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Variants of *MT-RNR2, MT-TI* and *MT-TL1* Genes in Hypertrophic Cardiomyopathy Families of Pakistan

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MK and TAK collected data, performed experiments and wrote the manuscript. MFK and KU critically reviewed the manuscript and helped in data analysis and management. ST and AUD designed and supervised the project. IU and ST proofread the manuscript.

Key words
Cardiomyopathy, Mitochondrial, Mutations, HCM, Pakistan

ABSTRACT

Hypertrophic cardiomyopathy is a condition in which the myocardial muscles become thickened without an apparent cause. It exhibits the prevalence of 1/500 globally. Eighteen samples from four families of maternally inherited HCM were collected, based on their clinical records. Three mitochondrial encoded genes *16S-rRNA* (MT-RNR2), $tRNA^{Ile}$ (MT-TI) and $tRNA^{Leu}$ (MT-TL1) were targeted for analysis of mutation. Genomic DNA was extracted from buccal swab using Phenol: Chloroform: protocol. The target genes were amplified via polymerase chain reaction (PCR). The amplified gene products were sequenced and compared with the revised Cambridge Reference Sequence (rCRS) Accession-No. N_012920.1. Four mutations in *16S-rRNA* gene have been identified *viz* mt-2552T>A, mt-1811A>G, mt-1888A>G and mt-2467A>T and two are novel, mt- 2552T>A and mt-2467A>T while others have been previously reported, in patients of German and French population. For the first time, the mt-1888A>G and mt-1811A>G mutation in MT-RNR2 gene have been observed in patients of HCM in the Pakistani population and even not found in the control group of the same families. No mutation in mt-DNA encoded $tRNA^{Ile,}$ and $tRNA^{Leu}$ genes were observed, the mutations detected in mt-DNA encoded *16S-rRNA* gene are the pathogenic mutations associated with HCM in Pakistani population.

INTRODUCTION

A disorder in which asymmetric thickening occurs in the walls and septum of the left ventricle of the heart is called hypertrophic cardiomyopathy (HCM). Every year it affects about 0.2 % (1/500) of the population worldwide (Shishi *et al*., 2018). In East Africa, its reported prevalence is 0.19% (Maron *et al*., 2006). In America, it affects about 0.02 to 0.2% of the total population. In China, about 0.16% of the entire population is affected by HCM (Zou *et al*., 2004). In Pakistan, hardly any reported data is available regarding the prevalence of HCM. Most of the individuals younger than 35 years die every year due to HCM (Shishi *et al*., 2018). About ten percent of the HCM patients progress to dilated cardiomyopathy (DCM) with the alteration of the left ventricle that Hypertrophic cardiomyopathy causes an atrioventricular causes several heart failures (Spirito *et al*., 1987). block in the left chamber of the heart that causes irregular conduction of blood; that is why it is considered to be the main reason for pacemaker implantation (Zhong *et al*., 2013). HCM has multiple causes, including genetic and environmental factors. Hereditary HCM may be caused due to a mutation in mitochondrial DNA or in nuclear DNA, which follows the dominant mode of inheritance (Morita *et al*., 2008). Approximately seventy-five percent of the hereditary HCM is caused by myosin-binding protein C, cardiac ß-myosin heavy chain, cardiac troponin T (TNNT2), and troponin I3 (TNNI3) (Morita *et al*., 2008). While in some families of the Chinese population, it is identified that mutation in mitochondrial DNA is also a core cause of HCM, which follows the maternal mode of inheritance (Shishi *et al*., 2018; Roşca and Pop, 2013). The first mutation in mitochondrial DNA was identified in tRNA Leu (UUR) gene associated with HCM (Zeviani *et al*., 1991). later a mutation in MT-TL1 gene (m.3260A>G) having an association with HCM was identified. Mutation

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of the mitochondrial genome in *t*RNA genes such as MT-TI (Taylor, 2003), MT-TK and MT-TH (Santorelli, 1995), associated with HCM had been reported. Few experimental studies determined that HCM may also occur due to any change in cellular metabolism (Shishi *et al*., 2018; Farrar *et al*., 2013).

This study was aimed to identify families suffering from HCM, draw their pedigrees, collect complete bio-data, buccal swabs samples and photographs, analyses of their mtDNA mutations and moreover, to assist the scientists in having a closer look while developing a therapeutic gene treatment. Genetic, molecular and clinical characterization of Pakistani families, with maternally inherited HCM, was performed to identify the association of MT-RNR2, MT-TI and MT-TL1 mutations with HCM in Pakistani Families and also focused on identifying the new pathogenic mutation and their clinical significance.

