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Sequencing Analysis of the Mitochondrial tRNA-Leucine Gene in Maternally Inherited Cardiovascular Disease Among the Human Population of Pakistan

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Abstract

Despite our increased understanding of its mechanisms, hypertension remains a modifiable risk factor for cardiovascular disease (CVD), a prevalent and chronic illness mostly affecting the elderly. The purpose of this research was to examine the mutation status of the mtDNA tRNALeu (UUR) gene in all Pakistanis. Twenty samples from persons with cardiovascular illness were collected from several hospitals in Swat, Pakistan, and DNA was extracted from their saliva. Polymerase Chain Reaction (PCR) was employed to amplify the mitochondrial tRNALeu (UUR gene using particular primers, and sequencing was conducted on 10 samples from various families. The sequencing results revealed a two-nucleotide discrepancy in the alignment. The A-to-G mutation at nucleotide pair 3243 of the mitochondrial gene was not initially detected in the subject of the pedigree. The patient did not demonstrate the A-to-G mutation at nucleotide position 3243 in the specified mitochondrial gene of 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. Recent research indicates that mitochondria function as both the target and origin of critical pathogenic pathways that facilitate the progression of cardiovascular disease (CVD). The alignment of the patient's R-1 sequencing data

disclosed a unique sample of findings, signifying the existence of a mutation in the mtDNA tRNALeu (UUR) gene in patients with cardiovascular disease (CVD).

Keywords: Cardiovascular diseases, Polymerase chain reaction, Blood pressure, Ethics committee, Essential hypertension.

Introduction

Cardiovascular diseases (CVD) remain a predominant cause of mortality and morbidity in several industrialized and developing nations, despite the extensive adoption of medical therapies in the previous decade (Khan et al., 2022). Cardiovascular disease (CVD) encompasses a collection of disorders that impact the circulatory system. Although these diseases manifest differently in individuals, they are strongly interconnected, since the damage caused by one sometimes precipitates the onset of another. Clinically silent forms of cardiovascular disease (CVD), such as hypertension and atherosclerosis, may deteriorate over time and result in acute ischemic conditions, including myocardial infarction and stroke (Go et al., 2014). Hypertension is an established risk factor for coronary artery disease, cerebrovascular accident, congestive heart failure, and renal dysfunction. Despite our enhanced understanding of hypertension, it remains a significant public health issue globally (El Shamieh et al., 2012). By 2025, it is anticipated that one-third of individuals globally would be hypertensive (Kearney et al., 2005). Various genes have been associated with hypertension in certain studies; however, these associations do not consistently persist throughout investigations including diverse populations. Nonetheless, most identified genetic variants were found in studies concentrating solely on the nuclear genome, resulting in very restricted understanding from the analysis of the mitochondrial genome (Oliphant et al., 2002; Padmanabhan et al., 2010). The molecular etiology of hypertension in the Pakistani population is insufficiently understood. This study involved the examination of mtDNA tRNALeu (UUR) gene mutations among a Pakistani population consisting of many families exhibiting signs of maternally inherited cardiovascular disease (CVD).

Materials & Methods

Saliva Collection

Twenty samples from individuals with cardiovascular disease were collected from various hospitals in Pakistan. Saliva samples were collected from all patients admitted to the heart ward of Swat Medical Complex Hospital due to concerns over elevated blood pressure and anxiety. The saliva samples were deposited in a sterile container containing 3-5 mL of a solution with a defined sugar content. The samples were transported to the molecular genetics laboratory, where they were stored at -20ºC for further analyses.

