the resulted sequence of sample F-1 with Revised Cambridge Reference Sequence (rCRS), accession number NC-012920.1. The underlined portion represents the Leu-tRNA gene. No mutation was detected in the Leu-tRNA gene of the CVD patient.

history showed that the patient mother and her sister were suffering from a heart problems. Both grandmother and grandfather were not suffering from heart disease as shown in Supplementary Material Fig S15. The recorded weight of the subject was 55 Kg and was also suffering from different other reported diseases were skin and eye problems. The medicines the patient used were a mother (2.5mg), Concor. Mutation in tRNA-*MT-L2* encoded by mitochondria was not found in this subject as shown in Supplementary Material Fig S16.

4. Discussion

Cardiovascular disease is the primary cause of morbidity and mortality globally despite the widespread use of medical therapies (Khan et al., 2022). Cardiomyopathies, heart failure (HF), congenital heart disease (CHD), coronary artery disease (CAD), cerebrovascular disease, abnormal

heart rhythms, peripheral artery disease (PAD), aortic disease, valvular heart disease (VHD), rheumatic heart disease (RHD), and various other cardiac and vascular conditions are all included under the umbrella term CVD. Millions of people worldwide pass away from CVD each year, accounting for about 31 percent of all fatalities and an annual cost of almost $1 trillion (Khan et al., 2022). With the patients with CVD in the area of Peshawar, Pakistan, we effectively explored the mutation in the mitochondrial DNA genes of Leu-tRNA MT-L1 and Leu-tRNA MT-L2. However, while examining patients with CVD, we did not discover any mutations in the mtDNA Leu-tRNA genes.

SNPs are increasingly serving as the foundation for genetic studies. The target sample is then genotyped for SNPs with a specific minimum allele frequency (for example, 0-10), and an association test is run. The goal of a case-control genetic association study is to determine whether there is a substantial correlation between n (n = 1, 2, 3, +...) genetic alterations and a particular phenotype. Furthermore, well-established power analysis methods for continuous and binary phenotypes with set sample sizes in two or more classes are available (Ait-Aider et al., 2007). Patients with myopathy, encephalopathy, lactic acidosis, and stroke-like events are reported to have the point mutation m. A3243G, which affects the tRNA leucine (MELAS). Patients with FSGS have also had this mutation documented in them (Dinour et al., 2004). The A3234G mutation in MELAS was discovered in a patient who had an end-stage renal illness with glomerulosclerosis and interstitial fibrosis (Mima et al., 2011). Given that it is the most prevalent mutation, this mutation is of particular importance to adults with mtDNA-related diseases (Hall et al., 2015). Patients generally have diabetes, hearing loss, or both. In addition to nephrotic syndrome, other renal manifestations can include proximal and/or distal impairment (Hall et al., 2015). A variety of point tRNA mutations can also cause FSGS (Lim et al., 2017; Meulemans et al., 2006). A 2,905bp deletion was discovered to cause FSGS in addition to point mutations, and it was followed by necrotizing nephritis and chronic interstitial nephritis (Becher et al., 1999).

In a study, a Leu-tRNA mutation is to blame for the mitochondrial problem seen in the donor patient and transform and hybrid systems provide clear evidence that genetic illnesses have a mitochondrial origin and must be taken into account when evaluating the harmful potential of mtDNA mutations (Caterina et al., 1994). The most prevalent apparent point mutation is the mtDNA A3243G mutation, which is also strongly related to diabetes (Shaag et al., 1997). Novel mitochondrial Leu-tRNA (UUR) gene mutation linked to mitochondrial encephalomyopathy (A3243T). All mitochondrial diseases commonly manifest as epilepsy. It appears prematurely in people with the A3243G mutation, who frequently have status epilepticus or stroke-like episodes. Patients who have a mutation in Leu-tRNA typically have a higher chance of experiencing stroke-like events (Roger and Bruce, 2015). A family with the Leu-tRNA (UUR) gene mutation A3288G has a maternally inherited form of mitochondrial myopathy. They verified that Leu-tRNA (UUR) is a hotspot for mtDNA mutations and that it is usually linked to the involvement of the respiratory muscle (Hadjigeorgiou et al., 1999). A3243G mutation in Leu-tRNA was found in the mitochondria of a patient with significant heart failure. Cardiomyopathy was recorded in these patients, either by itself or in conjunction with another multisystem disorder (Wu et al., 2002). Leu-tRNA UUR in mtDNA of a 46-year-old guy with lower leg edema, chest discomfort, and shortness of breath at rest was altered from A to G at nucleotide position 323 (Auer et al., 2016). Patients with the A3243G mtDNA mutation frequently experience increasing cardiac problems, and cardiac autonomic control is altered. CVDs and neuropsychiatric disorders were the most common reasons for mortality (Vanha-Majamaa et al., 2007).