MATERIALS AND METHODS

In the current study, eighteen samples from 4 families were collected, ascertain from different cities of the country based on radiographic and clinical phenotypes of HCM and mitochondrially inherited patterns. Informed written consent was obtained from the donors before sample collection. The current study was approved from the ethical review committee of the Department of Zoology, Hazara University, Mansehra. The affected individuals were subjected to medical evaluation, i.e. physical examination, family history, construction of family phylogeny, electrocardiography, and echocardiography. HCM was diagnosed based on New York Heart Association (NYHA).

Clinical evaluations

Clinical data of affected individuals and their relatives were furnished through various methods of consideration, such as physical assessment, laboratory tests (test from venous blood and routine urine), and medical record. Then ECHO (Doppler echocardiography), 12-lead echocardiograph, and two dimensional of M-mode analysis were also conducted. The medical diagnosis of HCM was based on echocardiography by demonstrating mysterious left chamber hypertrophy, that has maximum wall thickness ≥ 15mm of the left ventricle and classically asymmetric in circulation (IVS/left posterior wall thickness (LPW) ≥ 13mm. Patients of other cardiovascular disorders, i.e. aortic stenosis, heart failure, angina, and stroke at a young age were not included in this study.

DNA extraction and PCR amplification

Buccal swabs from 18 subjects of four diseased families of HCM were collected. DNA isolation was carried out through a modified protocol of Ralser *et al*. (2006). To check the eminence of mitochondrial DNA, 1%, agarose gel was prepared. The extracted DNA was amplified for *MT-TI* (mitochondrial encoded *tRNA^{Ile}*), *MT-RNR2* **(**mitochondrial encoded *16S-rRNA***)** and MT-TL1 (mitochondrial encoded *tRNA^{Leu}*) genes using F-Primer 5'ACT TCC TAC CAC TCA CCC TAG C3' and R-Primer 5'GAG TGT GCC TGC AAA GAT GG3' for *tRNA^{Ile}*. F-Primer 5' CTA AAC CTA GCC CCA AAC CC 3' and R-Primer CCT GGA TTA CTC CGG TCT GAA C 3' for *16S-rRNA* gene and F- Primer 5'CAA ATT CCT CCC TGT ACG AAA GG3' R-Primer 5'AAT GAG GAG TAG GAG GTT GGCC3' for the tRNA^{Leu} gene. The thermocycling was preceded at 94°C for 5 min, followed by thirty-five cycles of denaturation at 96°C for thirty seconds, annealing at 54°C for one min and extension at 72ºC for 45 seconds. The final elongation step was accomplished at 71°C for seven min. The amplified product was resolved by electrophoresis on one percent agarose gel run in 1× TAE. Then the samples were processed for thirty min at 70 volts. Then the amplicons were examined through UV Trans-illuminator

Sequencing and mutational analysis of mitochondrial genes

The purified amplicons of *16S-rRNA,* tRNA^{Ile,} and tRNA^{Leu} were sent for sequencing to Chengdu, China (TSINGKE Biological Technology). To detect any pathogenic mitochondrial mutation, the resulted sequences were aligned with rCRS (Revised Cambridge Reference Sequence) Accession No. N_012920.1. Through alignment, the *16S-rRNA*, *tRNA^{Ile,}* and *tRNA^{Leu}* genes were then analyzed to detect any pathogenic mutation and its association with HCM. All the identified mutations were recorded carefully and compared with control individuals of the families. Searched in the human mitochondrial databases such as Mito-map http://www.mitomap.org, mtDB http://www.genpat.uu.se/mtDB, and HmtDB http://www.hmtdb.uniba.it:8080/hmdb, mutations were confirmed for their novelty and also to find their significance in other diseases.

Structural analysis

The schematic secondary structure of the RNA with detected mutations was predicted through RNA fold software from the Vienna RNA package (Fig. 3) and compared with the normal sequence of the desired genes obtained from rCRS Accession No. N_012920.1.