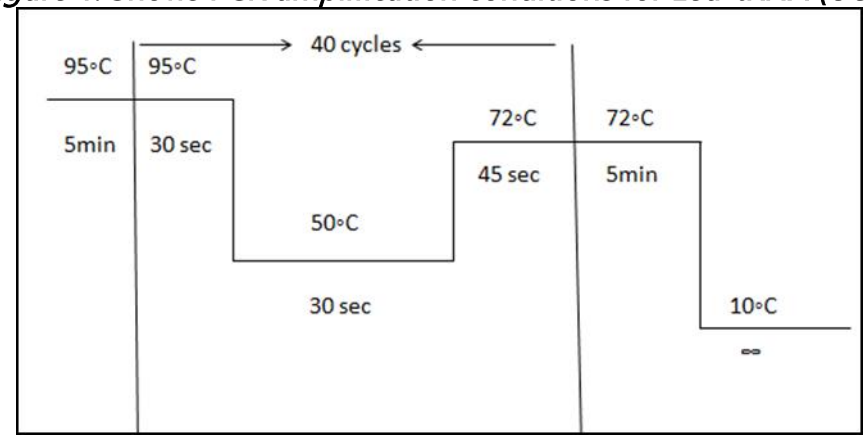
DNA Extraction

Aidar and Line (2007) state that the whole DNA of buccal cavity epithelial cells was extracted. To assess the quality of the extracted DNA, an agarose gel was prepared using 0.5g of agarose dissolved in 29.4 ml of double-distilled water, supplemented with 600 µl of 50X TAE buffer and 25 µl of ethidium bromide. Subsequently, 5µl of the extracted DNA was combined with 2µl of loading dye, and the gel electrophoresis was conducted for 30 minutes at 60V. The gel was subsequently imaged under ultraviolet light utilizing the gel documentation equipment depicted in figure 1. The DNA was thereafter preserved at -20°C until it was ready for use.

Polymerase Chain Reaction (PCR)

We used PCR to make the gene bigger that we were after. Figure 2 shows the 40 rounds of thermal cycling. The first step was to denature the protein at 95ºC for 5 minutes. The next step was to anneal it at 50ºC for 45 seconds. The last step was to extend it at 72ºC for 5 minutes.

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



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Ata & Ali - 2025

1% Agarose Gel

The conclusive PCR results were examined on gel. The solution was subsequently treated with 12 ml of ethidium bromide. The melted mixture was maintained at 25°C for cooling. The agarose mixture was applied to the gel plate and let to harden. We amalgamated 15 ml of PCR product with 2 ml of DNA loading blue dye and introduced it into the wells of the agarose gel. A 60 Volute was administered for 30 minutes in an electrophoresis method until DNA fragments migrated from left to right. The bands that were amplified were photographed and examined under ultraviolet light, as seen in Figure 3.

Figure 2: Genomic DNA Extracted from Patients Samples

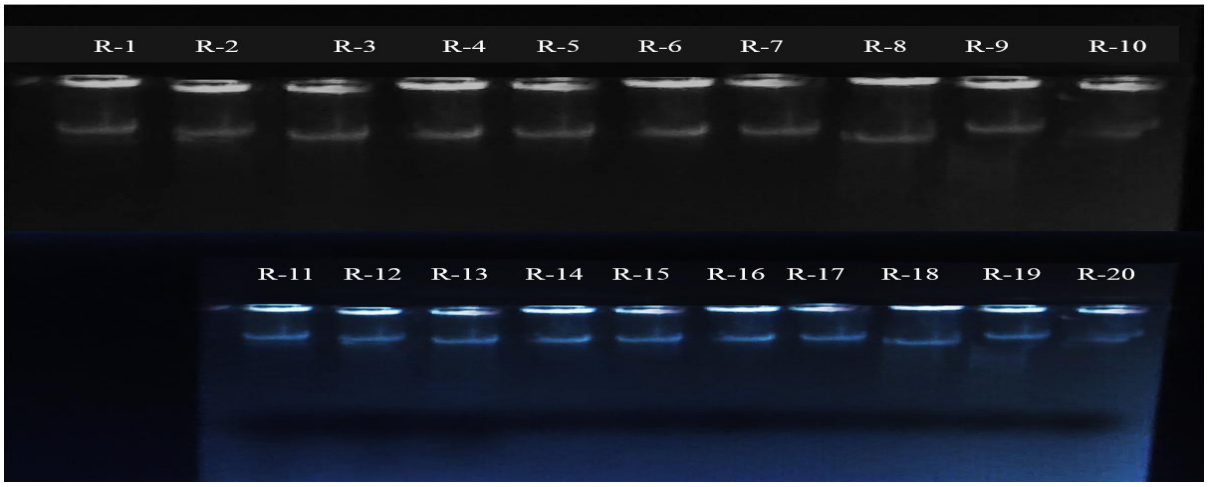
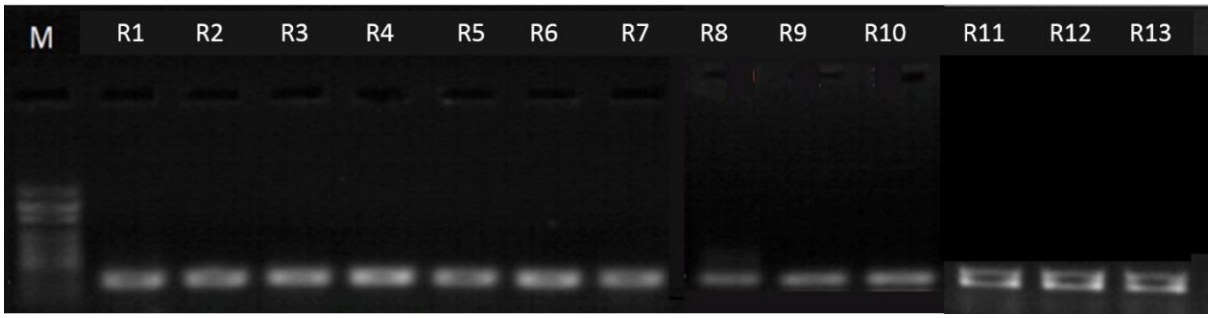


