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Original Article

Mitochondrial mutations in patients with congenital heart defects by next generation sequencing technology

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Abstract It has been shown that mitochondrial deoxyribo nucleic acid mutations may play an important role in the development of cardiomyopathy, and various types of cardiomyopathy can be attributed to disturbed mitochondrial oxidative energy metabolism. Several studies have described many mutations in mitochondrial genes encoding for subunits of respiratory chain complexes. Thus, recent studies confirm that pathologic mitochondrial deoxyribo nucleic acid mutations are a major reason of diseases and determining them by next-generation sequencing will improve our understanding of dysregulation of heart development. To analyse mitochondrial deoxyribo nucleic acid mutations, the entire mitochondrial deoxyribo nucleic acid was amplified in two overlapping polymerase chain reaction fragments from the cardiac tissue of the 22 patients with congenital heart disease, undergoing cardiac surgery. Mitochondrial deoxyribo nucleic acid was deep sequenced by next-generation sequencing. A total of 13 novel mitochondrial deoxyribo nucleic acid mutations were identified in nine patients. Of the patients, three have novel mutations together with reported cardiomyopathy mutations. In all, 65 mutations were found, and 13 of them were unreported. This study represents the most comprehensive mitochondrial deoxyribo nucleic acid mutational analysis in patients with congenital heart disease.

Keywords: Congenital heart defects; next-generation sequencing; mitochondrial mutations

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Congenital heart disease affects 19–75 babies in every 1000 live births and it is the most common cause of death in newborns.[1] Despite advances in medical and surgical care, the aetiology of congenital heart disease is still not completely understood and dysregulation of heart development is the underlying basis of the disease. Although many studies have demonstrated that environmental influences can affect the heart development,[2] major underlying pathology is thought to be genetic factors playing an important role in this manner.[3,4] Recent investigations and researches have taken different approaches to identify these genetic contributions. To identify novel genetic aetiologies by determining deoxyribo nucleic acid analyses with direct sequencing and/or microarray, Sanger capillary sequencing[5] method has been used as a gold standard for deoxyribo nucleic acid sequencing for the past 30 years.

In contrast to this, next-generation sequencing methods can generate vast amounts of sequencing data in less time and is cost-effective.[6] The goal of our study was to explore next-generation sequencing as a method to detect mitochondrial deoxyribo nucleic acid mutations in patients with congenital heart disease. Our aims were: (1) to identify all mitochondrial deoxyribo nucleic acid variants and potential mutations; (2) to evaluate other systemic effects; and (3) to correlate clinical data. In this report, we provide

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the results of mitochondrial deoxyribo nucleic acid analysis for 22 patients with congenital heart disease.

Materials and methods

Ventricular, atrial myocardial tissue samples were collected from 22 patients undergoing cardiac surgery at Istanbul Faculty of Medicine Department of Cardiovascular Surgery. Cardiac tissue was obtained intraoperatively and stored immediately for further examination. Clinical diagnoses as well as preoperative and postoperative clinical findings by next-generation sequencing of heart disease were reviewed. In addition, patients with suspected mitochondrial disease preoperatively were excluded to focus the study on mitochondrial pathology caused by the cardiac stress because of congenital heart malformation. All patients gave written informed consent and the Ethic Committee of Istanbul University (Istanbul, Turkey) approved the study. Clinical details of the patients were identified in Table 1.

Mitochondrial deoxyribo nucleic acid sample preparation

Genomic deoxyribo nucleic acid was extracted from the heart tissues of all patients with the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, California, United States of America) and mitochondrial deoxyribo nucleic acid was amplified in two overlapping Polymerase chain reaction fragments (9731 and 12,083 bp) using the Roche expand long-range polymerase chain reaction dNTPack (Roche Applied Science Indianapolis, Indiana, United States of America). Polymerase chain reaction conditions and sequence of the primers used are provided in Supplemental Table S1. Polymerase chain reaction products were cleaned with MinElute Polymerase chain reaction Purification Kit (Qiagen Inc.) and mixed in equimolar ratios for each patient.