RESULTS

Clinical findings

In this study, eighteen samples were collected from 4 families, ascertained from different cities of the country

(Table I). Among 18 samples, nine were male, and nine were female (9:9), and the mean of age range at the onset of disease was 30.27±13.64 years (12–55). The left ventricle wall thickness (LVWT) of the patients was 13.64± 6.50 mm (range 8.3-26.4), the intra-ventricle septum thickness (IVS) of the patients was 13.64± 6.65 mm (range 7-28.5), left posterior wall thickness of the patients was 11.31 ± 3.60 mm (ranges 7-16.2). The left ventricle ends diastolic diameter (LVEDD) of the patients was 54.83 ± 12.06 (55-31). The left ventricle ends systolic diameter (LVESD) of the patients was 45.72 ± 12.63. The left atrial volume index of the patients was 36.74± 7.92, as shown in Table I. The LVWT of the affected individuals in these families was much thicker than those in healthy individuals, which was not greater than 15mm, LPW from 06-11 mm, IVS from 06-11 mm, LAVI 19-38 mm, LVESD 25-41 mm, LVEDD 36-56 mm and EF 50-70%. This finally results in asymmetric hypertrophy. Furthermore, a specialist in neurology identified no neuromuscular deficits after an enough neuromuscular assessment. The lactic acid in whole venous blood, the urine, and blood routine test turned out to be negative. The family history was also obtained from the patients and shown in the schematic diagram of family pedigree (Fig. 1).

Figure 1 shows family pedigrees (a) family HCM-01 (b) family HCM-02 (c) family HCM-03 and (d) family HCM-04 (e) genomic DNA of the given samples (f) amplicons of *16S-rRNA (*g) amplicons of $tRNA^{Ile}$ and (h) amplicons of $tRNA^{Leu}$.

Mitochondrial DNA analysis

The maternal inheritance of HCM in these families recommended the mitochondrial association and led us to analyze the mt-DNA of the maternal members. The sequences of the mitochondrially encoded *16S-rRNA* (*mt-RNR2),* mitochondrially encoded *tRNA* Isoleucine (*MT-TI)* and mitochondrially encoded *tRNA* leucine (MT-TL1) genes exhibited four mutations in *16S-rRNA* at position mt-1811A>G, mt-2552T>A, mt-2467A>T and mt-1888A>G. Out of these mutations, mt-2467A>T and mt-2552T>A were declared novel mutations, while mt-1888A>G and mt-1811A>G mutations were formerly reported by Ruppert *et al*. (2004); Perucca-Lostanlen *et al*. (2000), in patients of maternally inherited diabetes and deafness syndrome and dilated cardiomyopathy in French and German population. These variants in *16S-rRNA* were further analyzed by allelic frequency in healthy members of the family according to the published data on http://www.mtdb.igp.uu.se and according to phylogenetic analysis encompassing 17 vertebrate species. Allelic frequency analysis showed that these mutations were absent in the 2704 controls http://www.genpat.uu.se/mtDB. Partial sequence chromatograms of the *16S-rRNA* gene of the affected and healthy individuals are shown in Figure 2. Schematic structures of all the mutant region of *16S-rRNA* were also designed using RNA fold software from the Vienna RNA package (Fig. 3). Sequence analysis showed that the *16S-rRNA* mt-1811A>G, mt-2552T>A, mt-2467A>T and

