Pakistan J. Zool., pp 1-9, 2024. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20240123131923

Studies on the Identification of Variants in Ribosomal RNA Genes in Maternal Diabetic Families: A Combined Approach Involving Nucleotide Sequence Analysis and Bioinformatics

Sajid ul Ghafoor[1]*, Aziz-ud-Din[1]*, Khushi Muhammad[1], Inamullah[1] and Abid ul Ghafoor[2]

[1]Department of Biotechnology and Genetic Engineering, Hazara University, Mansehra 21300, Khyber Pakhtunkhwa, Pakistan.
[2]Department of Medicine, Tehsil Headquarters Hospital, Khwaza Khela, District Swat, Khyber Pakhtunkhwa, Pakistan.

Article Information
Received 23 January 2024
Revised 05 March 2024
Accepted 17 March 2024
Available online 01 May 2024
(early access)

Authors' Contribution
ADD designed the study. SUG conducted the experimentation. SUG wrote the manuscript. KM, AUD and Inamullah edited and finalized the Draft for publication. AUG identified the families in the field and confirmed diabetic cases in the hospital.

Key words
***MT-RNR2* gene, 16*S* ribosomal RNA, Secondary structure, Oxidative phosphorylation, Diabetes mellitus, Mitochondrial dysfunction**

ABSTRACT

The *MT-RNR2* gene plays a crucial role in encoding 16*S* ribosomal RNA, which is essential for the mitoribosomal larger subunit (mt-LSU). Variations in this gene have the potential to disrupt the secondary structure of 16*S* rRNA, affecting the assembly of mt-LSU necessary for protein synthesis. These alterations can compromise oxidative phosphorylation (OXPHOS) complexes, which have been implicated in the development of diabetes mellitus (DM). In this study, we analyzed four maternal diabetic families to investigate *MT-RNR2* gene variation and its impact on 16*S* rRNA secondary structure. Next-generation sequencing (NGS) of the entire mitochondrial DNA (mtDNA) of sample "B3" revealed the presence of the m.1811A>G variation in the *MT-RNR2* gene, classified into haplogroup "U". Subsequent verification through Sanger sequencing identified the same m.1811A>G variation in fourteen members across the four families. The secondary structure of the entire 1559 bases 16*S* rRNA was constructed, incorporating the single variation "141A>G" corresponding to m.1811A>G. Analysis of maximum free energy (MFE ΔG) indicated -337.37 kcal/mol for the reference structure and -338.87 kcal/mol for the altered structure. Three-dimensional structure analysis of the entire reference and altered 16*S* rRNA showed a root mean square deviation (RMSD) of 85.390 Å. The presence of the m.1811A>G variation in fourteen members of the diabetic families, along with its verified impact on MFE and structures of mitochondrial 16*S* rRNA, suggests its potential association with DM within these families. Future investigations may further explore the functional consequences of identified variants through *in vitro* and *in vivo* experiments, providing deeper insights into the pathophysiological mechanisms underlying mitochondrial dysfunction in diabetes.

INTRODUCTION

Diabetes mellitus (DM) is a common metabolic disorder that affects 529 million people worldwide (Ong *et al*., 2023). Pakistan stands 3rd globally with 33 million reported cases of DM (Akhtar *et al*., 2019). Almost 90-95% of DM cases reported worldwide were represented by type 2 diabetes mellitus (T2DM) (Kaur *et al*., 2018; Ong *et al*., 2023). Genetic predisposition increased the risk of DM in people consuming unhealthy diets and sedentary lifestyles (Zheng *et al*., 2018).

Hereditary mitochondrial diabetes was caused by mutations in mitochondrial DNA (mtDNA), and so far 54 mutations have been discovered in different genes of mtDNA in DM patients (Dabravolski *et al*., 2021). Variations accumulated across different mitochondrial genomes classify them into different haplogroups (Tanaka *et al*., 2004) and can make a haplogroup more prone to a disease (Kofler *et al*., 2009; Gómez-Durán *et al*., 2010; Alwehaidah *et al*., 2020). Mitochondrial haplogroup "U" was found linked to maternal diabetes (Martikainen *et al*., 2015). A homoplasmic state of mitochondrial mutations across various tissues may increase the chance of failure of metabolic reactions and cause disease, particularly oxidative phosphorylation (OXPHOS). This failed state of

* Corresponding author: sajidulghafoor@gmail.com, geneticsaz@gmail.com
0030-9923/2024/0001-0001 $ 9.00/0

the OXPHOS reactions promotes elevated manufacturing of reactive oxygen species (ROS), reduces insulin responsiveness, less ATP production, and accumulation of glucose across tissues, eventually resulting in DM (Krako Jakovljevic *et al*., 2021). The OXPHOS complexes consist of proteins produced by thirteen protein-coding genes of mtDNA. The messenger RNAs (mRNAs) of these genes are translated by mitochondrial ribosomes, which are inherently present within the mitochondria (Brown *et al*., 2014; Amunts *et al*., 2015).

Mitoribosomes have a sedimentation coefficient of 55*S* and contain a large (mt-LSU, 39*S*) and small (mt-SSU, 28*S*) subunit. The mt-LSU and mt-SSU contain 16*S* rRNA and 12*S* rRNA, respectively. The *MT-RNR2* gene transcribes the 16*S* rRNA, while 12*S* rRNA was transcribed by the *MT-RNR1* gene of the mitochondrial genome. Inside the mitoribosomal units, the 12*S* and 16*S* rRNAs form secondary structures (Amunts *et al*., 2015; Hilander *et al*., 2021). The *MT-RNR2* gene consists of 1559 bp and was located between nucleotides m.1671-3229 in the rCRS (revised Cambridge Reference Sequence).