Table 1. Clinical information for the patients with cardiomyopathy.

Number of patients	22
Age (mean ± SD)	2.4 ± 0.98
Complete AV Block	1 – (P.4)
ALCAPA	3 – (P.6,8,11)
VSD	3 – (P.10,16,20
VSD + PA	2 – P.3,9)
Tetralogy of Fallot	9 – (P.1,2,5,12,14,15,17,19,22)
Tricuspit Atresia	1 – (P.7)
c-AVSD	1 – (P.13)
TAPVR	1 – (P.21)
PAPVR	1 – (P.18)

ALCAPA = anomali of the left coronary artery from the pulmonary artery; c-AVSD = complete atrioventricular septal defect; PAPVR = partial anomaly pulmonary venous return; TAPVR = total anomalous pulmonary venous return; VSD + PA = ventricular septal defect + pulmonary atresia

Complete mitochondrial deoxyribo nucleic acid sequencing and bioinformatics

Next-generation sequencing was performed at the Whole Genome Sequencing Laboratory, The Institute of Experimental Medicine. Mixed polymerase chain reaction samples (500 ng) for each patient were nebulised using nebulisers and reagents from the 454 Life Sciences (Roche) Genome Sequencer or Genome Sequencer FLX Shotgun Library Preparation kit following the manufacturer's instructions. Then, deoxyribo nucleic acid fragments were cleaned with the MinElute Kit (Qiagen Inc.) and a fragment size distribution ranging from 100 to 600 bp was verified via Agilent Bioanalyzer 2100 (Agilent Technologies, Anaheim, California, United States of America). Roche's 10-base multiplex identifier adaptors were used for tagging each nebulised and cleaned mitochondrial deoxyribo nucleic acid sample. Equimolar quantities of the tagged samples were pooled together. Single-strand libraries were prepared and the emulsion polymerase chain reaction was performed for pooled samples. Genome Sequencer FLX runs were performed in one picotiter sequencing plate divided into eight regions, including 10-base multiplex identifier tagged mitochondrial deoxyribo nucleic acid samples, and Titanium kits were used to produce ultra-deep pyrosequencing according to the manufacturer's protocol (Roche Diagnostics Co.).[7,8]

Genome Sequencer FLX Sequencing data were processed using Genome Sequencer Run Browser software to generate sequencing reads, base-call quality scores, and remove low-quality reads. The raw sequences obtained were sorted by barcodes Multiplex Identifier Adaptors and each individual sequence reads were determined. Then, the sequence reads were aligned to the revised Cambridge Reference Sequence (rCRS) (NC_012920), using CLCBIO Genomic Workbench v5.5 (CLCBIO, Denmark). Then, single-nucleotide polymorphisms and deletion–insertion polymorphisms of each patient were determined because of the following criteria: (i) variants must be detected in at least three unique (non-duplicate) sequencing reads with both forward and reverse reads; (ii) having at least 10% frequency among total unique sequencing reads at that location; and (iii) and high-quality scores (> Q20 for variants and > Q15 for three nucleotides at each side of variant). Moreover, deletion–insertion polymorphisms located at homopolymer regions (four to eight bases) were eliminated to prevent false-positive results.[9,10] Variants derived using these criteria were examined, novel, and reported as disease-associated mutations determined.

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Table 2. Novel and reported disease-associated mutations of 22 patients (P).