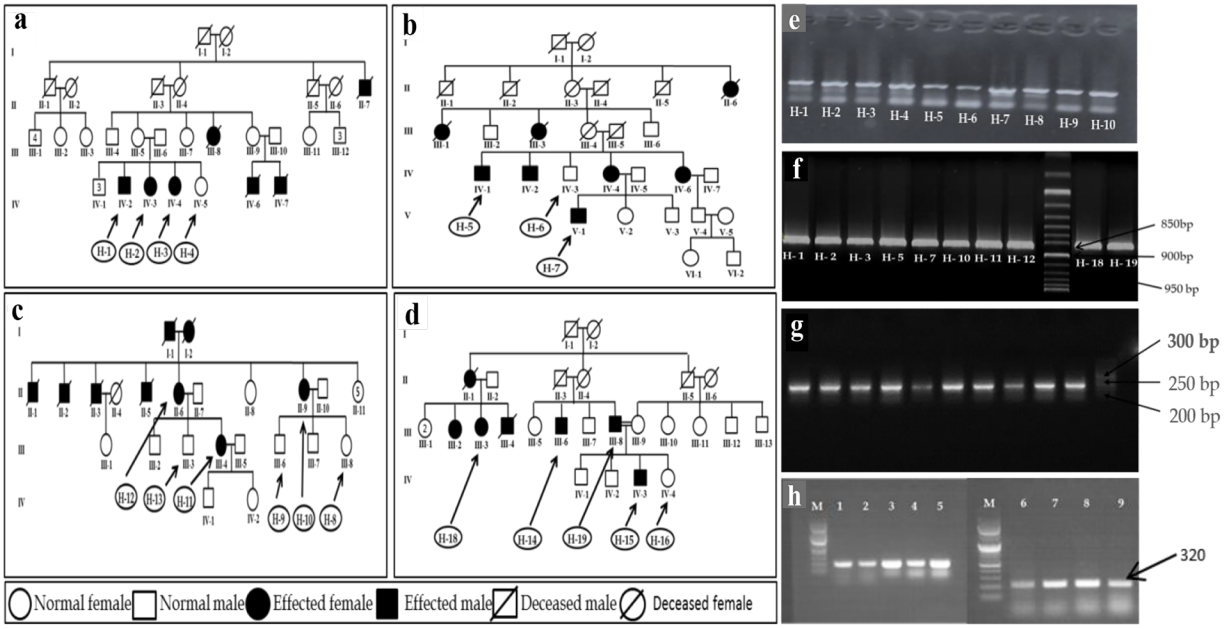

Fig. 1. Family pedigrees: a, family HCM-01; b, family HCM-02; c, family HCM-03 and d, family HCM-04; e, genomic DNA of the given samples; f, amplicons of *16S-rRNA;* g, amplicons of $tRNA^{Ile}$ and h, amplicons of $tRNA^{Leu}$.

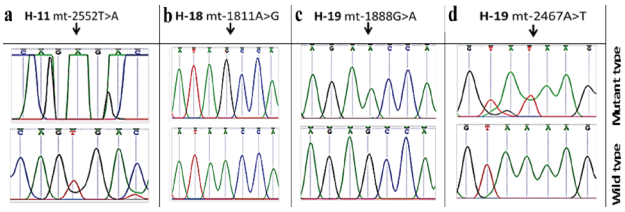

Fig. 2. Electrocardiogram sequence of mitochondrial genome variants detected in HCM patients. **a**, The upper section shows the mutant type nucleotide 'A' at position 2552 in the *16S-rRNA* gene while the lower section shows wild type nucleotide 'T.' **b**, The upper section shows the mutant type nucleotide 'G' at position 1811 in *16S-rRNA* gene, while the lower section shows wild type nucleotide 'A.' **c**, The upper section shows the mutant type nucleotide 'A' at position 1888 in *16r-RNA* gene, while the lower section shows wild type nucleotide 'G.' **d**, The upper section shows the mutant type nucleotide 'T' at position 2467 in *16r-RNA* gene, while the lower section shows wild type nucleotide 'A.'

mt-1888A>G mutations were homoplasmic in oral epithelial cells.

Figure 2 Electrocardiogram sequence of mitochondrial genome variants detected in HCM patients. a. The upper section shows the mutant type nucleotide 'A' at position 2552 in the *16S-rRNA* gene while the lower section shows wild type nucleotide 'T.' b. The upper section shows the mutant type nucleotide 'G' at position 1811 in *16S-rRNA* gene, while the lower section shows wild type nucleotide 'A.' c. The upper section shows the mutant type nucleotide 'A' at position 1888 in *16r-RNA* gene, while the lower section shows wild type nucleotide 'G.' d. The upper section shows the mutant type nucleotide 'T' at position 2467 in *16r-RNA* gene, while the lower section shows wild type nucleotide 'A.'

Figure 3 Schematic structures of mitochondrial genes variants detected in HCM patients, design by RNA fold software via the Vienna RNA package. Four pathogenic mt-DNA mutations associated with HCM are prescribed

Table I. Summary of the clinical data of affected and control individuals of the Families affected with HCM.