The gene was reported to have mutations linked to DM, *e.g.,* m.2706G>A, m.3010G>A, and m.3243A>G found in Chinese and Finnish diabetic communities (Liao *et al*., 2008; Soini *et al*., 2012). Similarly, five mutations (m.1811A>G, m.2706A>G, m.2831G>A, m.3010G>A, and m.3197T>C) were discovered in *MT-RNR2* gene which induced substantial changes in 16*S* rRNA structure in Leber's hereditary optic neuropathy (LHON) patients (Jiang *et al*., 2017; Rovcanin *et al*., 2020). The mutations were predicted to have structural variations in 16*S* rRNA (Liao *et al*., 2008; Soini *et al*., 2012; Jiang *et al*., 2017). The structures of rRNAs were found crucial for human mitoribosome assembly (Amunts *et al*., 2015), and their variation was reported to affect ATP metabolism from its substrates (Bar-Yaacov *et al*., 2016). It was also reported that a homoplasmic mtDNA mutation m.1811A>G was found to be related to monocyte activation levels in humans (Khotina *et al*., 2022). In clinical studies, 16*S* rRNA structural variations affected the performance of mitoribosomal mt-LSU (Elson *et al*., 2015a).

It is evident that mitochondrial variations in *MT-RNR2* gene can affect the structure of 16*S* ribosomal RNA and have an impact on protein making machinery of mitochondria as reported in LHON and diabetes (Bar-Yaacov *et al*., 2016; Rovcanin *et al*., 2020).

The study is crucial as it addresses the significant global burden of diabetes mellitus, particularly in Pakistan, by investigating the role of mitochondrial DNA variations, such as those in the *MT-RNR2* gene, in predisposing individuals to the disease. Understanding how these variations affect mitochondrial ribosomal RNA structure sheds light on the underlying molecular mechanisms contributing to diabetes, potentially leading to novel diagnostic and therapeutic strategies. In this investigation, we scrutinized the *MT-RNR2* gene for sequence variations and assessed their potential impact on the secondary structure of 16*S* rRNA in family members affected by maternal diabetes mellitus.

MATERIALS AND METHODS

Genetic factors within families that may lead to Diabetes mellitus can originate from either nuclear or mitochondrial genes. However, the study's focus was specifically on analyzing the mitochondrial *MT-RNR2* gene. Therefore, a meticulous selection process was necessary to identify familial mitochondrial diabetic families accurately. Criteria were developed to selectively exclude nuclear genes and include mitochondrial genes in the study. The criteria for family selection comprised of (i) adult diabetic patients whose data was available in the government hospitals. (ii) Families with diabetic patients whose familial diabetic status was confirmed from their clinical records (Weykamp, 2013) and World Health Organization criteria (Petersmann *et al*., 2019). (iii) Data on the ancestors of the patients was collected. Pedigrees of each family were built through an online tool (https://pedigree.progenygenetics.com/). To avoid nuclear genome involvement, the family who had diabetes in only female ancestors. The consent was obtained from the members for inclusion in the study. All the families with both paternal and maternal diabetic ancestors were excluded. The family with the maximum number of available members for DNA sampling was selected.

Based on the criteria mentioned, four families were selected that had only maternal ancestors involved in diabetes. Data of members from the selected families viz. Gender, disease status, age, and medicine used were collected (Supplementary Table I). Additionally, three non-related and non-diabetic samples A1, A4, and A5 were taken as a control for *MT-RNR2* gene sequencing. Venous blood was collected from all selected members in sterile commercial EDTA tubes. Total genomic DNA was extracted from all the samples using Phenol: Chloroform method (Grimberg *et al*., 1989).

Next-generation sequence (NGS) DNA sequencing and PCR amplification of MT-RNR2 *gene*

A single-family member (B3) of a family (B) was subjected to whole mtDNA NGS analysis. Variations at various locations were observed in the data, however as per the objective only *MT-RNR2* gene was considered for the study (Table I), and additional NGS data was provided in the

supplementary data file. The heteroplasmic/homoplasmic status of variations in *MT-RNR2* was sorted out in variant call format (VCF) files. The Mitomap database was searched for the most common variants and haplotypes of the Whole genome sequence of the sample (B3) from family B (Lott *et al*., 2013). The whole mtDNA Fasta file of B3 was put in Haplogrep 3 software to find out haplogroup (Schonherr *et al*., 2023). The variation found in NGS data was validated further through PCR and commercial Sanger sequencing (https://dna.macrogen.com) in all family members of family B and other selected families. The primers used for Sanger sequencing were designed through primer 3 software (https://primer3.ut.ee/).

The primer sequences were.

RNR2-F (1296…1315) AGTAAGCGCAAG-TACCCACG and

RNR2-R (3315…3336) AATGAGGAGTAGGAG-GTTGGCC.

Table I. NGS Data of *MT-RNR2* variations in sample B3 from Diabetic Family of Swat Khyber Pakhtunkhwa Pakistan.

Pos	Ref	Alt	GT	AD	DP	GQ	FRE	MF
1811	A	G	1/1	6/10574	10583	255	0.9991	7.8%
2706	A	G	1/1	11/32675	32687	255	0.9997	77.8%
3107	N	A	1/1	0/7	16688	35	0.875	No record

Pos refers to the position on the rCRS reference genome (NC_012920.1). Ref/reference, Base type of Reference genome, Alt/alteration, Base type of alteration (in B3 sample). GT/Genotype (Heteroplasmic 0/1, Homoplasmic 1/1). AD, Number of bases supporting the reference genome/Number of bases supporting the alteration. DP, Total depth of the position. GQ, Genotype quality value (must be more than 30). FRE, Alteration frequency, number of bases supporting alteration/ (number of bases supporting reference genome + number of bases supporting alteration). MF, Mitomap frequency.

