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ORIGINAL ARTICLE

High-resolution melting analysis of 15 genes in 60 patients with cytochrome-*c* oxidase deficiency

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Cytochrome-*c* oxidase (COX) deficiency is one of the common childhood mitochondrial disorders. Mutations in genes for the assembly factors *SURF1* and *SCO2* are prevalent in children with COX deficiency in the Slavonic population. Molecular diagnosis is difficult because of the number of genes involved in COX biogenesis and assembly. The aim of this study was to screen for mutations in 15 nuclear genes that encode the 10 structural subunits, their isoforms and two assembly factors of COX in 60 unrelated Czech children with COX deficiency. Nine novel variants were identified in exons and adjacent intronic regions of *COX4I2, COX6A1, COX6A2, COX7A1, COX7A2* and *COX10* using high-resolution melting (HRM) analysis. Online bioinformatics servers were used to predict the importance of the newly identified amino-acid substitutions. The newly characterized variants updated the contemporary spectrum of known genetic sequence variations that are present in the Czech population, which will be important for further targeted mutation screening in Czech COX-deficient children. HRM and predictive bioinformatics methodologies are advantageous because they are low-cost screening tools that complement large-scale genomic studies and reduce the required time and effort.
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INTRODUCTION

Cytochrome-*c* oxidase (COX) deficiency is a clinically heterogeneous group of disorders that range from isolated myopathy to severe multisystem disease that can develop at any age.[1] This defect is either biochemically isolated or combined with disruption of other components of the respiratory chain and can arise from mutations located either in mitochondrial genes or in nuclear genes encoding the structural subunits or corresponding assembly factors of the enzyme complex. The incidence of COX deficiency has been estimated to be 1:35000 births in the Slavonic population. The majority of the detected mutations are located in the *SCO2* and *SURF1* genes,[2] which may be accompanied with secondary impairment of the other respiratory chain complexes.[3–5] In several cases of primary COX defects, the normal borderline activity values of the other respiratory complexes were documented in *COX15*, *c2orf64*, *SCO2* and *SURF1* and *SCO1* patients.[4,6–8] To top it all, the secondary complex IV deficiency was observed in patients with primary complex I or complex III deficiency.[9,10]

Despite of the advances in the identification of an increasing number of mutations and genes involved in the disease *(SURF1, SCO1, SCO2, COX10, COX15, TACO1, LRPPRC, C2orf64, C20orf7, COX4I2* and *COX6B1)*,[7,11–14] the molecular basis of COX deficiency is unknown for many patients, which makes genetic counselling difficult. Over the last 20 years, we collected samples from a cohort of 106 Czech children with COX deficiency. In total, 51 of these patients have an isolated enzyme defect and 55 have a combined enzyme defect. COX deficiency was confirmed by biochemical and histochemical analyses. Sequencing of the *SURF1*, *SCO2* and *SCO1* genes was performed for all of the clinically relevant cases, and 25 patients were diagnosed with an isolated COX defect. By sequencing the mitochondrial DNA (mtDNA), the causative mutations were found in another seven patients with combined COX deficiency.[15] Because only an extremely limited amount of DNA samples was available, subsequent genetic analysis was not performed for 14 patients. Therefore, a total of 60 unrelated children without a known genetic cause of COX deficiency were included in this study. In all, 17 of these patients were diagnosed with an isolated defect and 43 were diagnosed with a combined defect.

High-resolution melting (HRM) analysis is a simple, sensitive and cost-effective method that is based on the measurement of changes in the stability of homoduplexes and/or heteroduplexes in the presence of a fluorescent dye while the temperature of the amplified PCR products is increasing.[16] Irrespective of the position of the base-pair variant within the PCR product, any type of homozygous or heterozygous sequence variant can be distinguished using this method.[17,18] The sensitivity and specificity of HRM analysis are

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better than many conventional methods used to detect mutations.[19] Because of these unique properties, we used HRM to screen for mutations in genes assumed to be a cause of COX defects in our group of patients.

The aim of this work was to find or exclude the presence of pathological mutations in all the exons of the nuclear genes encoding the 10 structural subunits *(COX4I1, COX4I2, COX5A, COX5B, COX6A1, COX6A2, COX6B1, COX6C, COX7A1, COX7A2, COX7B, COX7C* and *COX8A)* and two assembly factors *(COX10* and *COX15)* of COX using HRM analysis in a group of 60 unrelated Czech patients with biochemically confirmed COX deficiency. All new missense amino-acid substitutions identified in the exons of the studied genes were then analyzed using computational tools that can predict the effect of the mutation on protein function.