Case no.	Location	Base change	Amino-acid change	Frequency/coverage	Associated disease
P1	7501	T > C	–	11.1/90	Cardiomyopathy[9]
	8481	C > T	Pro39Leu	93.2/59	Novel mutation
	9804	G > A	Ala200Thr	96.9/64	LHON[11]
P2	716	T > C	–	12.5/88	Novel mutation
	2728	C > T	–	12.5/81	Novel mutation
	3010	G > A	–	100/62	CVS with migraine[12]
	4216	T > C	Tyr304His	100/186	LHON[13]
	10,398	A > G	Thr114Ala	97.2/71	Breast cancer risk[14]
	12,192	G > A	–	100/80	MIC[15]
	12,453	T > C	–	100/77	Novel mutation
	13,632	A > G	–	48.1/79	Novel mutation
	13,708	G > A	Ala458Thr	100/177	LHON[13]
	15,113	A > G	Thr123Ala	100/17	Novel mutation
P3	4917	A > G	Asn150Asp	100/64	LHON[13]
	16,519	T > C	–	16.7/60	CVS with migraine[12]
P4	13,810	G > A	Ala492Thr	100/89	Novel mutation
	15,927	G > A	–	100/78	MS/DEAF1555[16]
P5	10,398	A > G	Thr114Ala	100/25	Breast cancer risk[14]
	11,299	T > C	–	100/43	LHON[17]
	11,467	A > G	–	97.7/44	Altered brain pH[18]
	12,308	A > G	–	96.3/27	Cardiomyopathy[19,20]
	12,372	G > A	–	100/30	Altered brain pH[16]
	16,519	T > C	–	16.7/60	CVS with migraine[12]
P6	11,499	C > T	Thr247Met	100/58	Novel mutation
P7	3010	G > A	–	100/89	CVS with migraine[12]
P8	4902	A > G	Ile145Val	100/93	Novel mutation
P9	10,398	A > G	Thr114Ala	100/35	Breast cancer risk[14]
	11,467	A > G	–	98.4/64	Altered brain pH[18]
	12,308	A > G	–	90.6/32	Cardiomyopathy[19,20]
	12,372	G > A	–	100/31	Altered brain pH[18]
P10	11,467	A > G	–	97.7/44	Altered brain pH[18]
	12,308	A > G	–	100/110	Cardiomyopathy[19,20]
	12,372	G > A	–	100/41	Altered brain pH[18]
	15,693	T > C	Met316Thr	100/78	LVNC[21]
P11	9088	T > C	Ser188Pro	100/45	Novel mutation
	11,467	A > G	–	100/53	Altered brain pH[18]
	12,308	A > G	–	95.7/47	Cardiomyopathy[19,20]
	12,372	G > A	–	100/45	Altered brain pH[18]
P12	8902	G > A	Ala126Thr	99.5/219	Novel mutation
	11,467	A > G	–	100/47	Altered brain pH[18]
	12,308	A > G	–	98/49	Cardiomyopathy[19,20]
	12,372	G > A	–	100/50	Altered brain pH[18]
P13	11,499	C > T	Thr247Met	100/87	Novel mutation
P14	11,467	A > G	–	100/26	Altered brain pH[18]
	12,192	G > A	–	100/31	MIC[15]
	12,308	A > G	–	100/98	Cardiomyopathy[19,20]
	12,372	G > A	–	100/98	Altered brain pH[18]
P15	10,398	A > G	Thr114Ala	100/91	Breast cancer risk[14]
	10,454	T > C	–	100/23	DEAF helper mutation[22]
	15,043	G > A	–	100/30	MDD-associated[18]
P16	3010	G > A	–	100/30	CVS with migraine[12]
	4216	T > C	Tyr304His	100/33	LHON[13]
	10,398	A > G	Thr114Ala	100/19	Breast cancer risk[14]
	13,708	G > A	Ala458Thr	100/40	LHON[13]
P17	4216	T > C	Tyr304His	100/32	LHON[13]
	4917	A > G	Asn150Asp	100/14	LHON[13]
	15,928	G > A	–	100/53	Multiple sclerosis
P18	12,308	A > G	–	100/87	Cardiomyopathy[19,20]
	12,372	G > A	–	100/110	Altered brain pH[18]
P19	4216	T > C	Tyr304His	100/36	LHON[13]
	10,398	A > G	Thr114Ala	100/120	Breast cancer risk[14]
	13,708	G > A	Ala458Thr	100/39	LHON[13]
P20	12,073	C > T	–	100/120	Novel mutation
P21	11,874	C > A	–	78/141	Novel mutation
P22	123,72	G > A	–	100/6	Altered brain pH[18]