The Sanger sequenced (ab1) files received from a commercial sequencing firm were aligned to the reference genome (NC_012920.1) obtained from the NCBI GenBank in Ugene software (http://ugene.net/) for confirmation of variation identified in NGS data. After the variation was confirmed, the whole *MT-RNR2* reference and altered gene sequences were converted to 16*S* rRNA sequences in Ugene (Okonechnikov *et al*., 2012). The 2-dimensional (2D) secondary structure of complete (1559 bases) 16*S* rRNA having the m. 1811 A>G altered and reference (NC_012920.1) was predicted through "RNAfold" webserver with a minimum free energy option (MFE) and partition function. The MFE was calculated for both structures (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) (Gruber *et al*., 2008) and the data obtained was put in Table III. The same sequences were used to create three dimensional (3D) model of both 16*S* reference and altered rRNA in Xiao lab servers (http://biophy.hust.edu.cn/new/3dRNA) (Zhang *et al*., 2022). The top-scoring 3D PDB files were downloaded from the servers and visualized in ChimeraX software (https://www.cgl.ucsf.edu/chimerax/) (Pettersen *et al*., 2021). The Matchmaker tool of the software was used to make pair-wise alignment by superimposing both models (Meng *et al*., 2006). For pair-wise alignment Needleman-Wunsch algorithm was used and root mean square deviation (RMSD) was calculated. All other options were kept on default. The distance tool of ChimeraX was used to calculate the distance between the altered and reference base in angstroms (Å).

Table II. Free energy assessments of 16*S* rRNA 2D models.

	Minimum free energy (ΔG) prediction kcal/mol	Thermodynamic ensemble prediction of free energy (ΔG) kcal/mol
Reference	-337.37	-363.09
Altered	-338.87	-364.15
Difference	-1.5	-1.06

ΔG refers to Gibbs free energy

Table III. Superimposed model comparison of reference and mutated 16*S* rRNA molecules.

141 A to G residues distance	RMSD	
	8 pruned atoms	1559 residues
86.004 Å	0.992 Å	85.390 Å

RMSD refers to root means square deviation.

RESULTS

Sample B3 was obtained from Family B, which was selected for Whole mtDNA sequencing and belonged to district Swat, Khyber Pakhtunkhwa. The other three families included in the study were Family C, Family G1, and Family G2. Family C was from District Okara, Punjab, while the latter two families were from District Mansehra, Khyber Pakhtunkhwa. Detailed information regarding the samples from these families is provided in Supplementary Table I.

The pedigree of Family B, consisting of ten samples is shown in Figure 1, whereas the pedigrees of the other three families are provided in the supplementary files. These pedigrees exhibited a maternal lineage of the disease, which was essential for the investigation of the mitochondrial *MT-RNR2* gene.

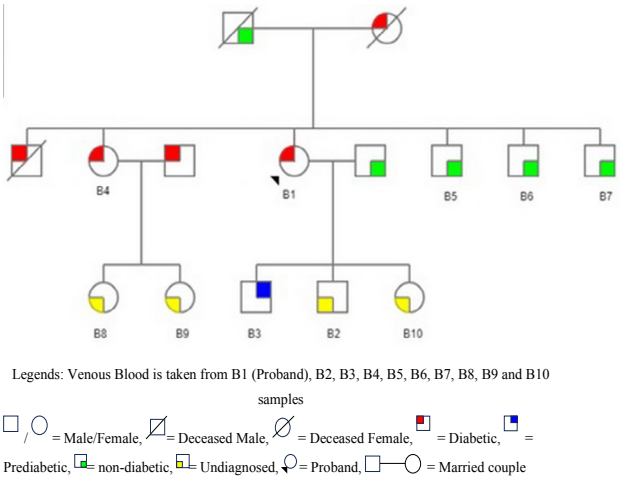

Fig. 1. Pedigree of diabetic family from Swat Khyber Pakhtunkhwa Pakistan.

The *MT-RNR2* gene region (1671-3229) of the B3 sample (Table I) was successfully amplified through NGS (Supplementary data is available for further reference). Within this gene region, a single variation was identified at position m.1811A>G, showing high quality with a Genome Quality (GQ) score of 255 and a frequency of 0.9991. The reference nucleotide "A" was observed six times, while the altered base "G" was present 10,574 times, indicating that this variation was homoplasmic.

In the same gene, two other variations were noted, namely 2706A>G and 3107N>A. The m.1811A>G and m.2706A>G variations had Mitomap frequencies of 7.8% and 77.8%, respectively, while m.3107N>A had no recorded frequency (NR).

The variation m.1811A>G, along with m.2706A>G, was homoplasmic within the sample B3 mitochondrial population. However, m.3107N>A was detected with a very low-Genotype Quality (GQ) score of 35, supported by only seven good-quality bases.

Contrarily, m.2706A>G was detected with high quality, but this variation was present in 77.8% of the genome sequences reported in Mitomap, suggesting it as a common variant not specific to any disease. Consequently, further analysis focused on m.1811A>G, which had a 7.8% frequency in reported sequences.

Using the Mitomap tool, Haplogrep 3, the consensus sequence of the whole mtDNA genome sequence of B3 was identified, corresponding to the "U" haplogroup. Within the "U" haplogroup, the m.1811A>G variation was identified as the determining factor of the "U2'3'4'7'8'9" sub-haplotype.

To screen the m.1811A>G in other samples of the four selected families, PCR amplification was conducted in samples A1, A4, A5, B1, B3, B4, B7, B8, B10, C1, C2, C5, G2, G13, G3, G5, and G6. The obtained bands were located at the expected 2041 bp location. After successful PCR, the product was sequenced through Sanger sequencing in samples A1, B1, B3, B4, B7, B8, and B10. The results presented in Figure 2, confirmed the presence of the altered "G" nucleotide at position 1811 in samples B1, B3, B4, B7, B8, and B10, consistent with the NGS data from sample B3. Similarly, the m.1811 A>G variation was confirmed in samples C1, C2, C5, G2, G13, G3, G5, and G6 from families C, G1, and G2.