MATERIALS AND METHODS

Clinical information on the patients

Altogether, 60 unrelated children without a known genetic cause of COX deficiency were included in this study. Both patients with isolated COX deficiency and combined deficiency of COX with other respiratory chain complexes were involved in the study group.

The onset of mitochondriopathy was noticed at birth in 35 patients, during the first year of life in 14 patients, at the age of 1–5 years in six patients, at the age of 5–10 years in two patients and at the age of 10–15 years in three patients. The 60 patients included in this study presented with the following signs of mitochondrial disorders: failure to thrive (30/60), delay of psychomotor development (29/60), encephalopathy (28/60), hypotonia (26/60), visual impairment (25/60), myopathy (19/60), dysmorphia (15/60), cardiomyopathy (14/60), hepatomegaly (14/60), intrauterine growth retardation (12/60), spasticity (10/60), defects in motor skills (9/60), hearing impairment (9/60), epilepsy (7/60), dystrophy (7/60), microcephaly (7/60), nephropathy (6/60) and diabetes mellitus (2/60). Routine metabolic workup showed lactate acidosis (23/60), anemia (23/60) and hepatopathy (22/60). A total of 30 patients died prior to the beginning of this study; their survival ranged from 4 days to 13 years with a median of 1.1 years.

Patient consent

This study was approved by the Ethics Committee of the General University Hospital in Prague. All the samples were analyzed with the informed consent of the patients or their parents.

mtDNA analysis

Prior to the start of HRM mutation screening, mtDNA of all the 60 patients was sequenced. Briefly, whole-mtDNA molecule was amplified from muscle or fibroblast total DNA using PCR in 34 overlapping fragments. All the fragments were sequenced in both direction on ABI PRISM 3100/3100-Avant Genetic Analyser (Applied Biosystems, Carlsbad, CA, USA) and obtained sequences were compared with revised Cambridge Reference Sequence (rCRS) of the human mtDNA (NC_012920, http://www.mitomap.org/bin/view.pl/MITOMAP/HumanMitoSeq).

PCR design

As we did not know the exact occurrence and distribution of common single-nucleotide polymorphisms (SNPs) in the examined Czech population, the mutation screening was performed with probe-free HRM. This approach is especially suitable for large-scale genetic studies.[20–22] Primers were designed, using the software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), to amplify the coding regions of *COX4I1, COX4I2, COX5A, COX5B, COX6A1, COX6A2, COX6B1, COX6C, COX7A1, COX7A2, COX7B, COX7C, COX8A, COX10* and *COX15*. Genomic DNA was amplified using PCR in the presence of LCGreen Plus Melting Dye (Idaho Technology Inc., Salt Lake City, UT, USA). Primer sequences and specific PCR conditions are listed in Supplementary Table 1. Genomic DNA was extracted from peripheral blood using the standard procedures with protease digestion, phenol-chloroform extraction and ethanol precipitation. For genetic and subsequent HRM analyses, a total of 15–50 ng of genomic DNA was amplified (NanoDrop ND-1000 UV-Vis Spectrophotometer, Nano-Drop Technologies Inc., Wilmington, DE, USA).

HRM analysis

The size of the scanned amplicons ranged from 191–565 bp. The HRM analysis was performed using a LightScanner instrument (Idaho Technology Inc.) according to the instructions in the LightScanner's manual. A total of 70 amplicons covering 65 coding regions were analyzed. The melting profiles of 60 patient samples were analyzed blindly along with 14 reference control samples. Provided a new sequence variant was found by HRM analysis, all the remaining exons of the suspected gene were then sequenced (ABI PRISM 3100/3100-Avant Genetic Analyser, Applied Biosystems). Variants of both *COX4I2* (rs6088855) and *COX10* (c.1291C>T, rs113058506) were clearly distinguishable from the wild type when the DNA was mixed at a 1:1 ratio. The use of High Sensitivity Master Mix (Idaho Technology Inc.) allowed superior resolution of all genotypes for exon 5 of the *COX10* gene and exon 9 of the *COX15* gene. All the other common variants were readily identified by HRM during the first experiment.