Figure 3: PCR Results of Samples R-1 to R-13. A Fragment of 279bp was Amplified. The Ladder Sequence is Denoted by M =1000bp Marker



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

Table 1 shows that the average age of the patients was (60.15 ± 12.50). After that, all the samples that had been gathered were sent to the molecular lab for more work.

Table 1: *Characteristics of Studied Subjects.*

Sample	Diagnosis	Sex	Age	BMI	DNA	PCR	Seq
R-1	Myocardial infarction	Male	54	19.5	+	+	+
R-2	Myocardial infarction	Male	51	25.5	+	+	-
R-3	Myocardial infarction	Male	48	24.5	+	+	-
R-4	Myocardial infarction	Male	75	18.1	+	+	-
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	80	18.4	+	+	+
R-10	Acute Coronary syndrome	Male	54	25.7	+	+	+
R-11	Chronic coronary syndrome	Female	78	28.9	+	+	-
R-12	Chronic coronary syndrome	Female	62	21	+	+	-
R-13	Chronic coronary syndrome	Female	66	24.9	+	+	-
R-14	Chronic coronary syndrome	Female	70	26.2	+	-	-
R-15	Chronic coronary syndrome	Female	45	23.4	+	-	-
R-16	Chronic coronary syndrome	Female	36	25.7	+	-	-
R-17	Acute coronary syndrome	Female	67	26.7	+	-	-
R-18	Acute coronary syndrome	Female	48	21.3	+	-	-
R-19	Acute Coronary syndrome	Female	68	23.2	+	-	-
R-20	Chronic coronary syndrome	Female	62	16.5	+	-	-

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

Sequencing Analysis

Among 20 samples, only 10 were sent to Microgen Inc. Korea for nucleotide sequencing study. Nonetheless, we obtained sequencing findings for only three of those samples. The acquired sequencing data was subsequently compared to the whole mitochondrial sequence, namely the revised Cambridge Reference Sequence (rCRS) Accession number NC-012920.1. An extensive analysis of the tRNALeu (UUR) gene alignment was performed to evaluate mutations.

Pedigree 1

A cardiac condition (valve issue) was identified in a 55-year-old male patient. You can see in figure 4A that the patient's dad and two siblings all experienced heart problems. Figure 4B shows that there was a two-nucleotide mismatch in the alignment, as demonstrated in the presentation of the sequencing data. Analysis of the chromatogram from the sequencing results indicated that the two-nucleotide mismatch seen in the alignment was attributable to a technical error. This prompted us to conclude that there was not a genuine mismatch (figure 4C). This individual lacked the A-to-G mutation at nucleotide pair 3243 of the mitochondrial gene.

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 tRNALeu (UUR) gene. (C) depicts the sequencing process by Macrogen Inc. in South Korea, resulting in a chromatogram for sample R-1.