CVS = cyclic vomiting syndrome; LHON = Leber hereditary optic neuropathy; LVNC = left ventricular non-compaction; LVNC = left ventricular non-compaction; MDD = major depressive disorder MS/DEAF1555 = multiple sclerosis/DEAF1555 increased penetrance (helper mutation); MIC = maternally inherited cardiomyopathy

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Results

A total of 10 newborn and 12 infant patients with cardiomyopathy were studied using next-generation sequencing system and bioinformatics analysis of the sequence data allowed determination of new and reported variation for each individual. In total, 22.208.222 fragments were sequenced from 22 samples, with an average read length of 27,693 bp.

Initially, we globally aligned all sorted fragments to the revised Cambridge Reference Sequence using CLCBIO Genomic Workbench and mitochondrial deoxyribo nucleic acid variants derived, using the criteria mentioned above that were examined for each patient listed at Table 2. Novel and reported disease-associated mutations determined are shown at Table 2.

There were three kinds of cardiomyopathy mutations – 7501 T > C, 12,308 A > G, 12,192 G > A –reported previously that were detected in eight patients by using *Vienna RNA Web Services* (Vienna RNA Web Services Institute for Theoretical Chemistry, http://rna.tbi.univie.ac.at/). Of the patients, two, P2 and P14, have the same mutation (12,192 G > A), which was reported as a homoplasmic point mutation related to maternally inherited cardiomyopathy.[14] The mutation 7501 T > C was in the TRNS1 gene. Whereas the free energy of the thermodynamic ensemble for the secondary structure of wild-type TRNS1 RNA was –12.43 kcal/mol (Fig 1a), in the presence of 7501 T > C mutation free energy was – 12.02 kcal/mol (Fig 1b). The mutation 12,308 A > G was in the TRNL2 gene. Whereas the free energy of the thermodynamic ensemble for the secondary structure of wild-type TRNL2 RNA was –15.45 kcal/mol (Fig 1c), in the presence of 12,308 A > G mutation free energy was –15.32 kcal/mol (Fig 1d). The mutation 12,192 G > A was in the presence of TRNH gene. Whereas the free energy of the thermodynamic ensemble for the secondary structure of wild-type TRNH RNA was – 10.69 kcal/mol, (Fig 1e), in the presence of 12,192G > A mutation free energy was – 10.61 kcal/mol (Fig 1f).

In all, 13 different novel mitochondrial deoxyribo nucleic acid mutations – 8481 C > T, 716 T > C, 2728 C > T, 12,453 T > C, 13,632 A > G, 15,113 A > G, 13,810 G > A, 11,499 C > T, 4902 A > G, 9088 T > C, 8902 G > A, 12,073 C > T, and 11,874 C > A – were identified at 10 patients. Of these mutations, 10 are caused by amino-acid changes – Pro39Leu, Thr123Ala, Ala492Thr, Thr247Met, Ile145Val, Ser188Pro, and Ala126Thr. The mutations Pro39Leu, Ser188Pro, and Ala126Thr are in ATP6 gene. The other mutations such as Thr123Ala is in CYTB gene, Ala492Thr is in ND5, Thr247Met is in ND4, and Ile145Val is in ND2 gene. The frequency of these mutations was over 93.2 and commonly equals to 100.

Of the patients, three – P1, P11, and P12 – had novel mutations together with reported cardiomyopathy mutations. Of the different novel mutations, five – 716 T > C, 2728 C > T, 12,453 T > C, 13,632 A > G, and 15,113 A > G – were observed (determined) in one patient, P2. Leber's hereditary optic neuropathy mutations – 9804 G > A, 4216 T > C, 13,708 G > A, and 4917 A > G– were detected in six patients – P1, P2, P3, P16, P17, and P19. In addition, Cyclic vomiting syndrome with migraine, multiple sclerosis, and breast cancer risk-associated mutations also observed in some patients – P2, P3, P5, P7, P16, P15, and P17. Of the patients, seven – P6, P7, P8, P9, P20, P21, and P22 – had only one mutation; five of them had novel mutation and others had mutations related with cyclic vomiting syndrome with migraine and altered brain pH.