In silico analysis

The web servers SIFT, SNAP, PolyPhen-2, MutPred, PMut and PANTHER were used to evaluate the possible pathogenicity of the identified missense substitutions as described elsewhere.[23–28]

Molecular genetic analysis

The frequency of rare sequence variants in the Czech population was ascertained by PCR-restriction fragment length polymorphism and/or HRM analysis. RNA was isolated from human cell fibroblasts and transcribed to cDNA as described previously.[29] cDNA and promoter analysis of the *COX4I2, COX5A, COX7A1* and *COX10* genes were performed in patients P8, P12, P17 and P29 (Expand Long Template PCR System, Roche Diagnostics GmbH, Mannheim, Germany). The nuclear DNA of these patients was analyzed using a Genome-Wide Human SNP 6.0 microarray chip (Affymetrix, Santa Clara, CA, USA).

RESULTS

Mutation analysis of mtDNA in the investigated group of patients

In 59 of the patients, sequencing of the mtDNA revealed only known common polymorphisms listed in Mitomap

Table 1 New intronic sequence variants identified by high-resolution melting (HRM) analysis

Gene	*Site of variation*	*Comments*
COX10	c.929-8_-7insCCC	Heterozygous in 11 patient and 1 control samples
COX4I2	c.*63C>A	Heterozygous in 1 patient sample
COX6A1	c.-45G>A	Heterozygous in 1 patient sample
	c.*21C>T	Homozygous in 1 patient sample and heterozygous in 2 patient and 2 control samples
	c.*147C>T	Heterozygous in 1 patient sample
COX7A1	c.102+16G>C	Heterozygous in 1 patient sample
COX7A2	c.-6T>A	Heterozygous in 2 patient and 2 control samples

(http://www.mitomap.org/MITOMAP) or mtDB—Human Mitochondrial Genome Database (http://www.mtdb.igp.uu.se/index.html). However, in patient P25, a homoplasmic variant, m.15866A>G (p.N374D), in *MT-CYB* gene was detected that has not been yet reported. It was found to be homoplasmic in a patient skeletal muscle, blood and cultivated fibroblasts and was not present among the 200 healthy controls, which was confirmed by BbsI-restriction fragment length polymorphism analysis. Because we lost the contact with the patient family, the presence of m.15866A>G (p.N374D) in *MT-CYB* gene could not be tested in maternal relatives. The protein alignment showed that the Asp374 of cytochrome-*b* is not evolutionary conserved. Because the western blot assembly profile and the activity of complex III performed on patient fibroblast were within the physiological range, we assumed that this base-pair exchange was a polymorphic variant. Considering the highly polymorphic nature of mitochondrial genome and the strict application of postulated pathogenicity criteria,[30,31] we assessed the m.15866G variant as polymorphic. These data suggested a nuclear genetic origin of the COX deficiency in the patient. However, subtle modification in the rate of complex III biogenesis might occur, as documented by others.[32,33] With the exception of the new homoplasmic m.15866G variant, the patient P25 carried only common SNPs in her mtDNA.

HRM analysis

Our findings expanded the known spectrum of mutations and SNPs in the *COX* genes. We documented nine new sequence variants; two were located in exons of *COX7A1* (c.91_93delAAG, p.K31del) and *COX6A2* (c.34T>G, p.L12V), whereas the remaining seven were located in introns of COX-related genes (Table 1). All the newly identified nucleotide sequence data are available in the EMBL database under the WEBIN ID accession numbers HE647854–HE647864. Furthermore, the HRM technique directly detected 52 known homozygous and/or heterozygous sequence variants located in *COX*-related genes (Supplementary Table 2). In all, 33 amplicons had a wild-type profile and did not have any heterozygous sequence variants. The 175 homozygous and/or heterozygous genetic variants, which when combined resulted in 152 distinct genotypes, were correctly detected.

