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MITOCHONDRIAL LEUCINE tRNA GENE MUTATION AND MATERNALLY INHERITED CARDIOVASCULAR DISEASE (CVD) IN PAKISTANI POPULATION

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Abstract
Hypertension is a frequent, chronic, age-related disorder, which remains a major modifiable risk factor for cardiovascular disease (CVD) despite important advances in our understanding of its pathophysiology. In the present study, we performed mtDNA tRNA$^{Leu\ (UUR)}$ gene mutation analysis in Pakistani population. Twenty samples from CVD patients were collected at various hospitals in Swat, Pakistan and DNA was extracted from their saliva. The mitochondrial tRNA$^{Leu(UUR}$ gene was amplified using Polymerase Chain Reaction (PCR) using specified primers and ten samples from different families were sequenced. The sequencing data presentation showed a two-nucleotide mismatch in the alignment. The A-to-G mutation in nucleotide pair 3243 of the mitochondrial gene was not found in the subject of pedigree first. Notably, this subject did not display the A-to-G mutation at nucleotide pair 3243 in the mitochondrial gene shown in the third pedigree. The present research concluded, that mitochondria as the target and origin of major pathogenic pathways which lead to the progression of CVD. The alignment of the patient's R-1 sequencing data and showed one sample of results, because we find any mutation in the mtDNA tRNALeu $^{(UUR)}$ gene in the patients with CVD.

Introduction
Despite the extensive use of medical therapy over the past ten years, cardiovascular diseases (CVD) remain one of the primary causes of mortality and morbidity in many industrialized and developing nation (Khan et al., 2022). CVD refers to a class of related disorders that affect the circulatory system. Despite diverse clinical manifestations, these diseases are nonetheless profoundly connected in that the damage generated by one often causes the onset of another. For example, clinically silent forms of CVD, such as hypertension and atherosclerosis, often develop over time and can trigger the onset of acute ischemic diseases, such as myocardial infarction and stroke (Go et al., 2014). Hypertension is an established risk factor for coronary heart disease, stroke, congestive heart failure, and renal dysfunction. Despite significant advances in our understanding of the pathophysiology of hypertension, it remains to be one of the world's greatest public health issues (El Shamieh et al., 2012). It is estimated that one-third of the world's adult population will be

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hypertensive by 2025 (Kearney et al., 2005). Variations in a variety of genes have shown an association with hypertension in certain studies; however, these associations are often not reproducible in studies on other populations. However, most of the reported genetic variants were identified in studies of the nuclear genome only limited insights have been gained from the investigation of the mitochondrial genome (Oliphant et al., 2002; Padmanabhan et al., 2010). The molecular pathogenesis of hypertension in the Pakistani population remains poorly understood. In the present study, we performed mtDNA $tRNA^{Leu (UUR)}$ gene mutation analysis in Pakistani population with different families with suggestive maternally transmitted CVD.

Materials & Methods

Ethical approval

All samples were obtained with the informed consent of patients and the experiment was approved by the Ethics Committee of Hazara University Mansehra, Khyber Pakhtunkhwa (KP) Pakistan.

Saliva collection

Twenty samples from CVD patients were collected at various hospitals in Swat, Pakistan. The saliva samples were collected from all those patients who admitted in the cardiac word of Swat medical complex hospital with complaint of hypertension and anxiety. The saliva samples were put in a sterile cup with 3-5 mL of a solution having a certain percentage of sugar. The samples were transported to the molecular genetics laboratory at Hazara University Mansehra, where the samples were stored at -20◦C for further analysis.