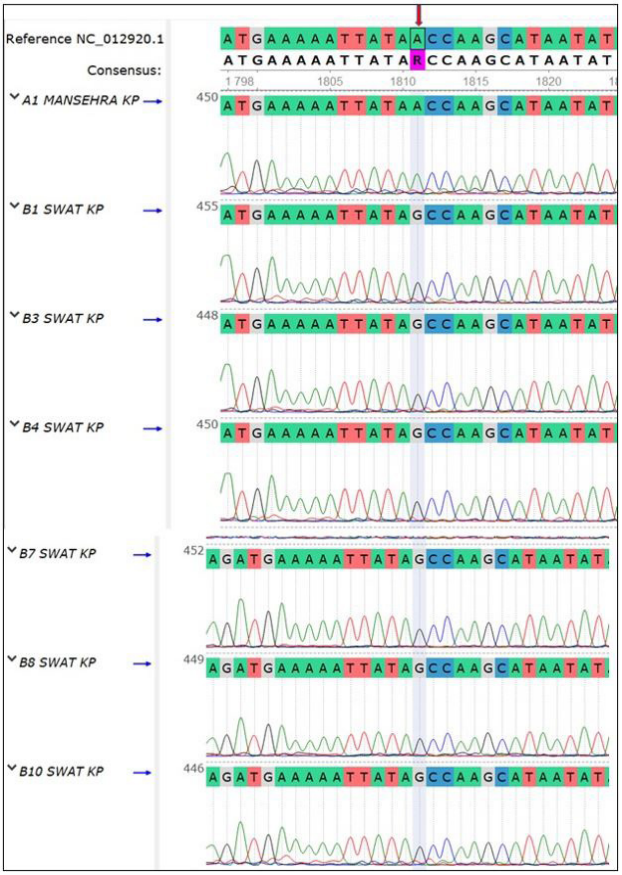

Fig. 2. *MT-RNR2* gene alteration status at genomic position m.1811. Samples A1, B1, B3, B4, B7, B8, and B10 of a Diabetic Family from Swat Khyber Pakhtunkhwa Pakistan. A>G Variation was highlighted with a vertical arrow at "1811".

Subsequently, the sequences of the *MT-RNR2* gene were converted to 16*S* rRNA sequences, with reference to 16*S* rRNA nucleotide sequence "A" and the altered version "G" at position 141 depicted in Figure 3. The alteration impacted an area extending approximately from position 100 to 250 nucleotide bases of 16*S* RNA.

Significant differences were observed between both structures, affecting the free energy of the models (Fig. 4). The reference and altered structures had minimum free energies (MFE) of -337.37 kcal/mol and -338.87 kcal/mol, respectively (Table III).

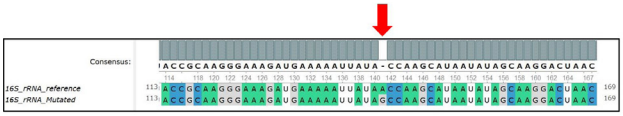

Fig. 3. Reference and altered 16*S* rRNA at position 141A>G. A>G variation was highlighted with a vertical arrow at "141".

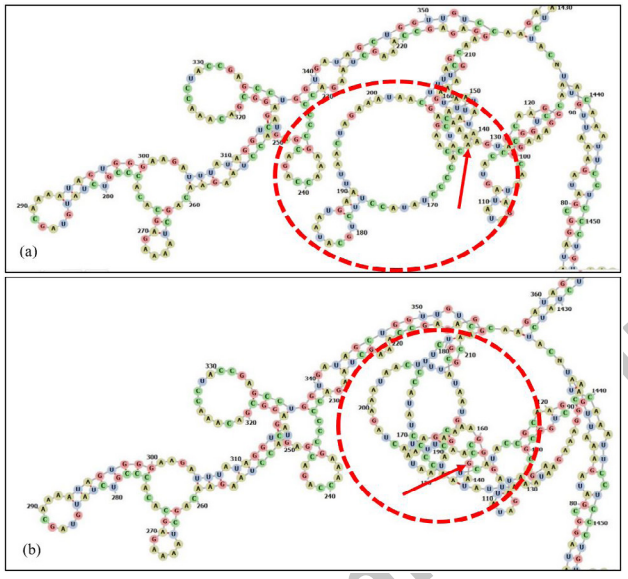

Fig. 4. RNA Fold structure of 16*S* rRNA molecule and impact of a single 141A>G variation.
(a) Reference structure; (b) Altered structure. The red encircled area specifies the structural variation.

Following visual confirmation of the variations in the 2D models, 3D models of the two 16*S* rRNA molecules were constructed and aligned (as illustrated in Fig. 5a). These models featured a yellow chain representing the reference structure and a blue chain representing the altered structure of 16*S* rRNA, with nucleotides 141A and 141G, respectively. Each chain consisted of 1559 bases. A section of the same model was magnified to determine the distance between the two bases on different chains (as shown in Fig. 5b). The comparison of these superimposed models yielded results, including root mean square deviation (RMSD) data (Table III). Furthermore, the ChimeraX distance tool was utilized to calculate the distance between residues.