Of the patients, two, P2 and P14, have the same mutation, which was reported as a homoplasmic point mutation related to maternally inherited cardiomyopathy.[14]

In this report, we provided the results of mitochondrial deoxyribo nucleic acid analysis for 22 paediatric patients with congenital heart disease (Table 2).

Discussion

Human mitochondrial deoxyribo nucleic acid is circular, 16,569 base sequences that encode for 13 proteins, 22 transfer RNAs (tRNAs), and two ribosomal RNAs.[4] By evolving "next-generation" sequencing technologies, novel genetic abnormalities in children with congenital heart disease have started to identify. Next-generation sequencing methods can generate vast amounts of sequencing data in less time and are cost-effective.[5] Therefore, several studies have started to describe mutations in mitochondrial deoxyribo nucleic acid -located genes encoding for subunits of respiratory chain complexes. Thus, recent studies confirm that pathologic mitochondrial deoxyribo nucleic acid mutations are a major reason of diseases[6] and determining them by next-generation sequencing will improve our understanding of dysregulation of heart development. Pathogenic mutations in mitochondrial deoxyribo nucleic acid cause mitochondrial dysfunction, and therefore these mutations are related to cardiomyopathy and heart failure.[23,24]

The presence of multiple mutations in a number of different genetic cardiovascular disorders suggests that the association of multiple mutations has an additive effect in terms of molecular pathogenesis with a more severe clinical phenotype.

We analysed mitochondrial deoxyribo nucleic acid mutation from the cardiac tissue with varying degrees in the paediatric patients with congenital heart disease. We found several mutations in mitochondrial