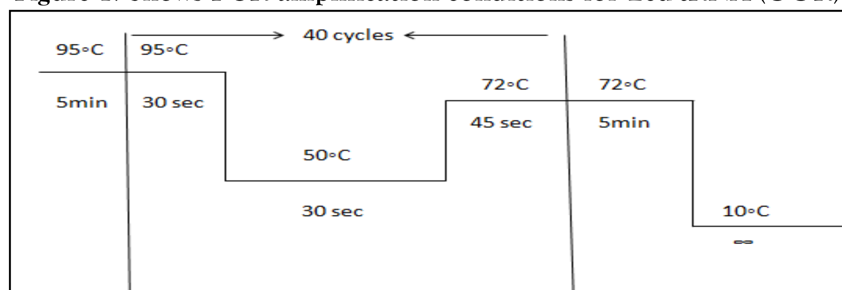
DNA extraction

The whole DNA of buccal cavity epithelial cells was extracted, according to Aidar & Line (Aidar & Line, 2007). [7] For checking the quality of extracted DNA, agarose gel (0.5g of agarose dissolved in 29.4 ml double distal water with 600ul of 50X TAE mixture) with 25ul ethidium bromide was used; then, 5µl of extracted DNA was dissolved with 2µl of loading dye, and the gel was run for 30 minutes at 60V before being photographed under ultraviolet light using the gel documentation system showed in figure 1. After, the DNA was kept at -20°C until it was time to process it.

Polymerase Chain Reaction (PCR)

PCR was used to amplify the desired gene. Initial denaturation was at 95◦C for 5 minutes, followed by denaturation at 95◦C for 5 minutes, annealing at 50◦C for 45 seconds, extension at 72◦C for 5 minutes, and final extension at 72◦C for 5 minutes; these were the thermal cycling conditions followed for 40 cycles showed in figure 2.

Figure 1: Shows PCR amplification conditions for Leu-tRNA (UUR).



1% Agarose gel

The final PCR findings were analyzed on gel. The mixture was then treated with 12ml of ethidium bromide. For cooling, the melted mixture was kept at 25◦C. The agarose mixture previously had been put on the gel plate and allowed to solidify. We combined 15ml of PCR product with 2ml of DNA loading blue dye and put it into the agarose gel wells. A 60 Volute was supplied for 30 minutes in an electrophoresis approach until DNA fragments move from left to right. The bands that had been magnified were photographed and inspected under ultraviolet light showed in figure 3.

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Figure 2: Genomic DNA extracted from patients samples.