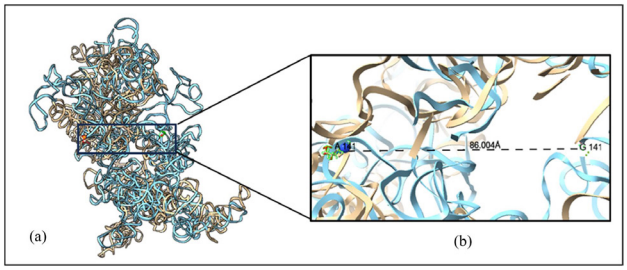

Fig. 5. Zoomed superimposed 3D structures of whole 16*S* rRNA specifying position 141A>G.
(a) Superimposed chains reference yellow and altered blue. (b) Zoomed 141A>G with distance *i.e.,* 86.004Å.

DISCUSSION

Diabetes mellitus is a chronic disease present commonly in Pakistan. Various environmental and genetic factors are responsible for the widespread occurrence of the disease. The study's objective was to discover variations in 1559 bp long *MT-RNR2* gene and its impact on the secondary structures of 16*S* rRNA in maternal diabetic families. A careful selection criterion was adopted to ensure the selection of mitochondrially affected families (Supplementary Table I). NGS sequencing of "B3" a pre-diabetic patient revealed an alteration A>G at position 1811 of *MT-RNR2* Gene, which encodes 16*S* rRNA. A genotype frequency of 0.999 was detected for the nucleotide "G" with a genotype quality (GQ) of 255, comprising 10574 instances of the "G" base. The same position had only 6 "A" bases. This proves that m.1811A>G alteration was homoplasmic in mtDNA population of B3 (Table I) and has a high probability of causing an impact (Swalwell *et al*., 2008). Further analysis through Sanger sequencing (Baudhuin *et al*., 2015) confirmed the presence of the same alteration throughout fourteen other sequenced samples of the four families, except A1, which was a member of a non-diabetic family (Fig. 2).

Several mitochondrial haplogroups exhibit a predisposition to certain diseases and a greater susceptibility when compared to other haplogroups (Farha *et al*., 2016; Strobbe *et al*., 2018). In the present research, the whole mtDNA sequenced, belonged to haplogroup "U2'3'4'7'8'9," a subgroup of haplogroup "U". A review of the existing literature unveiled that haplogroup U was more prevalent among individuals with maternal family history of diabetes mellitus (Fig. 1) in comparison to those who had no such family history (Martikainen *et al*., 2013, 2015).

Direct experiments to correlate mitochondrial ribosomal RNA variations with a disease were not available (Elson *et al*., 2015b). Hence a careful structural

evaluation approach was carried out to understand the impact of 1811A>G variation on the structure of mitochondrial 16*S* rRNA.

In the first step a structure with the variation was obtained through RNAfold server which revealed deviations from standard reference 16*S* rRNA structure. Interestingly a single alteration of 1811A>G considerably changed the structure at several rings and shape of the molecule was altered (Figs. 4, 5). MFE and thermodynamic ensemble evaluation (Ding *et al*., 2005) of both structures have a difference of -1.5 and -1.06, respectively. The altered 16*S* rRNA has more free energy for reactions in both algorithms (Table II). This revealed the impact of variation on the MFE of the 16*S* rRNA.

In the second step 3D modelling was carried out through Xiao lab server for 3D prediction of 16*S* rRNA (Zhang *et al*., 2022). ChimeraX was found to be very useful and user-friendly 3D model analysis software. The "Matchmaker" tool of ChimeraX (Pettersen *et al*., 2021) analyzed the two models 16*S* rRNA (Reference and altered) downloaded from Xiao labs and superimposed both very easily without much computational load (Fig. 5). The results of Matchmaker analysis reveal average deviation of corresponding residues present in both structures at 85.390 Å (RMSD). The actual A and G bases were at 86.004 Å. Eight pruned residues in 16*S* rRNA structures were at an average distance of 0.992 Å, which shows least impact of the structural variation at distance (Table III). It proves that single base variation from A to G made structural difference and put all residues at a considerable distance. It also suggests the sensitive nature of 16*S* rRNA molecules. The variations examined in 16*S* rRNA 2D and 3D structures as well as the estimates of ΔG values prove the impact of m.1811A>G variation. These structural variations could impact the binding pattern of rRNA to proteins in the larger subunit of mitoribosome, which can affect its performance during translation and might have initiated diabetes mellitus in the families as reported previously in other studies (Rackham *et al*., 2012; Jiang *et al*., 2017). The study also demonstrated that in absence of paternal mtDNA involvement, the maternal mtDNA variation was passed from mother to children, exhibiting a characteristic pattern of mitochondrial and maternal inheritance (Deluca and O'farrell, 2012).

CONCLUSION

We conducted a sequence analysis of the *MT-RNR2* gene in four maternally inherited diabetic families from Pakistan. Our analysis revealed a novel variation of A>G at position 1811 of the mtDNA genome within the *MT-RNR2* gene and placed the mtDNA in haplogroup "U2'3'4'7'8'9," a subgroup of haplogroup "U". Haplogroup "U" has been associated with maternal diabetes in previous studies. Structural and free energy analysis of the resulting altered 16*S* rRNA at position 141A>G demonstrated significant variations, impacting both minimum free energy (MFE) and root mean square deviation (RMSD). The presence of the m.1811A>G variation across fourteen family members, along with subsequent structural alterations, likely compromised the functionality of the mitoribosome, potentially triggering diabetes mellitus in the selected families from Pakistan. Our findings establish a foundation for further investigation across diverse populations worldwide, aiming to validate this identified variation as a potential biomarker for early diagnosis of maternal diabetes mellitus.

Subsequent research efforts could probe into the functional implications of the identified variants through both in vitro and in vivo experiments, offering more profound insights into the pathophysiological mechanisms associated with mitochondrial dysfunction in diabetes.

ACKNOWLEDGEMENT

All family members were highly acknowledged for providing blood samples and health-related data, which will add to the scientific information and understanding of DM from Pakistan.