	Age		ECHO							
Patient tag	**At onset**	**BP Mm/Hg**	**LVWT (mm)**	**IVS (mm)**	**LPW (mm)**	**LVEDD (mm)**	**LVESD (mm)**	**EF (%)**	**LAVI (mL/ m2)**	**Physical status**
HCM 01										
IV-2 H-1	17	98-70	20.3	24.2	16.2	69	57	38	43.04	Patients
IV-3 H-2	19	95-70	17	18.7	13.3	62	51	60	38.12	Patients
IV-4 H-3	26	100-70	23	28.5	15.2	68	59	46	39.15	Patients
IV-5 H-4	29	120-80	9.6	9.6	7.8	41	31	70	ND	Normal
HCM 02										
IV-1 H-5	30	110-75	25.7	21.7	12.6	60	49	41	37.54	Patients
IV-3 H-6	50	150-90	8.3	12.2	7.9	44	36	72	ND	Normal
V-1 H-7	17	90-65	26.4	23	12.9	68	64	45	36.92	Patients
HCM 03										
III-8 H-8	18	95-65	10.7	8.4	8.4	37	25	75	ND	Normal
III-6 H-9	23	100-70	9	9.1	7.4	38	27	75	ND	Normal
II-9 H-10	47	120-90	18	17.3	15.4	58	47	43	37.3	Patients
III-4 H-11	34	110-70	22	21.2	13.3	57	45	46	39.4	Patients
II-6 H-12	55	125-70	16	13.2	14.2	60	50	57	44	Patients
III-3 H-13	25	100-70	11	8.9	7.4	48	36	75	ND	Normal
HCM 04										
III-6 H-14	37	120-75	24.8	15	16.2	55	49	65	38.64	Patients
IV-3 H-15	17	100-65	14	19	12.9	66	62	66	36.2	Patients
IV-4 H-16	12	93-62	9.3	8.2	6.4	31	26	75	ND	Normal
III-3 H-18	35	90-60	ND	7.0	7.0	58	52	20	14	Patients
III-8 H-19	54	130-90	ND	ND	ND	67	57	23	ND	Patients
Normal values										
----	-	120-80	>15	06-11	06-11	36-56	25-41	50-70	19-38	

BP, Blood pressure; ECHO, Echocardiography; EF, Ejection fraction; F, Female; HCM, Hypertrophic cardiomyopathy; IVS, Interventricular septum thickness; LPW, Left posterior wall thickness; LAVI, Left atrial volume index; M, Male; LVWT, Left ventricular wall thickness; LVEDD, Left ventricular End diastolic diameter; LVESD, Left ventricular systolic diameter.

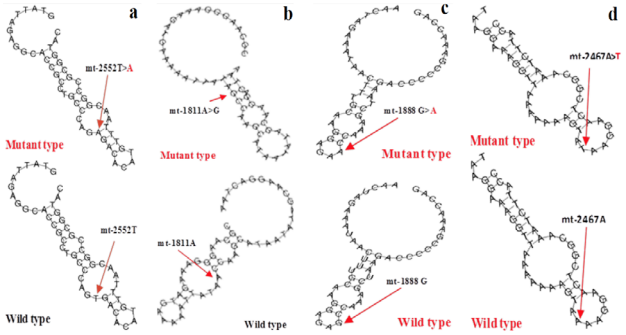

Fig. 3. Schematic structures of mitochondrial genes variants detected in HCM patients, design by RNA fold software via the Vienna RNA package. Four pathogenic mt-DNA mutations associated with HCM are prescribed in the upper panel as shown in figure a, c and d while the lower panel shows the wild type of nucleotides sequences.

in the upper panel as shown in figure a, c and d while the lower panel shows the wild type of nucleotides sequences.