In silico analysis

Seven sequence variants leading to missense amino-acid substitutions were identified in a total of eight patients (P4, P8, P12, P17, P25, P29, P33 and P39) located in four of the nuclear-encoded subunits, *COX4I2*, *COX5A*, *COX6A2* and *COX7A1*, in an assembly factor *COX10* (Figure 1, Table 2) and in mtDNA. In the 52 remaining patients, common SNPs were merely found (Supplementary Table 2). The onset of disease, symptoms and biochemical findings of four patients P8, P12, P17 and P29 harboring the four potentially pathogenic heterozygous mutations in nuclear DNA and of one patient P25 carrying the new homoplasmic variant in mtDNA are listed in Table 3.

The heterozygous variant of the *COX4I2* gene that was identified in patient P8 was predicted to be pathological by SNAP, PolyPhen-2, MutPred and PMut. The SIFT software uses the protein alignment only, which may explain why p.R85W *(COX4I2)* was scored as tolerable, as opposed to the four other algorithms. Owing to the lack of data, PANTHER could not assign the status of this substitution.

Although SIFT determined the *MT-CYB* (p.N374D) and *COX10* (p.R431W) substitutions to be non-tolerable, both predictions had low confidence scores. Additionally, the latter variant was classified as having low reliability by SNAP. The use of a different data library and