Funding

This study was supported by the Department of Biotechnology and Genetic Engineering and the lab facilities were used. The chemicals and sequencing analysis were self-funded.

IRB approval

The study was approved by Advanced Study and Research board, Hazara University Mansehra under notification number Dir A&R/Notifications/HU/2021/270.

Ethical statement

Ethical principles as mentioned in "World Medical Association Declaration of Helsinki" were followed (World Medical, 2013) and approval of the study was obtained from Hazara University Ethical Research Committee under letter number HU/11 dated 8 Nov 2023. The study design included subjects' privacy, and informed consent was obtained by explaining the purpose of the research.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20240123131923

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Akhtar, S., Nasir, J.A., Abbas, T. and Sarwar, A., 2019. Diabetes in Pakistan: A systematic review and meta-analysis. *Pak. J. med. Sci.,* **35**: 1173-1178. https://doi.org/10.12669/pjms.35.4.194

Alwehaidah, M.S., Bakhiet, M. and Alfadhli, S., 2020. Mitochondrial haplogroup reveals the genetic basis of diabetes mellitus type 2 comorbidity in psoriasis. *Med. Principles Pract. Int. J. Kuwait Univ., Hlth. Sci. Centre*, https://doi.org/10.1159/000509937

Amunts, A., Brown, A., Toots, J., Scheres, S.H.W. and Ramakrishnan, V., 2015. The structure of the human mitochondrial ribosome. *Science,* **348**: 95-98. https://doi.org/10.1126/science.aaa1193

Bar-Yaacov, D., Frumkin, I., Yashiro, Y., Chujo, T., Ishigami, Y., Chemla, Y., Blumberg, A., Schlesinger, O., Bieri, P. and Greber, B., 2016. Mitochondrial 16S rRNA is methylated by tRNA methyltransferase TRMT61B in all vertebrates. *PLoS Biol.,* **14**: e1002557. https://doi.org/10.1371/journal.pbio.1002557

Baudhuin, L.M., Lagerstedt, S.A., Klee, E.W., Fadra, N., Oglesbee, D. and Ferber, M.J., 2015. Confirming variants in next-generation sequencing panel testing by sanger sequencing. *J. mol. Diagn.,* **17**: 456-461. https://doi.org/10.1016/j.jmoldx.2015.03.004

Brown, A., Amunts, A., Bai, X.C., Sugimoto, Y., Edwards, P.C., Murshudov, G., Scheres, S.H.W. and Ramakrishnan, V., 2014. Structure of the large ribosomal subunit from human mitochondria. *Science,* **346**: 718-722. https://doi.org/10.1126/science.1258026

Dabravolski, S.A., Orekhova, V.A., Baig, M.S., Bezsonov, E.E., Starodubova, A.V., Popkova, T.V. and Orekhov, A.N., 2021. The role of mitochondrial mutations and chronic inflammation in diabetes. *Int. J. mol. Sci.,* **22**: 6733. https://doi.org/10.3390/ijms22136733

Deluca, S.Z. and O'farrell, P.H., 2012. Barriers to male transmission of mitochondrial DNA in sperm development. *Dev. Cell,* **22**: 660-668. https://doi.org/10.1016/j.devcel.2011.12.021

Ding, Y., Chan, C.Y. and Lawrence, C.E. 2005. RNA secondary structure prediction by centroids in a Boltzmann weighted ensemble. *RNA,* **11**: 1157-1166. https://doi.org/10.1261/rna.2500605

Elson, J.L., Smith, P.M., Greaves, L.C., Lightowlers, R.N., Chrzanowska-Lightowlers, Z.M.A., Taylor, R.W. and Vila-Sanjurjo, A., 2015a. The presence of highly disruptive 16S rRNA mutations in clinical samples indicates a wider role for mutations of the mitochondrial ribosome in human disease. *Mitochondrion,* **25**: 17-27. https://doi.org/10.1016/j.mito.2015.08.004

Elson, J.L., Smith, P.M. and Vila-Sanjurjo, A., 2015b. Heterologous inferential analysis (HIA) as a method to understand the role of mitochondrial rRNA mutations in pathogenesis. *Methods mol. Biol.,* **1264**: 369-383. https://doi.org/10.1007/978-1-4939-2257-4_32

Farha, S., Hu, B., Comhair, S., Zein, J., Dweik, R., Erzurum, S.C. and Aldred, M.A., 2016. Mitochondrial haplogroups and risk of pulmonary arterial hypertension. *PLoS One,* **11**: e0156042. https://doi.org/10.1371/journal.pone.0156042

Gómez-Durán, A., Pacheu-Grau, D., López-Gallardo, E., Díez-Sánchez, C., Montoya, J., López-Pérez, M.J. and Ruiz-Pesini, E., 2010. Unmasking the causes of multifactorial disorders: OXPHOS differences between mitochondrial haplogroups. *Hum. mol. Genet.,* **19**: 3343-3353. https://doi.org/10.1093/hmg/ddq246

Grimberg, J., Nawoschik, S., Belluscio, L., Mckee, R., Turck, A. and Eisenberg, A., 1989. A simple and efficient non-organic procedure for the isolation of genomic DNA from blood. *Nucl. Acids Res.,* **17**: 8390. https://doi.org/10.1093/nar/17.20.8390

Gruber, A.R., Lorenz, R., Bernhart, S.H., Neuböck, R. and Hofacker, I.L., 2008. The vienna RNA websuite. *Nucl. Acids Res.,* **36**: W70-W74. https://doi.org/10.1093/nar/gkn188

Hilander, T., Jackson, C.B., Robciuc, M., Bashir, T. and Zhao, H., 2021. The roles of assembly factors in mammalian mitoribosome biogenesis. *Mitochondrion,* **60**: 70-84. https://doi.org/10.1016/j.mito.2021.07.008