DISCUSSION

In the current research, we have studied clinical, genetic and molecular characterizations of four Pakistani families with mitochondrially inherited HCM. By studying medical record of HCM patients, shows a comparatively wide range of LVWT which was not greater than 15 mm, left posterior wall thickness (LPW) from 06-11 mm, intraventricular septum thickness (IVS) from 06-11mm, left atrial volume index (LAVI) from 19-38 mm, left ventricular systolic diameter (LEVSD) from 25-41 mm, left ventricle end diastolic diameter (LVEDD) from 36-56 mm, and ejection fraction (EF) from 50-70% which was previously studied by Zhong *et al*. (2013); Bashyam *et al*. (2012); Hirota *et al*. (2010). In our study, the LVWT of the patients was 13.64± 6.50 mm (range 8.3-26.4), LPW of the patients was 11.31 ± 3.60 mm (ranges 7-16.2), IVS of the patients was 13.64± 6.65 mm (range 7-28.5), LVEDD of the patients was 54.83 ± 12.06 (range 31-55), LVESD of the patients was 45.72 ± 12.63 (range 25-64), LAVI of the patients was 36.74 ± 7.92 (range 14-44) and EF of the patients was 55.11± 17.99 (range 20-75), that are comparatively much higher than those found in the general HCM individuals (Zhong *et al*., 2013). Moreover, about 25% of the HCM patients have faced left ventricular outflow tract obstruction, while rarely presenting in HCM associated with mt-DNA variations. After enough neuromuscular examination in neurology, no neuromuscular deficits were recognized by a specialist, which may be because of the tissue-specific defect of the mitochondrial disease. A similar result was previously reported by Zhong *et al*. (2013) and Guan *et al*. (2006). Out of these associations, the genetic significance is not clearly understood. However, such type of findings is not unexpected given the common source of particular conduction system elements and the running myocardium. However, the determination of mitochondrial DNA mutation, which can originate these abnormalities might provide vital information on their pathogenicity. In our study, the recognition of pathogenic mutations in HCM members with mitochondrial DNA determined *MT-TI, mt-RNR2,* and MT-TL1 genes were sequenced. Four families of HCM were analyzed, to find out their relation or association with mt-DNA mutation. In each family more than two samples were sequenced for 16S-rRNA, $tRNA^{Ile}$ and $tRNA^{Leu}$ in which only 4 mutations were determined in *16S-rRNA* at position mt-1811 A>G patient H-18, mt-2552T>A patient H-11, mt-2467A>T, and mt-1888 patient H-19, in the mentioned 4 mutations at mt-2467A>T and mt-2552T>A positions could be declared novel mutations. The mutation mt-1811A>C was previously identified in affected individuals of dilated cardiomyopathy in the population of Germany (Ruppert *et al*., 2004).

Moreover, mutation mt-1888A>G was previously detected in patients of mitochondrial inherited diabetes and deafness syndrome in the French population (Perucca-Lostanlen *et al*., 2000). The schematic secondary structure of the detected mutations was also predicted through RNA fold software from the Vienna RNA package (Fig. 3), as it is previously described by Zarrouk-Mahjoub *et al*. (2015); Burk *et al*. (2002) and Mears *et al*. (2006). For the first time, the mutation in mt-1811A>G and mt-1888A>G have been recorded in HCM patients while no mutation was detected in $tRNA^{Ile}$ and $tRNA^{Leu}$ genes. The detected mutations were definite by their privation in all the control members of the families. Generally, the cardiovascular abnormalities occur due to mutations in mitochondrial-*tRNA* and mitochondrial genes, which codes proteins while mutations in *mt-RNR2* gene are relatively less responsible for cardiovascular disorder. This is the first report of *mt-RNR2* gene mutation linked with HCM in the population of Pakistan. In a recent study, it is recognized that mitochondrial mutation originated changes in ultra-structure of mitochondria, which has been recognized to be linked with HCM. In the population of China, it is anticipated from microscopic analysis of lymphoblast cell that both the number and size are increased in HCM individuals than those in control, which show that less adenosine triphosphate production is main cause of HCM (Ashrafian *et al*., 2013).

Furthermore, the irregular shape of cristae was also recognized in affected persons (Zhong *et al*., 2013).

Moreover, studies on physiological aspects of mt-1811 A>G, mt- 2552T>A, mt-2467A>T and mt-1888A>G mutations are desired to expose the link of these mutations with HCM. Our results afford new insight into the pathogenesis of HCM.

CONCLUSION

After experimentation and sequencing analysis, four mutations were detected in MT_RNR2 gene, so it could be concluded that these mutations might have a strong association with the onset of the disorder. Out of these four mutations, two mutations could be declared novel in association with the HCM in Pakistani families. On the other hand, as no mutation was detected in MT-TI gene and MT-TLI gene so, it could be concluded that in the subjects investigated in the present study, a mutation in these genes might not be the reason of etiology of the disease. This is the 1st ever report of such type of mutation linked with HCM, and it confirms the impact of these pathological mutations in the onset of HCM in Pakistani families.

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Ethical consideration

The current study was approved from the ethical review committee of the Department of Zoology, Hazara University Mansehra. Informed written consent was obtained from the donors before sample collection. In an instance of aged patients, the informed consent was obtained from a legal guardian or parent of the patients.

Conflict of interest

Authors declare that there is no known conflict of interest associated with this publication and the manuscript has been read and approved by all named authors and that there is no other person who satisfied the criteria for authorship but is not listed.

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