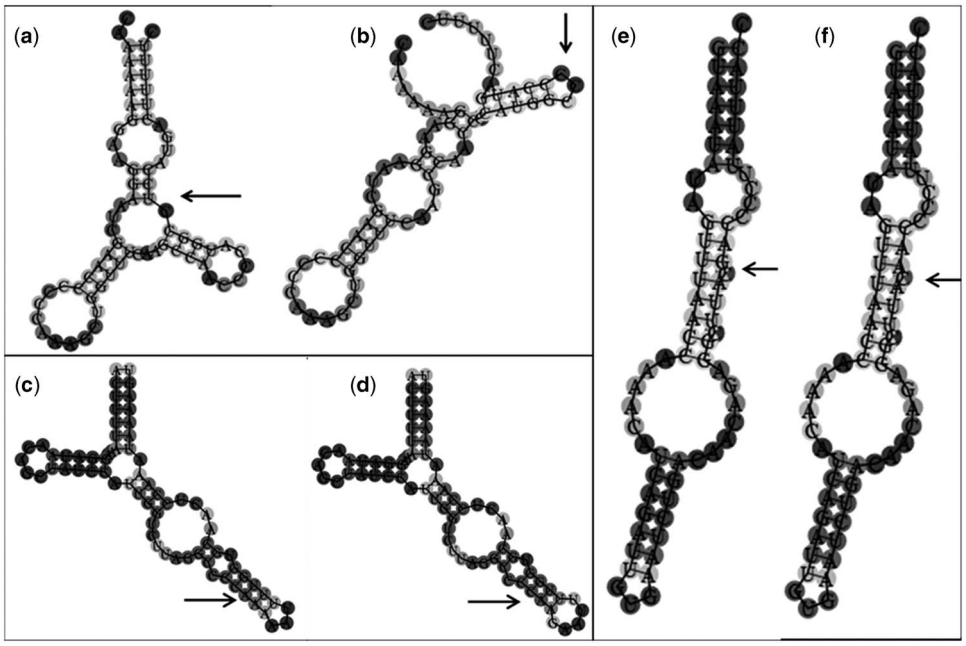

Figure 1.
*The secondary structures of mtRNAs that were found to have cardiomyopathy related mutation. (**a**) Secondary structure of wild-type TRNS1 gene RNA. (**b**) Secondary structure of TRNS1 gene RNA that has the mutation 7501 T > C. (**c**) Secondary structure of wild-type TRNL2 gene. (**d**) Secondary structure of TRNL2 gene RNA that has the mutation 12,308 A > G. (**e**) Secondary structure of wild-type TRNH gene. (**f**) Secondary structure of TRNH gene RNA that has the mutation 12,192 G > A.*

deoxyribo nucleic acid of cases. The mutation-related cardiomyopathy were in the tRNA coding genes: TRNS1, TRNL2, and TRNH. Therefore, these mutations are not expected to cause any change at protein sequence. However, these mutations were found to change the free energy for the thermodynamic ensemble of the secondary structures of RNA. The alteration of energy in the secondary structure of RNA may affect the stability or accessibility of the tRNA. This incompatibility may decrease the activity of oxidative phosphorylation complexes.

The identified mutations were in the RN1, 16 S RNA, ATP6, ATP8, COX3, CYTB, ND1, ND2, ND3, ND4, ND5, TRNH, TRNL2, TRNR, TRNS1, and TRNT genes. The five different mutations were detected in CYTB, ND4, and ND5. These genes play role-transferring electron in the mitochondria during the synthesis of ATP. The mutations of the genes may cause problems for the production of energy in the cardiac cell, and therefore it may induce the development of cardiomyopathy and/or cardiac disorders or prognosis.

In our study, we applied this approach to a large number of mitochondrial deoxyribo nucleic acid sequences from patients with congenital heart disease or another potential mitochondrial disease. There is a possibility that the content and the regulation mitochondrial deoxyribo nucleic acid transcription and replication of the infant hearts are different from older children. Patients with congenital heart disease have age overlap with the control group, and we did not observe any age-dependent changes of mitochondrial deoxyribo nucleic acid content and replication in control hearts. The importance of mitochondrial deoxyribo nucleic acid depletion or mutation in the development of mitochondrial dysfunction and cardiomyopathy has been well documented in mitochondrial diseases.[25] The results in this study allowed us to identify potential mutations with mitochondrial cardiomyopathy.

In this study, we detected 13 novel mitochondrial deoxyribo nucleic acid mutations – 8481 C > T, 716 T > C, 2728 C > T, 12,453 T > C, 13,632 A > G, 15,113 A > G, 13,810 G > A, 11,499 C > T, 4902 A > G, 9088 T > C, 8902 G > A, 12,073 C > T, and 11,874 C > A – in 10 of 22 patients. For patient 1, the novel mutation of 8481 C > T was associated with the cardiomyopathy mutation of 7501 T > C. For patient 11, the novel mutation of 9088 T > C and for patient 12, the novel mutation of 8902 G > A was associated with the cardiomyopathy mutation of 12,308 A > G. These findings in next-generation sequencing point use these mutations can be candidate mutations or have an additive effect in terms of molecular pathogenesis for the development of cardiomyopathy. On the contrary, we have observed same novel mutation of 11,499 C > T in patients 6 and 13 who are clinically diagnosed as anomaly of the

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left coronary artery from the pulmonary artery and atrioventricular septal defect. Despite the cardiac defect of these pathologies being completely different each other, clinical findings show the cardiac failure in both patients may indicate this mutation also has an additive effect to molecular pathogenesis. It is shown that the analysing of these mutations, which are mostly homoplasmic, might be worthy to investigate the large patient and control populations.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human experimentation (Mitochondrial mutations in patients with congenital heart defects by next generation sequencing technology) and with the Helsinki Declaration of 1975, as revised in 2008, and has been approved by the institutional committees (Istanbul Faculty of Medicine Clinical Research Ethics Committee).

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1047951114000754.

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