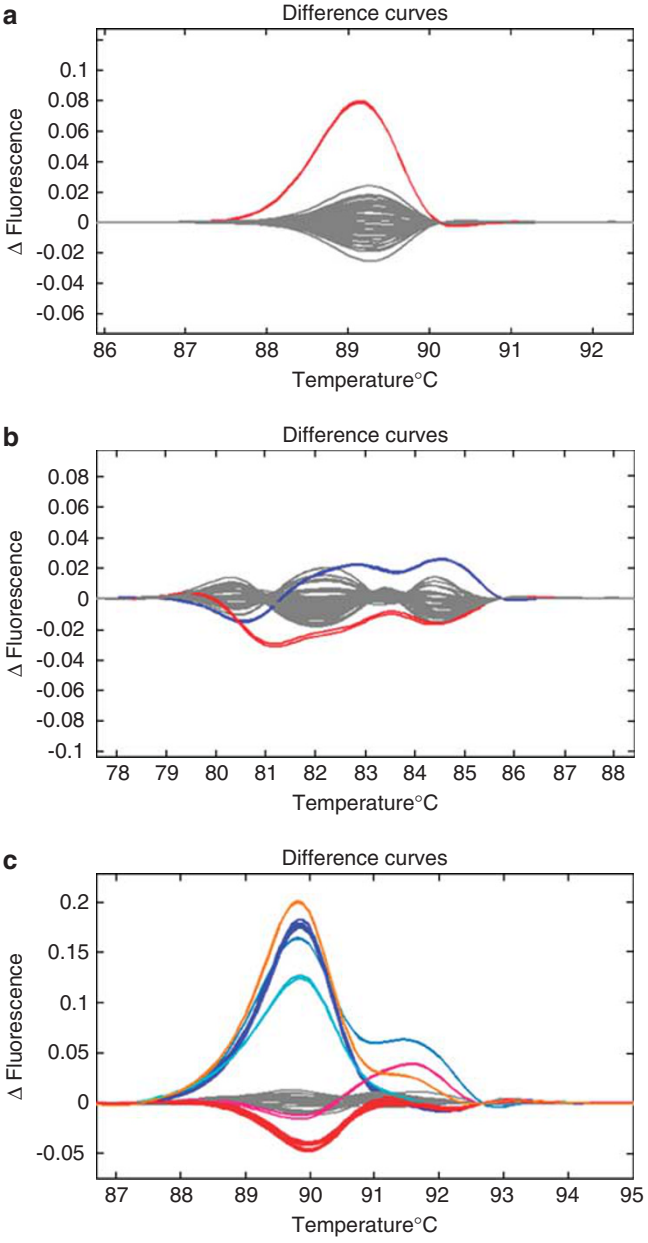

Figure 1 Melting curve plots for the amplicons covering three exons of *COX4I2*, *COX5A* and *COX7A1*. Melting curves detected for exon 4 of *COX4I2*. The grey line depicts the wild type and the red line depicts the heterozygous variant c.253C>T (p.R85W) (**a**). Melting curves detected for exon 2 of *COX5A*. The grey line depicts the wild type, the red line depicts the heterozygous variant c.101–63G>A and the blue line depicts the heterozygous variant c.212G>A (p.R71H) (**b**). Melting curves detected for exon 2 of *COX7A1*. Seven distinct sequence genotypes were detected in these amplicons, which demonstrates the efficiency of the HRM assay. The orange line depicts rs68159832 (G), rs2285599 (G) and rs2285598 (G/C). The dark-blue line depicts rs68159832 (G), rs2285599 (G) and rs2285598 (G). The middle blue line depicts rs68159832 (G), rs2285599 (T/G) and rs2285598 (G/C). The light-blue line depicts heterozygous c.91_93delAAG (p.K31del), rs68159832 (G), rs2285599 (G) and rs2285598 (G). The grey line depicts rs68159832 (delG/G), rs2285599 (T) and rs2285598 (G). The rose line depicts rs68159832 (delG), rs2285599 (T), rs2285598 (G) and c.102+16G>C. The red line depicts rs68159832 (delG), rs2285599 (T) and rs2285598 (G) (**c**).

Table 2 Non-synonymous variants identified in 60 investigated patients

			Prediction of pathogenicity					
Patient	*Site of variation*	*Type of variation*	*SIFT*	*SNAP*	*PolyPhen-2*	*MutPred*	*PMut*	*PANTHER*
P25	***MT-CYB*** m.15866A>G (p.N374D)	Homoplasmic	***Non-tolerated?***	**Neutral**	**Possibly damaging**	**Possibly pathogenic**	**Pathological**	**Neutral**
P8[a]	***COX4I2*** c.253C>T (p.R85W) **rs149245323**	Heterozygous	**Tolerated**	**Non-neutral**	**Probably damaging**	**Pathological**	**Pathological**	**Not assessed**
P17	***COX5A*** c.212G>A (p.R71H) **rs150174803**	Heterozygous	**Non-tolerated**	**Non-neutral**	**Possibly damaging**	**Possibly pathogenic**	**Pathological**	**Pathological**
P4, P33	***COX6A2*** c.34T>G (p.L12V)	Heterozygous	**Tolerated**	**Neutral**	**Benign**	**Neutral**	**Neutral**	**Neutral**
P8[a], P39	***COX10*** c.1096G>T (p.V366L) **rs111541535**	Heterozygous	**Tolerated**	**Neutral**	**Benign**	**Neutral**	**Neutral**	**Neutral**
P12[b], P29	***COX10*** c.1291C>T (p.R431W) **rs113058506**	Heterozygous	***Non-tolerated?***	**Non-neutral**	***Benign? Probably damaging?***	**Neutral**	**Pathological**	**Pathological**

[a]In patient P8, two diverse heterozygous missense substitutions were identified in *COX4I2* (p.R85W) and *COX10* (p.V366L).
[b]Patient P12 harbored two distinct heterozygous variants of *COX7A1* (p.K31del) and *COX10* (p.R431W). Patients, affected genes and types of pathogenicity predictions are in bold. Uncertainty of pathogenicity is indicated by question mark and in italics.

algorithm may be the reasons for the neutrality of p.R431W *(COX10)* that was predicted by MutPred. Contradictorily, PolyPhen-2, which uses the two modus operandi algorithm, indicated that p.R431W *(COX10)* was probably damaging (HumDiv: score = 0.986; sensitivity = 0.72; specificity = 0.96) and benign (HumVar: score = 0.365; sensitivity = 0.85; specificity = 0.77), which implies that this variation might be pathogenic.

Two variations, p.L12V *(COX6A2)* and p.V366L *(COX10)*, were each determined to be benign. Therefore, they are most likely to be neutral SNPs. Moreover, both variants occur at sites that are not evolutionary conserved. The pathogenicity classification of the *MT-CYB* missense substitution is ambiguous. Unfortunately, the prediction methodology, which would be able to infallibly evaluate missense variations located in highly polymorphic vital proteins, such as cytochrome-*b*, is not available at present. To the best of our knowledge, there are no predictive online servers that would be able to score proteins with deletions, such as the p.K31del (*COX7A1*).