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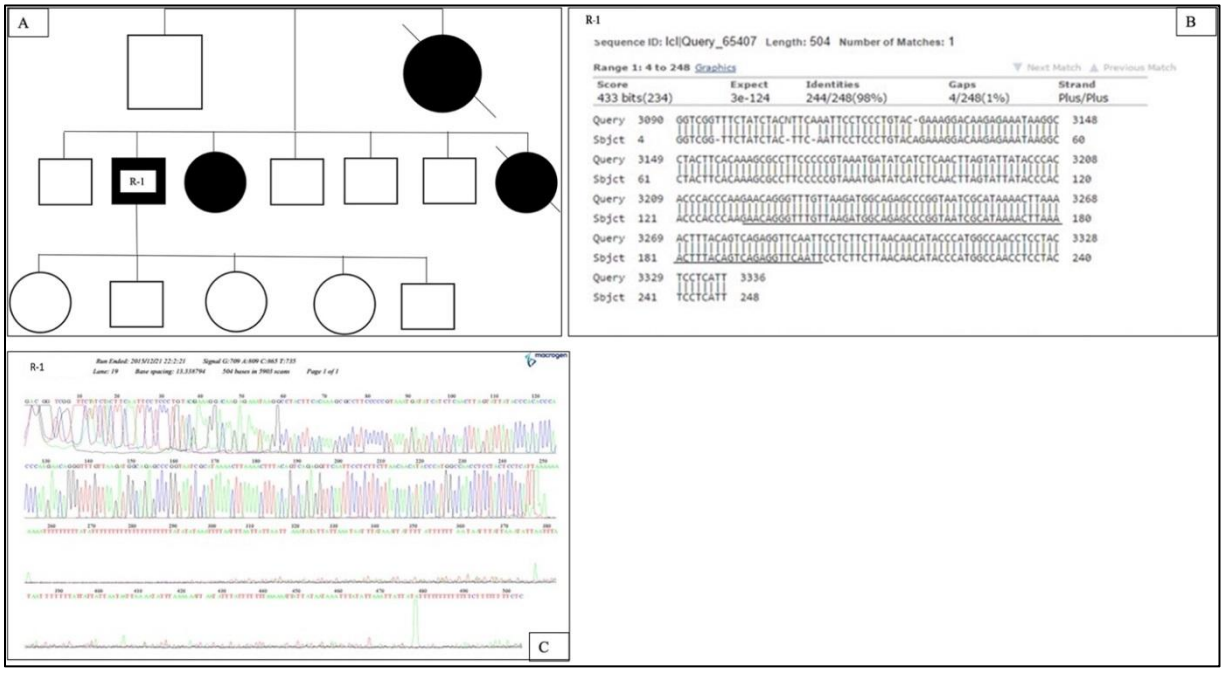

Pedigree 2

The patient had coronary heart disease at the age of 82. The familial history indicated that his mother, two brothers, sister, and niece all had a history of cardiovascular disease (CVD) (figure 5A). The R-9 alignment sequencing data of the patient is presented in figure 5B, while the chromatogram of the sequencing results is illustrated in figure 5 C. This person lacked the A-to-G mutation at nucleotide position 3243 in the mitochondrial gene.

Figure 5: (A) illustrates the patient's family tree by depicting males as squares, females as circles, and those who have passed away due to cardiovascular disease as lines. (B) Based on the Cambridge reference accession number, it displays the alignment sequence data for sample R-1. Concerning the tRNALeu (UUR) gene, the most crucial portion is that... (C) Displayed the chromatogram for sample R-9, which was generated by the sequencing procedure carried out in South Korea by Macrogen Inc.

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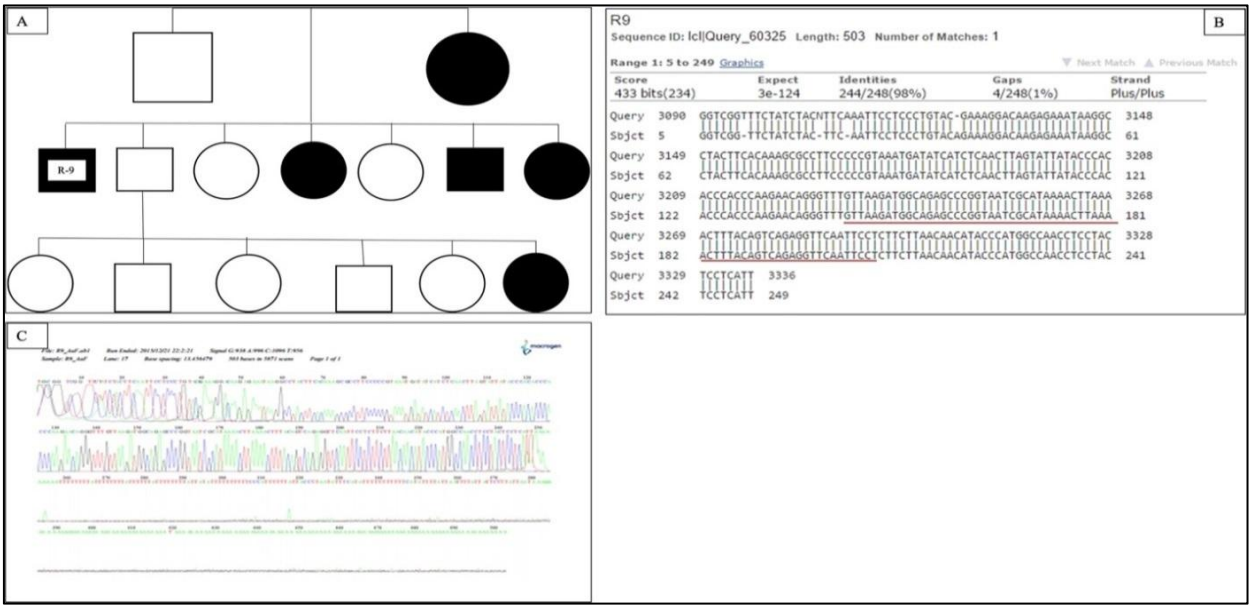