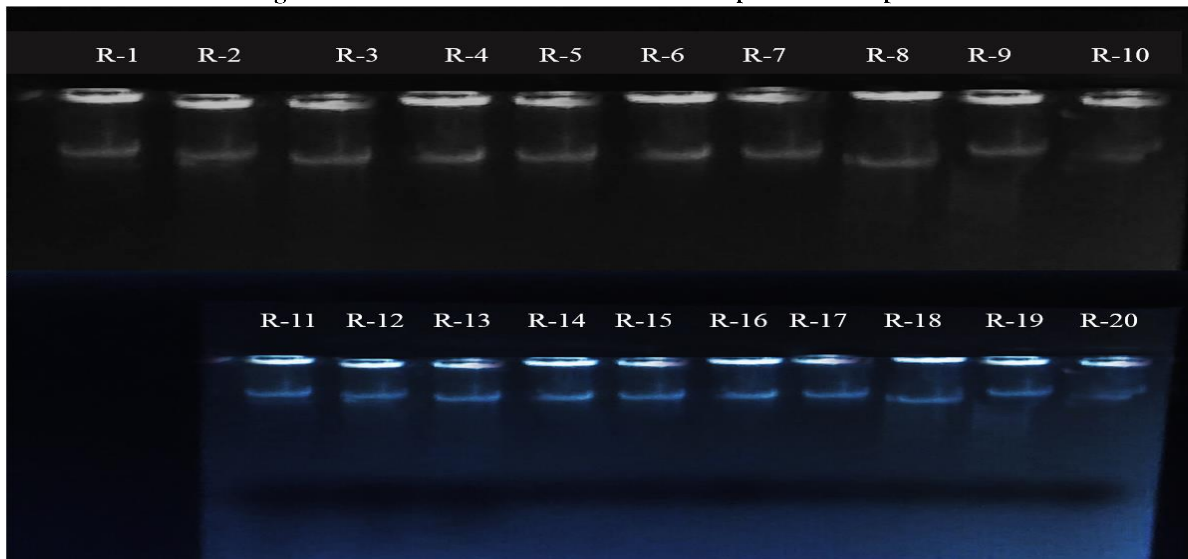
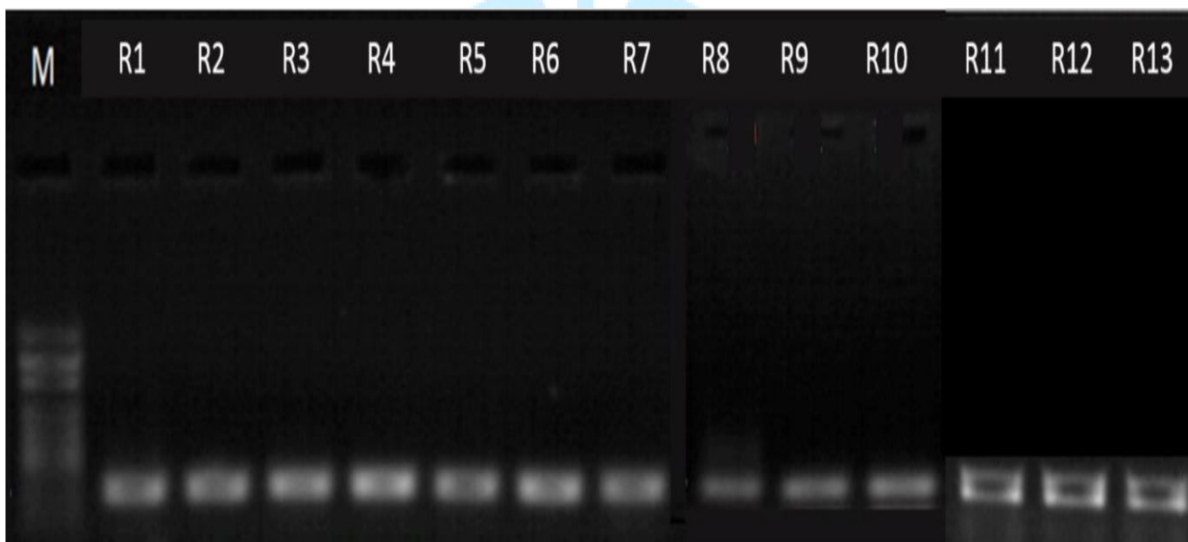


Figure 3: PCR results of samples R-1 to R-13. A fragment of 279bp was amplified. The ladder sequence is denoted by M =1000bp marker.



Primer designing

The common primer pair (Forward 5'-CAAATTCCTCCCTGTACGAAAGG-3'; Reverse 5'-AATGAGGAGTAGGAGG-TTGGCC-3') was used to amplify the mitochondrial tRNA$^{Leu\ (UUR)}$ gene. A 279-base-pair fragment's amplification is depicted. Ten eluted DNA samples were sent for sequencing to Macrogen Inc. in Korea. The resulting sequencing information was contrasted with the whole mitochondrial sequence, rCRS Accession number NC-012920.1. The tRNA$^{Leu\ (UUR)}$ gene alignment was examined to check for any mutations.

Results

Characteristic of studies subjects

The mean age of the patients was (61.25 ± 13.49) shown in table 1. Subsequently, all collected samples were delivered to the molecular lab at Hazara University Mansehra for further processing.

Table 1: Characteristics of studied subjects.