Frequency of rare missense variants

Three heterozygous missense variations in *COX4I2* (c.253C>T, p.Arg85Trp), *COX5A* (c.212 G>A, p.Arg71His) and *COX7A1* (c.91_93delAAG, p.K31del) were found in both the group of 60 patients and a set of 100 healthy control samples, which was confirmed by PCR-restriction fragment length polymorphism analysis. This finding was further confirmed by PCR-restriction fragment length polymorphism analysis. Using HRM, the variant c.1291C>T (p.Arg431Trp, rs113058506) was found to be a heterozygous variant in two patients (2/60) and in two control samples (2/250). No homozygotes for c.1291T of *COX10* were detected. Additionally, the protein alignment showed that the affected codons are evolutionary conserved. Even though all the remaining exons and adjacent intronic regions of the suspected genes were sequenced in P8 (COX4I2), P17 (COX5A), P29 (COX10) and P12 (COX7A1 and COX10), only common SNPs were identified. No mutations in the promoter regions and/or alternative cDNA splicing products were detected in *COX4I2*, *COX5A*, *COX7A1* and *COX10* of the patients P8, P12, P17 and P29. Besides, the deletions overlapping *COX4I2*, *COX5A* and *COX7A1* genes were proved to be absent in all the four investigated patient genomic DNAs by microarray analysis.

DISCUSSION

HRM analysis is capable of detecting homozygous and/or heterozygous sequence variations in amplified PCR products through monitoring differences in their thermal stability and evaluation of the shape and/or shift in their melting curves.[34,35] Although HRM analysis makes it possible to screen the entire amplicon region, sequencing is still needed to determine the precise sequence variation that is present in an amplicon. The accuracy of HRM analysis is dependent on the salt concentration, GC content, length and the primary sequence of the duplex. Additionally, it could be affected by the presence of many melting domains.[36–38] However, several strategies can be used to achieve better resolution: genotyping using small amplicons, unlabelled probes, snapback primers, internal temperature calibrators and/or mixing patient samples with the reference control genotype.[39–44]

We used internal calibrators and DNA mixing to improve the resolution of individual genotypes for four amplicons of the COX genes. However, the majority of examined amplicons did not require these adjustments. Our findings expanded the known spectrum of SNPs in *COX* genes. Using the optimized HRM methodology, we identified two new variants with altered codons, *COX6A2* (p.L12V) and *COX7A1* (p.K31del), that had not been previously described. Moreover, we discovered seven new non-coding variations in *COX4I2, COX6A1, COX7A1, COX7A2* and *COX10*.

Because it is important to distinguish newly identified neutral variants from those that affect protein function, we applied online computational prediction tools to classify the variants. Nevertheless, proper interpretation of the results that were extracted from the web must take into account the differences in criteria and in the sequence and structural data that was used as the standard for the functional comparison of the analyzed mutant protein.[45–47] The reliability of pathogenicity-prediction methods has been verified by several comparative studies to be approximately 81–92%.[48,49] Despite the mentioned drawbacks, the current predictive tools are an invaluable resource for genetic testing, especially because of their ability to determine when a rare sequence variant may be the cause of a Mendelian disorder. However, the application of only one predictive algorithm could be misleading.

Six rare heterozygous base-pair variations were found in five nuclear-encoded genes that affect codons of *COX4I2* (p.R85W), *COX5A* (p.R71H), *COX6A2* (p.L12V), *COX7A1* (p.K31del) and *COX10* (p.V366L, p.R431W) and were classified by predictive bioinformatics tools. To the best of our knowledge, this is the first time that these missense variants were detected in COX-deficient

Table 3 Phenotype–genotype comparison of the patients with COX deficiency and heterozygous potentially pathogenic mutations