Pedigree 3

A 56-year-old individual was diagnosed with coronary heart disease. The family history revealed that his mother, sister, and brother also had a history of cardiovascular illness, as seen in figure 6A. Figure 6B presents the alignment sequencing data for the patient's R-9 sample, while Figure 6C illustrates the chromatogram flow of the sequencing findings. This individual did not have 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 tRNALeu (UUR) gene. (C) Illustrates the sequencing process conducted by Macrogen Inc. in South Korea, generating a chromatogram for sample R-10.

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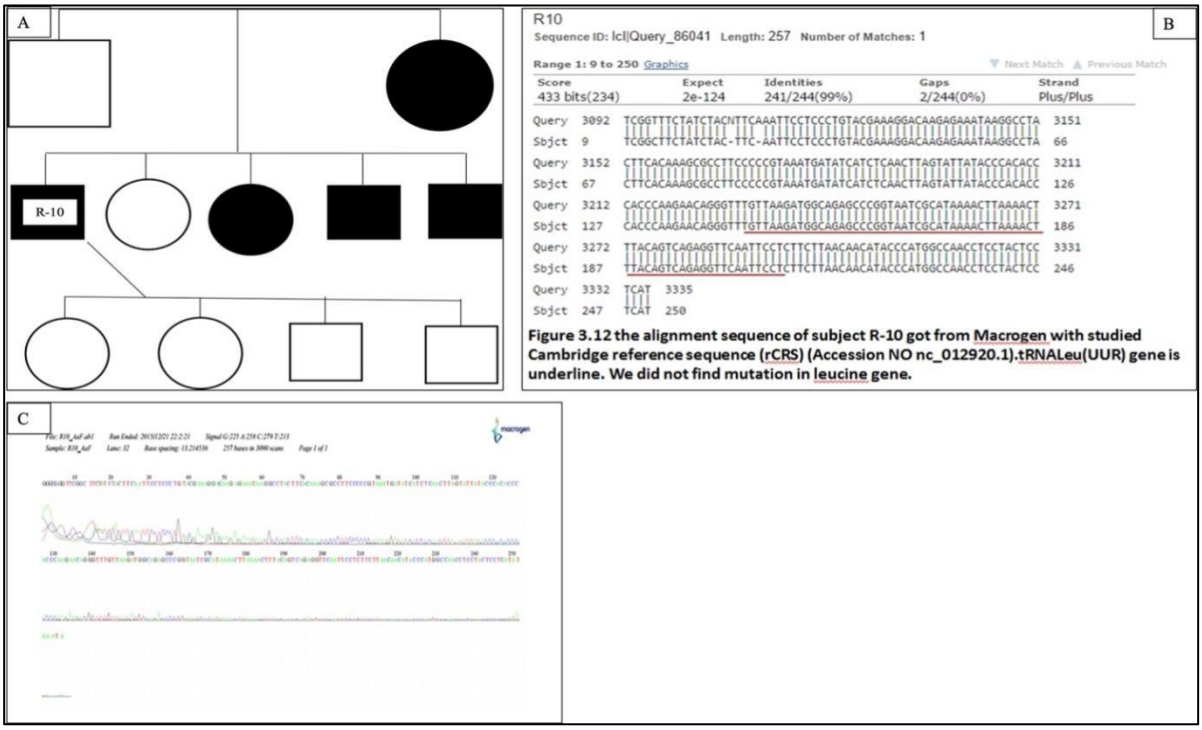


Their medical histories indicated that the individuals had hypertension. On any patient's $tRNA^{Leu\ (UUR)}$ gene, there were no mutations identified. Even though there is a two-nucleotide mismatch in the alignment, we were able to determine from this sequencing result's chromatogram that it was a technical 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.