Sample	Diagnosis	Sex	Age	BMI	DNA	PCR	Seq
R-1	Myocardial infarction	Male	55	20.8	+	+	+
R-2	Myocardial infarction	Male	52	26	+	+	-
R-3	Myocardial infarction	Male	49	25.4	+	+	-
R-4	Myocardial infarction	Male	77	20.1	+	+	-
R-5	Myocardial infarction	Male	47	26	+	+	-
R-6	Acute coronary syndrome	Male	44	24.7	+	+	-
R-7	Acute coronary syndrome	Male	82	23.6	+	+	-
R-8	Acute coronary syndrome	Male	57	26	+	+	-
R-9	Acute coronary syndrome	Male	82	18.4	+	+	+
R-10	Acute Coronary syndrome	Male	56	25.7	+	+	+
R-11	Chronic coronary syndrome	Female	81	28.9	+	+	-
R-12	Chronic coronary syndrome	Female	64	21	+	+	-
R-13	Chronic coronary syndrome	Female	67	24.9	+	+	-
R-14	Chronic coronary syndrome	Female	73	26.2	+	-	-
R-15	Chronic coronary syndrome	Female	47	23.4	+	-	-
R-16	Chronic coronary syndrome	Female	39	25.7	+	-	-
R-17	Acute coronary syndrome	Female	68	26.7	+	-	-
R-18	Acute coronary syndrome	Female	50	21.3	+	-	-
R-19	Acute Coronary syndrome	Female	70	23.2	+	-	-
R-20	Chronic coronary syndrome	Female	65	16.5	+	-	-

BMI: Body mass index; PCR: Polymerase chain reaction; Seq: sequencing.

Sequencing analysis

Among the 20 samples, only 10 were forwarded to Microgen Inc. Korea for nucleotide sequence analysis. Nevertheless, we received sequencing results for just 3 of those samples. The obtained sequencing information was then compared with the entire mitochondrial sequence, specifically the revised Cambridge Reference Sequence (rCRS) Accession number NC-012920.1. A careful examination of the tRNA^{Leu} $^{(UUR)}$ gene alignment was conducted to assess mutations.

Pedigree 1

A male patient was diagnosed with heart disease (Valve complication) having the age of 55 years. Family history showed that the patient's father and his two siblings suffered from heart disease, as shown in figure 4A. The sequencing data presentation showed a two-nucleotide mismatch in the alignment as depicted in figure 4B. While examining the flow of the sequencing results' chromatogram, it became evident that the two-nucleotide mismatch observed in the alignment was attributable to a technical error, leading us to the conclusion that it was not a genuine mismatch figure 4C. The A-to-G mutation in nucleotide pair 3243 of the mitochondrial gene was not found in this subject.