Due to the involvement of numerous components, including heteroplasmy in the cell, post-mitotic tissues, and rapidly proliferating cells, the risk factors of mitochondrial illness are exceedingly challenging to uncover. Leu-tRNA genes MT-L1 and MT-L2 encoded mitochondria from 10 CVD patients were amplified and sequenced as part of our study. We did not find any mutation in the aforementioned subjects' tRNAs by aligning them with the reference sequence (rCRS). We suggest that future research on the mtDNA mutation linked to CVD include a large number of participants. To identify mutations in additional genes encoded by mtDNA, full genome sequencing of mtDNA in cardiovascular individuals is required because the variety of mtDNA mutations also depends on the ethnicity of the human population. One of the main drawbacks of the current study is that a sample size of three digits was not obtained. Sadly, no genotype frequencies were found in any studies looking at mitochondrial mutations. All of the subjects chosen were of Pakistani descent.

5. Conclusion

As there is no correlation between CVD and mitochondrial mutation in the Pakistani population, this study concludes. To accurately depict CVD and mitochondrial mutations from the cross-national studies, a meta-analysis might be undertaken again. To enable effective treatments, deeper comprehension of the precise illness pathways is required.

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Supplementary Material

Supplementary material accompanies this paper.

Fig S1. Pedigree of Sample F-3. Circles represent females while squares represent male members. Circles or squares filled with black color show patients while the white color shows healthy individuals.

Fig S2. Chromatogram of sample F-3.

Fig S3. Pedigree of Sample F-4. Circles represent females while squares represent male members. Circles or squares filled with black color show patients while the white color shows healthy individuals. The line on circles/squares are shows the deceased members.

Fig S4. Chromatogram of sample F-10.

Fig S5. Pedigree of Sample F-5. Circles represent females while squares represent male members. Circles or squares filled with black color show patients while the white color shows healthy individuals. The line on circles/squares are shows the deceased members.

Fig S6. Chromatogram of sample F-12.

Fig S7. Pedigree of Sample F-6. Circles represent females while squares represent male members. Circles or squares filled with black color show patients while the white color shows healthy individuals.

Fig S8. Chromatogram of sample F-1.

Fig S9. Pedigree of Sample F-7. Circles represent females while squares represent male members. Circles or squares filled with black color show patients while the white color shows healthy individuals. The line on circles/squares are shows the deceased members.

Fig S10. Chromatogram of sample F-2.

Fig S11. Pedigree of Sample F-8. Circles represent females while squares represent male members. Circles or squares filled with black color show patients while the white color shows healthy individuals. The line on circles/squares are shows the deceased members.

Fig S12. Chromatogram of sample F-9.

Fig S13. Pedigree of Sample F-9. Circles represent females while squares represent male members. Circles or squares filled with black color show patients while the white color shows healthy individuals. The line on circles/squares are shows the deceased members.

Fig S14. Chromatogram of sample F-14.

Fig S15. Pedigree of Sample F-10. Circles represent females while squares represent male members. Circles or squares filled with black color show patients while the white color shows healthy individuals. The line on circles/squares are shows the deceased members.

Fig S16. Chromatogram of sample F-22.

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