Jiang, W., Li, R., Zhang, Y., Wang, P., Wu, T., Lin, J., Yu, J. and Gu, M., 2017. Mitochondrial DNA mutations associated with type 2 diabetes mellitus in Chinese uyghur population. *Sci. Rep.,* **7**: 16989. https://doi.org/10.1038/s41598-017-17086-7

Kaur, R., Kaur, M. and Singh, J., 2018. Endothelial dysfunction and platelet hyperactivity in type 2 diabetes mellitus: molecular insights and therapeutic strategies. *Cardiovasc. Diabetol.,* **17**: 121. https://doi.org/10.1186/s12933-018-0763-3

Khotina, V.A., Bagheri Ekta, M., Baig, M.S., Wu, W.K., Grechko, A.V. and Sukhorukov, V.N., 2022. Challenges of mitochondrial DNA editing in mammalian cells: Focus on treatment of

cardiovascular disease. *Vessel Plus,* **6**: 65. https://doi.org/10.20517/2574-1209.2022.28

Kofler, B., Mueller, E.E., Eder, W., Stanger, O., Maier, R., Weger, M., Haas, A., Winker, R., Schmut, O. and Paulweber, B., 2009. Mitochondrial DNA haplogroup T is associated with coronary artery disease and diabetic retinopathy: A case control study. *BMC med. Genet.,* **10**: 1-7. https://doi.org/10.1186/1471-2350-10-35

Krako-Jakovljevic, N., Pavlovic, K., Jotic, A., Lalic, K., Stoiljkovic, M., Lukic, L., Milicic, T., Macesic, M., Stanarcic Gajovic, J. and Lalic, N.M., 2021. Targeting mitochondria in diabetes. *Int. J. mol. Sci.,* **22**: 6642. https://doi.org/10.3390/ijms22126642

Liao, W.Q., Pang, Y., Yu, C.A., Wen, J.Y., Zhang, Y.G. and Li, X.H., 2008. Novel mutations of mitochondrial DNA associated with type 2 diabetes in Chinese Han population. *Tohoku J. exp. Med.,* **215**: 377-384. https://doi.org/10.1620/tjem.215.377

Lott, M.T., Leipzig, J.N., Derbeneva, O., Xie, H.M., Chalkia, D., Sarmady, M., Procaccio, V. and Wallace, D.C., 2013. mtDNA variation and analysis using mitomap and mitomaster. *Curr. Protoc. Bioinf.,* **44**: 21-26. https://doi.org/10.1002/0471250953.bi0123s44

Martikainen, M.H., Ronnemaa, T. and Majamaa, K., 2013. Prevalence of mitochondrial diabetes in southwestern Finland: A molecular epidemiological study. *Acta Diabetol.,* **50**: 737-741. https://doi.org/10.1007/s00592-012-0393-2

Martikainen, M.H., Rönnemaa, T. and Majamaa, K., 2015. Association of mitochondrial DNA haplogroups and vascular complications of diabetes mellitus: A population-based study. *Diabetes Vasc. Dis. Res.,* **12**: 302-304. https://doi.org/10.1177/1479164115579007

Meng, E.C., Pettersen, E.F., Couch, G.S., Huang, C.C. and Ferrin, T.E., 2006. Tools for integrated sequence-structure analysis with UCSF Chimera. *BMC Bioinf.,* **7**: 339. https://doi.org/10.1186/1471-2105-7-339

Okonechnikov, K., Golosova, O., Fursov, M. and Team, U., 2012. Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics,* **28**: 1166-1167. https://doi.org/10.1093/bioinformatics/bts091

Ong, K.L., Stafford, L.K., Mclaughlin, S.A., Boyko, E.J., Vollset, S.E., Smith, A.E., Dalton, B.E., Duprey, J., Cruz, J.A. and Hagins, H., 2023. Global, regional, and national burden of diabetes from 1990 to 2021, with projections of prevalence to 2050: A systematic analysis for the global burden of disease study 2021. *Lancet*, **402**: 203–234. https://doi.org/10.1016/S0140-6736(23)01301-6

Petersmann, A., Muller-Wieland, D., Muller, U.A., Landgraf, R., Nauck, M., Freckmann, G., Heinemann, L. and Schleicher, E., 2019. Definition, classification and diagnosis of diabetes mellitus. *Exp. Clin. Endocrinol. Diabetes Off. J. German Soc. Endocrinol. German Diabetes Assoc.,* **127**: S1-S7. https://doi.org/10.1055/a-1018-9078

Pettersen, E.F., Goddard, T.D., Huang, C.C., Meng, E.C., Couch, G.S., Croll, T.I., Morris, J.H. and Ferrin, T.E., 2021. UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci.,* **30**: 70-82. https://doi.org/10.1002/pro.3943

Rackham, O., Mercer, T.R. and Filipovska, A., 2012. The human mitochondrial transcriptome and the RNA-binding proteins that regulate its expression. *Wiley Interdisciplin. Rev. RNA,* **3**: 675-695. https://doi.org/10.1002/wrna.1128

Rovcanin, B., Jancic, J., Samardzic, J., Rovcanin, M., Nikolic, B., Ivancevic, N., Novakovic, I. and Kostic, V., 2020. *In silico* model of mtDNA mutations effect on secondary and 3D structure of mitochondrial rRNA and tRNA in Leber's hereditary optic neuropathy. *Exp. Eye Res.,* **201**: 108277-108277. https://doi.org/10.1016/j.exer.2020.108277

Schonherr, S., Weissensteiner, H., Kronenberg, F. and Forer, L., 2023. Haplogrep 3- an interactive haplogroup classification and analysis platform. *Nucl. Acids Res.,* **51**: W263-W268. https://doi.org/10.1093/nar/gkad284