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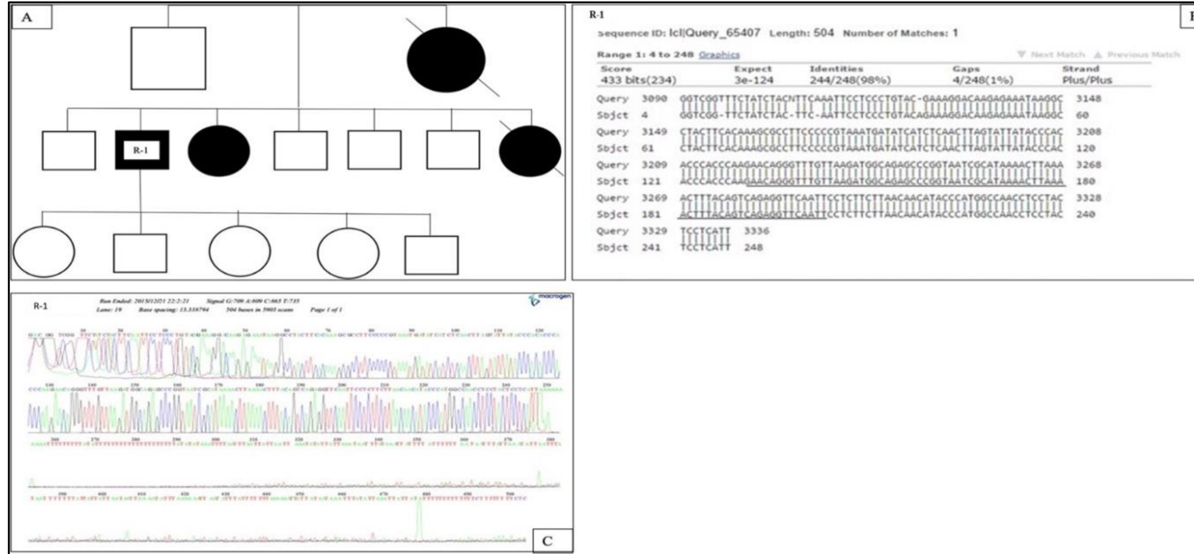
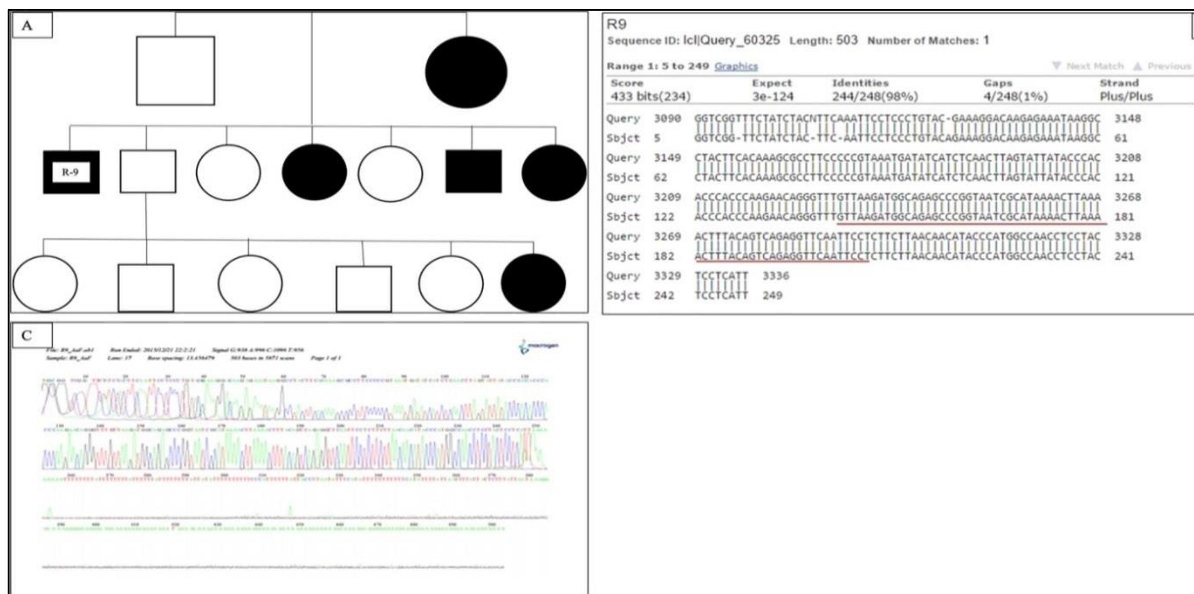
Figure 4: (A) represents the family history of the patient, with squares representing males, circles representing females, and lines indicating individuals deceased from cardiovascular disease. (B) Indicated the alignment sequence data for the R-1 sample, aligning with the Cambridge reference accession number, highlighting the region corresponding to the tRNA$^{Leu\ (UUR)}$ gene. (C) depicts the sequencing process by Macrogen Inc. in South Korea, resulting in a chromatogram for sample R-1.



Pedigree 2

The patient underwent coronary heart disease at the age of 82 years. The family history revealed that his mother, two brothers, sister and niece also had a history of CVD figure 5A. The patient's R-9 alignment sequencing data, figure 5B, and the flow of the sequencing results' chromatogram as shown in figure 5C. This subject did not exhibit the A-to-G mutation at nucleotide pair 3243 in the mitochondrial gene.

Figure 5: (A) illustrates the patient's family history, using squares for males, circles for females, and lines to signify individuals who have passed away due to CVD. (B) Represents the alignment sequence data for sample R-1, aligned with the Cambridge reference accession number, emphasizing the section related to the tRNA$^{Leu\ (UUR)}$ gene. (C) Indicated the sequencing process conducted by Macrogen Inc. in South Korea, yielding a chromatogram for sample R-9.