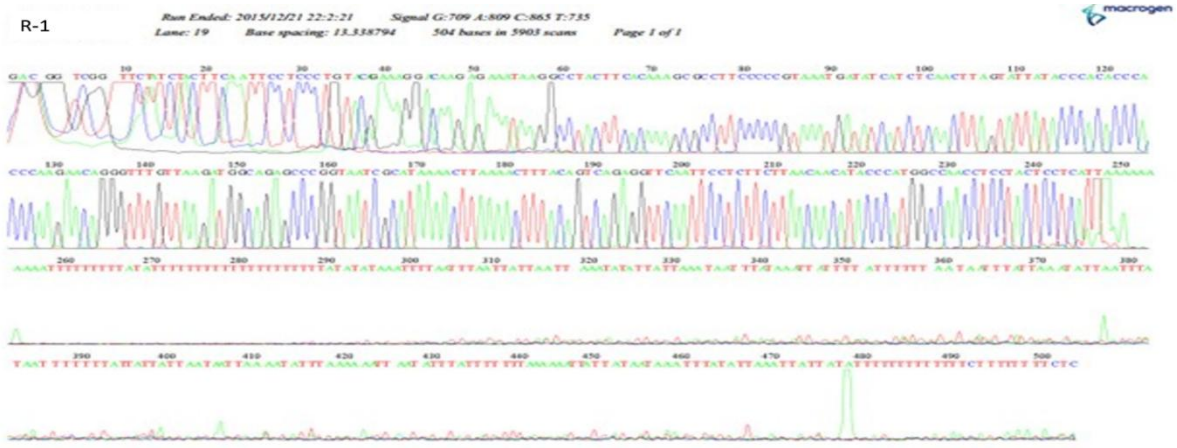
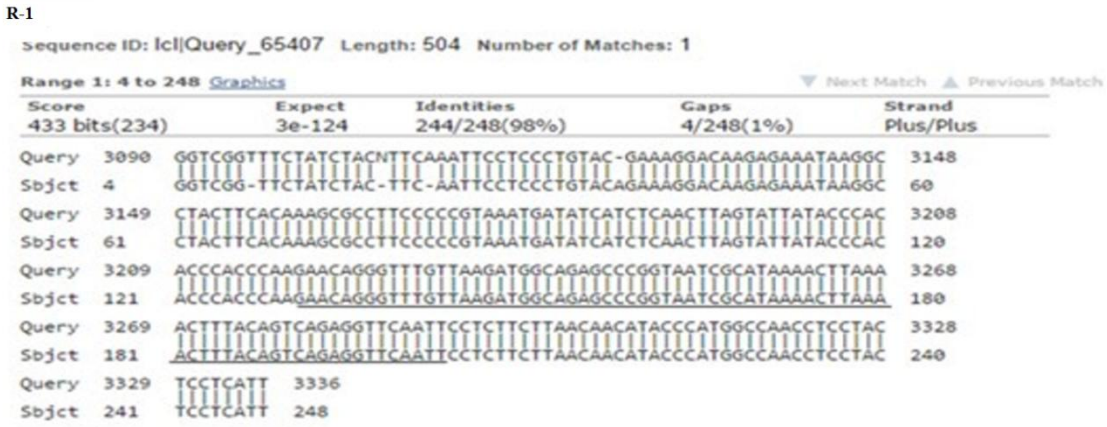


Figure 8: Data from the subject R-1 sequencing are aligned. The Cambridge reference accession number was used to compare the sequence. The highlighted region indicates the tRNALeu (UUR) gene.



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 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 investigation established that twenty samples from CVD patients were collected from various hospitals in Pakistan. The mean age of the patients was 60.15 years, accompanied by a standard deviation of 12.50 years. The sequencing results revealed a two-nucleotide mismatch in the alignment. The A-to-G mutation at nucleotide pair 3243 of the mitochondrial gene was initially not present in the subject of the pedigree. This individual did not exhibit the A-to-G mutation at nucleotide position 3243 in the mitochondrial gene mentioned in the second pedigree. The patient did not display the A-to-G mutation at nucleotide position 3243 in the specified mitochondrial gene of the third pedigree. The two-nucleotide mismatch in the alignment was identifiable from sequencing in hypertension. The alignment of the patient's R-1 sequencing data identified a singular sample of findings, revealing a mutation in the mtDNA tRNALeu (UUR) gene in patients with cardiovascular disease (CVD).

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