Patient	*P8*	*P12*	*P17*	*P29*	*P25*		
Genotype							
Found potentially pathogenic base-pair exchange	c.253C>T (*COX4I2*)	c.91_93delAAG (*COX7A1*); c.1291C>T (*COX10*)	c.212G>A (*COX5A*)	c.1291C>T (*COX10*)	m.15866A>G		
Investigated heterozygous variants in parents[a]	NA	not present in mother (*COX7A1*); heterozygous in mother (*COX10*)	NA	heterozygous in mother (*COX10*)	NA		
Phenotype							
Sex	M	F	M	F	F		
Age at onset of disease	neonatal	neonatal	6 months	neonatal	1 year		
Intrauterine growth retardation		+		+			
Failure to thrive	+	+	+	+	+		
Delay of psychomotor development	+		+		+		
Encephalopathy	+	+	+	+	+		
CT/MRI of the brain	cortical atrophy		cortical atrophy	cortical atrophy			
Epilepsy	West syndrome				West syndrome		
Hypotony	+	+	+	+	+		
Myopathy		+		+	+		
Cardiomyopathy				hypertrophic			
Hepatopathy	+	+	+	+			
Anemia	+	+	+	+	+		
Lactate acidosis	+	+			+		
Further signs	Visual impairment	Digestive dilatation, nephropathy	Hepatosplenomegaly, skoliosis, kyphosis	Hepatomegaly, dysmorphia	Hypertrichosis, hepatosplenomegaly, dermatitis seborrhoica, recurrent respiratory infection		
Age of death	10 months	1 month	26 months	12 months	Loss of contact with the patient family		
Respiratory chain analysis (nmol min^{-1} per mg of protein)[b]	Muscle mitochondria	Fibroblasts	Muscle homogenate	Liver biopsy—histochemistry	Thrombocytes	Fibroblasts	Muscle biopsy—histochemistry
CIV (COX)	**127.9** (285–1080)	**13.68** (18–40)	**50.9** (68–213)	Marked CIV deficiency	**5.71** (20–40)	23.9 (18–40)	Moderate CIV deficiency
CS	324 (200–640)	41.53 (45–70)	97.4 (48–128)		42.37 (20–40)	47.94 (45–70)	
CIV/CS	**0.39** (1.11–2.21)	0.33 (0.33–0.69)	**0.52** (0.74–4.08)		**0.13** (0.23–0.35)	0.49 (0.33–0.69)	
CI	**133.4** (195–355)	**10.4** (15–50)	nd		21 (21–55)	30.8 (15–50)	
CII	25 (10–105)	10 (5–20)	nd		6.7 (5–15)	13.75 (5–20)	
CIII	**29** (100–260)	nd	nd		11 (9.5–18)	16.27 (7–30)	

Abbreviations: COX, cytochrome-*c* oxidase; CT, computed tomography; F, female; M, male; MRI, magnetic resonance imaging; NA, not analyzed; nd, not done.
[a]Parents' DNA samples for genetic analyses were available only in mother of patient P12 and P29. Consanguinity of the parents was not present in any case.
[b]Enzyme activities below the reference range are in bold. Reference values are in brackets.

patients. With regard to the findings of the NHLBI-ESP (https://esp.gs.washington.edu/drupal) and the 1000 Genomes (http://www.1000genomes.org/home) research projects, the homozygous c.253T (*COX4I2*), c.212A (*COX5A*) and c.1291T (*COX10*) variants are extremely rare and could be pathogenic. Because the defects caused by mutations in nuclear-encoded COX-related genes are autosomal recessive, patients P8, P12, P17 and P29 should be considered heterozygous carriers of pathogenic mutations. Thus, it is evident that every human individual is a complex variable mosaic of potential pathogenic variants, which is in accordance with the results from exome sequencing and whole-genome microarray analyses.[50–52] Nevertheless, an additional study of the four non-synonymous variations, p.R85W *(COX4I2)*, p.R71H *(COX5A)*, p.K31del *(COX7A1)* and p.R431W *(COX10)*, should be performed to evaluate their pathogenicity, significance and severity. Cells with stable down-regulated expression of individual subunits may be utilized.[53]

The clarification of complexity of COX defect is exceptionally challenging, especially because of the phenotype–genotype variability in COX-deficient patients. Because the existence of new uncharacterized assembly factors taking part in biogenesis of individual respiratory complexes are highly probable, a whole-exome sequencing is the optional approach to find the molecular bases of COX defect in our patients.[54] As reported, high-throughput next-generation sequencing has enabled to sequence the whole DNA or RNA in a cost-efficient

way, which may improve diagnosis and counselling in affected families.[55] Hence, next-generation sequencing has opened discovery of disease-causing or disease-associated variants not available before but also new challenges, particularly in processing, analyzing and interpreting data.[56]

In conclusion, we designed and validated an HRM assay of 15 nuclear-encoded genes of COX that were possible causes of COX deficiency. We demonstrated that HRM and predictive methodologies are suitable low-cost screening tools. In accordance with the results obtained by the employed prediction tools, we documented nine new variants of COX-related genes, which updated the contemporary spectrum of known genetic sequence variations present in the Czech population. These variants will be important for future targeted mutation screening in Czech COX-deficient children.

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