Pedigree 3

The individual, aged 56 was diagnosed with coronary heart disease. Family history revealed that his mother, sister and brother also had a history of CVD, as depicted in figure 6A. The figure 6B displays the alignment sequencing data for the patient's R-9 sample, while figure 6C illustrates the sequencing results' chromatogram flow. Notably, this subject did not display the A-to-G mutation at nucleotide pair 3243 in the mitochondrial gene.

Figure 6: (A) indicated the patient's family history, using squares for males, circles for females and lines to denote individuals who have passed away due to cardiovascular disease. (B) Shown the alignment sequence data for the R-1 sample, aligned with the Cambridge reference accession number, emphasizing the region associated with the tRNA$^{Leu\ (UUR)}$ gene. (C) Illustrates the sequencing process conducted by Macrogen Inc. in South Korea, generating a chromatogram for sample R-10.

Their medical histories indicated that the individuals had hypertension. On any patient's tRNA$^{Leu\ (UUR)}$ gene, there were no mutations identified. Even though there is a two-nucleotide mismatch in the alignment, we were able to determine from this sequencing result's chromatogram that it was a technical error rather than an exact miss-match (Figure 7). Indicated here is the alignment of the patient's R-1 sequencing data (Figure 8). Herein, we only showed one sample of results because we find any mutation in the mtDNA tRNA$^{Leu\ (UUR)}$ gene in the patients with hypertension.

Figure 7: Macrogen Inc. of South Korea provided a chromatogram of sample R-1 as a result of the sequencing.

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Figure 8: Data from the subject R-1 sequencing are aligned. The Cambridge reference accession number was used to compare the sequence. The highlighted region indicates the tRNA$^{Leu\ (UUR)}$ gene.