Soini, H.K., Moilanen, J.S., Finnila, S. and Majamaa, K., 2012. Mitochondrial DNA sequence variation in Finnish patients with matrilineal diabetes mellitus. *BMC Res. Notes*, **5**: 1-12. https://doi.org/10.1186/1756-0500-5-350

Strobbe, D., Caporali, L., Iommarini, L., Maresca, A., Montopoli, M., Martinuzzi, A., Achilli, A., Olivieri, A., Torroni, A. and Carelli, V., 2018. Haplogroup J mitogenomes are the most sensitive to the pesticide rotenone: relevance for human diseases. *Neurobiol. Dis.,* **114**: 129-139. https://doi.org/10.1016/j.nbd.2018.02.010

Swalwell, H., Blakely, E.L., Sutton, R., Tonska, K., Elstner, M., He, L., Taivassalo, T., Burns, D.K., Turnbull, D.M. and Haller, R.G., 2008. A homoplasmic mtDNA variant can influence the phenotype of the pathogenic m. 7472Cins MTTS1 mutation: Are two mutations better than one? *Eur. J. Hum. Genet.,* **16**: 1265-1274. https://doi.org/10.1038/ejhg.2008.65

Tanaka, M., Cabrera, V.M., González, A.M., Larruga,

J.M., Takeyasu, T., Fuku, N., Guo, L.J., Hirose, R., Fujita, Y., Kurata, M., Shinoda, K., Umetsu, K., Yamada, Y., Oshida, Y., Sato, Y., Hattori, N., Mizuno, Y., Arai, Y., Hirose, N., Ohta, S., Ogawa, O., Tanaka, Y., Kawamori, R., Shamoto-Nagai, M., Maruyama, W., Shimokata, H., Suzuki, R. and Shimodaira, H., 2004. Mitochondrial genome variation in eastern Asia and the peopling of Japan. *Genome Res.,* **14**: 1832-1850. https://doi.org/10.1101/gr.2286304

Weykamp, C., 2013. HbA1c: A review of analytical and clinical aspects. *Annls Lab. Med.,* **33**: 393. https://doi.org/10.3343/alm.2013.33.6.393

World Medical, A., 2013. World medical association declaration of Helsinki: Ethical principles for medical research involving human subjects. *J. Am. med. Assoc.,* **310**: 2191-2194. https://doi.org/10.1001/jama.2013.281053

Zhang, Y., Xiong, Y. and Xiao, Y., 2022. 3dDNA: A computational method of building DNA 3D structures. *Molecules,* **27**. https://doi.org/10.3390/molecules27185936

Zheng, Y., Ley, S.H. and Hu, F.B., 2018. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. *Nat. Rev. Endocrinol.,* **14**: 88-98. https://doi.org/10.1038/nrendo.2017.151

Pakistan J. Zool., pp 1-9, 2024. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20240123131923

Supplementay Material

Studies on the Identification of Variants in Ribosomal RNA Genes in Maternal Diabetic Families: A Combined Approach Involving Nucleotide Sequence Analysis and Bioinformatics

Sajid ul Ghafoor[1]*, Aziz-ud-Din[1]*, Khushi Muhammad[1], Inamullah[1] and Abid ul Ghafoor[2]

[1]Department of Biotechnology and Genetic Engineering, Hazara University, Mansehra 21300, Khyber Pakhtunkhwa, Pakistan.
[2]Department of Medicine, Tehsil Headquarters Hospital, Khwaza Khela, District Swat, Khyber Pakhtunkhwa, Pakistan.

Supplementary Table I. Details of samples collected from selected diabetic families.

ID	Gender	Disease status	Age	Onset	Medicine used
Family B Swat Khyber Pakhtunkhwa					
B1	Female	Diabetic/ Proband	55	47	Tagamet, Insulin, Various Anti-diabetic drugs
B2	Male	Undiagnosed/No symptoms	19	NA	None
B3	Male	Prediabetic	38	NA	Diet Control
B4	Female	Diabetic	51	36	Insulin, Various Anti-diabetic drugs
B5	Male	Normal	70	40	Antihypertensive
B6	Male	Normal	58	NA	None
B7	Male	Normal	63	NA	None
B8	Female	Undiagnosed/no symptoms	30	NA	None
B9	Female	Undiagnosed/no symptoms	29	NA	None
B10	Female	Undiagnosed/no symptoms	35	NA	None
Family C Okara Punjab					
C1	Female	Normal	48	NA	None
C2	Male	Diabetic	67	65	Metformin
C3	Male	Diabetic	53	48	Metformin
C4	Female	Diabetic	60	55	Metformin, Glucophage
C5	Male	Diabetic	35	31	Diet Control

Table continued on next page.....

* Corresponding author: sajidulghafoor@gmail.com, geneticsaz@gmail.com
0030-9923/2024/0001-0001 $ 9.00/0

ID	Gender	Disease status	Age	Onset	Medicine used
C6	Male	Diabetic	52	49	Glucophage
C7	Male	Normal	69	NA	None
C8	Female	Diabetic	58	56	Glucophage
C9	Male	Normal	49	NA	None
Family G1 Mansehra Khyber Pakhtunkhwa					
G1	Female	Diabetic	52	40	Diet control
G2	Female	Diabetic	65	30	Glucophage
G4	Male	Normal	48	NA	NA
G12	Female	Normal	38	NA	NA
G13	Female	Diabetic	80	50	Insulin
Family G2 Mansehra Khyber Pakhtunkhwa					
G3	Female	Prediabetic	50	40	Diet control
G5	Male	Prediabetic	55	38	Diet control
G6	Female	Diabetic	74	35	Glucophage
G7	Female	Normal	23	NA	NA
G11	Male	Diabetic	58	40	Insulin`