R-1

```
Sequence ID: lcl|Query_65407  Length: 504  Number of Matches: 1

Range 1: 4 to 248  Graphics                     Next Match  Previous Match
Score           Expect    Identities      Gaps        Strand
433 bits(234)   3e-124    244/248(98%)    4/248(1%)   Plus/Plus

Query  3090  GGTCGGTTTCTATCTACNTTCAAATTCCTCCCTGTAC-GAAAGGACAAGAGAAATAAGGC  3148
Sbjct  4     GGTCGG-TTCTATCTAC-TTC-AATTCCTCCCTGTACAGAAAGGACAAGAGAAATAAGGC  60
Query  3149  CTACTTCACAAAGCGCCTTCCCCCGTAAATGATATCATCTCAACTTAGTATTATACCCAC  3208
Sbjct  61    CTACTTCACAAAGCGCCTTCCCCCGTAAATGATATCATCTCAACTTAGTATTATACCCAC  120
Query  3209  ACCCACCCAAGAACAGGGTTTGTTAAGATGGCAGAGCCCGGTAATCGCATAAAACTTAAA  3268
Sbjct  121   ACCCACCCAAGAACAGGGTTTGTTAAGATGGCAGAGCCCGGTAATCGCATAAAACTTAAA  180
Query  3269  ACTTTACAGTCAGAGGTTCAATTCCTCTTCTTAACAACATACCCATGGCCAACCTCCTAC  3328
Sbjct  181   ACTTTACAGTCAGAGGTTCAATTCCTCTTCTTAACAACATACCCATGGCCAACCTCCTAC  240
Query  3329  TCCTCATT  3336
Sbjct  241   TCCTCATT  248
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Discussion

In the present study, two-nucleotide mismatch in the alignment were able to determine from sequencing. The alignment of the patient's R-1 sequencing data and showed one sample of results, because we find any mutation in the mtDNA tRNA$^{Leu\ (UUR)}$ gene in the patients with CVD. A previous study found that there was a significant correlation between maternal hypertension and hypertension in progeny and observed that some diseases caused by mtDNA mutation were accompanied by hypertension (Fuentes et al., 2000). Watson et al, also found that the incidence of the m. 10398A>G mutation in the mitochondrial ND3 gene and the mutation 6620T>C/ mutation 6260G>A (OMIM516030) double point mutation in the HaeIII CO1 gene was significantly higher in patients with hypertension-associated end-stage renal disease than in the control group (Watson et al., 2001). Liu et al, compared and analyzed gene changes in the D-loop region that regulates hypertension and normal blood pressure and found that the frequency of variation in the hypertension group was higher than that seen in the normal blood pressure group (Liu et al., 2021). These studies suggest that essential hypertension with maternal genetic characteristics may be related to mtDNA mutations. Furthermore, a patient with severe heart failure was analyzed as having a mitochondrial 3243A>G mutation lies in tRNA$^{Leu\ (UUR)}$. Such patients were reported with cardiomyopathy, either alone or as part of a multisystem disorder (Wu et al., 2002). Cardiac abnormalities are common and progressive in patients with the 3243A>G mtDNA mutation and cardiac autonomic regulation is changed. The most frequent causes of death were neuropsychiatric and CVD (Vanha-Majamaa et al., 2007). The mitochondrial tRNA$^{Leu\ (UUR)}$ contains five mutations initiate in tissues from patients using the symptoms of MELAS (3243A>G; 3258T>C; 3244G>A; 3271T>C and 3291T>C) lacked normal taurine modification (5-taurinomethyluridine) (Yohei, 2005). The 3260A>G transition in tRNA$^{Leu(UUR)}$ encoded by the mitochondrial genome is certainly responsible for the mitochondrial disorder recognized in the donor patient, gives the origin for evidence of a mitochondrial origin of a genetic disorder, and could be an evaluation of the pathogenic potential of the mtDNA mutations (De Caterina et al., 1994). It has also been reported that the mtDNA encoded cytochrome c oxidase I (COX I) and COX II exist exclusively with the correct amino acid sequences in 3243A>G cells in a misassembled complex IV (Janssen et al., 2007). Patients hang are at a higher risk of developing stroke-like episodes with the mutation 3243A>G mutation (Boehme et al., 2017). The cardiac autonomic regulation is disturbed and cardiac abnormalities are regular and progressive in patients with the 3243A>G mtDNA mutation in the tRNA$^{Leu\ (UUR)}$ gene (Vanha-Majamaa et al., 2007). Patients with the 3243A>G mutation and their first-degree maternal relatives died younger than expected. The most common causes of death were neuropsychiatric among

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cardiovascular diseases (Vanha-Majamaa et al., 2007). Furthermore, patients diagnosed as having a mitochondrial 3243A>G mutation presented with severe heart failure, and cardiomyopathy in patients, alone or as part of a multisystem disorder (Wu et al., 2002).

Conclusion

The present research concluded, the twenty samples from CVD patients were collected at various hospitals in Swat, Pakistan. The mean age of the patients was (61.25 ± 13.49). The sequencing data presentation showed a two-nucleotide mismatch in the alignment. The A-to-G mutation in nucleotide pair 3243 of the mitochondrial gene was not found in the subject of pedigree first. This subject did not exhibit the A-to-G mutation at nucleotide pair 3243 in the mitochondrial gene indicated in the second pedigree. Notably, this subject did not display the A-to-G mutation at nucleotide pair 3243 in the mitochondrial gene shown in the third pedigree. In hypertension, the two-nucleotide mismatch in the alignment was able to determine from sequencing. The alignment of the patient's R-1 sequencing data and showed one sample of results, because we find any mutation in the mtDNA $tRNA^{Leu (UUR)}$ gene in the patients with CVD.

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Data availability statement: The sequences have been uploaded to the National Center for Biotechnology Information using accession number: NC-012920.1

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Conflict of interest: The authors declare that they have no competing interests.

Author contribution

Conduct research and drafted the manuscript: I. K;
Supervise and edited the manuscript: S.U. G;
Pedigree analysis: J. S;
Formal analysis: S. K;
Edited the manuscript: F. K;
Correct grammatically mistakes: H. A. H;
Formal analysis: M;
Checked the manuscript: A. M. H. S;

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