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ORIGINAL INVESTIGATION

Observation of novel COX20 mutations related to autosomal recessive axonal neuropathy and static encephalopathy

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Abstract

Cytochrome c oxidase 20 (COX20)/FAM36A encodes a conserved protein that is important for the assembly of COX, complex IV of the mitochondrial respiratory chain. A homozygous mutation (p.Thr52Pro) in COX20 gene has been previously described to cause muscle hypotonia and ataxia. In this study, we describe two patients from a non-consanguineous family exhibiting autosomal recessive sensory-dominant axonal neuropathy and static encephalopathy. The whole-exome sequencing analysis revealed that both patients harbored compound heterozygous mutations (p.Lys14Arg and p.Trp74Cys) of COX20 gene. The pathogenicity of the variants was further supported by morphological alternations of mitochondria observed in sural nerve and decreased COX20 protein level of peripheral blood leucocytes derived from the patients. In conclusion, COX20 might be considered as a candidate gene for the complex inherited disease. This observation broadens the clinical and genetic spectrum of COX20-related disease. However, due to the limitation of a single-family study, additional cases and studies are definitely needed to further confirm the association.

Introduction

Cytochrome c oxidase 20 (COX20, also known as FAM36A) encodes a conserved protein that is important for the assembly of COX, complex IV of the mitochondrial respiratory chain (Bourens et al. 2014; Herrmann and Funes 2005; Szklarczyk et al. 2013). Defects in the assembly of COX are a frequent cause of oxidative phosphorylation disorders in humans. Patients suffering from COX-related mitochondrial diseases present with heterogeneous clinical phenotypes ranging from encephalomyopathy, hypertrophic cardiomyopathy and liver disease to Leigh's syndrome (Bourens et al. 2014). While only a number of COX deficiencies can be traced to mutations in the mitochondrial and nuclear-encoded subunits, the majority of disease-causing mutations can be found in genes encoding assembly factors. COX20 is a critical COX2-specific assembly factor that acts as a chaperone in the early steps of COX2 maturation (Bourens and Barrientos 2017). In the absence of COX20, COX2 is inefficiently incorporated into early COX subassemblies (Bourens et al. 2014; Szklarczyk et al. 2013). In humans, a homozygous mutation (c.154A>C; p.Thr52Pro) in the COX20 gene has been linked to muscle hypotonia and ataxia from separate consanguineous families. Muscle biopsies and protein analysis of fibroblasts from the patients showed decreased COX activity, and an absence of COX20 protein, respectively (Doss et al. 2014; Szklarczyk et al. 2013).

In this study, we describe two patients from one family exhibiting the same phenotype with features that include sensory-dominant axonal neuropathy and static encephalopathy. The whole-exome sequencing (WES) identified the same novel compound heterozygous mutations (p.Lys14Arg and p.Trp74Cys) of the COX20 gene, co-segregated with the

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disease state. Sural nerve biopsy of the proband confirmed an axonal neuropathy and morphological alternations of the mitochondria to some extent. Protein analysis of the peripheral blood leucocytes (PBL) from both affected individuals revealed significantly decreased level of COX20 protein compared to the normal control.

Materials and methods

Patients

All family members were evaluated at the Neurology Department of The First Affiliated Hospital of Zhengzhou University. They were independently examined by two senior neurologists who were long engaged in neuromuscular disorders. This study was approved by the Local Institutional Review Board (IRB). A written informed consent was obtained from all participants or their guardians. Detailed clinical, physical and biochemical investigations were performed for affected individuals. Electromyography (EMG)/nerve conduction studies (NCS) data were acquired using conventional methods for all family members, with limb temperature maintained at 36 °C.

Nerve biopsy

The sural nerve biopsy was performed in the proband after obtaining informed consents. The paraffin-embedded specimens were processed for hematoxylin and eosin (HE), modified Gomori trichrome (MGT) and Congo red stain. Semithin sections were stained with toluidine blue and viewed under light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate, and were examined by electron microscope (EM).

Whole-exome sequencing

Genomic DNA was extracted from peripheral blood samples of all family members. We carried out WES on DNA using the Agilent Exome kit for exon enrichment. The reads were aligned for single-nucleotide polymorphism (SNP) and indel calling. Sanger sequencing with specific primers was conducted to confirm the identical variants of the proband and his parents. The frequency in the general population of the identified variants was checked using the single-nucleotide polymorphism (dbSNP) database and the 1000 Genomes Project. PolyPhen-2 and Mutation Taster was used to predict the possible protein functional changes caused by the variant. The American College of Medical Genetics (ACMG) standards and guidelines were applied to determine the pathogenicity of each variation. To exclude the possibility that the identical variants represent polymorphisms, identical genomic fragments from 200 healthy controls of Chinese origin were examined for the presence of the mutations.

Western blotting

PBL from the affected individuals were used for measuring the expression level of COX20 protein. Protein samples were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in phosphate buffered saline (PBS) containing 0.1% [v/v] Tween-20 (PBST) prior to incubations with primary antibodies. The following primary antibodies were used: rabbit anti-COX20 (Eterlife, Edgbaston Birmingham, UK), mouse anti-β-actin (Proteintech, Wuhan, Hubei, China). Immunoreactive bands were visualized using the enhanced chemiluminescence kit (Thermo Scientific) and detected using the Chemidoc XRS + system (Biorad). The densitometric values of the bands were normalized to β-actin levels. Western blot was independently performed three times with comparable results.

Results

Patients exhibit sensory-dominant axonal neuropathy and static encephalopathy

The proband (II-3, Fig. 1a) was a 19-year-old male who complained of inability to walk and intractable dysesthesia in his extremities. He was born by normal delivery as the third child of two healthy non-consanguineous parents, and showed slightly delayed development. He was unable to walk until 14 months old, and he could not run fast. He developed mild muscle weakness of bilateral legs and began to trip and had an occasionally fall at the age of 9. At 13 years of age, symptoms of dysesthesia, dysarthria and intellectual disability were noted. It was difficult for him to keep up with peers and he quit middle school at age 14. He was inadequately nourished, and his weakness had progressed steadily. On examination, performed by the authors at age 19, inspection revealed the presence of dysarthria, distal limb muscle hypotrophy, claw hands, pes cavus, hammer toes and shortened heel tendons (Fig. 1b). Strength examination was significant for moderate reduced power in his legs symmetrically, with proximal and distal muscle strength of lower limbs grading 4/5 and 3/5 on the Medical Research Council (MRC) score, respectively. Distal muscle strength of upper limbs was graded 4/5, whereas the rest muscles were relatively preserved (5/5). Stretch reflexes were diminished symmetrically throughout. Sensory examination was abnormal because of reduced temperature and pinprick sensation to the ankles bilaterally and reduced vibratory perception from the toes

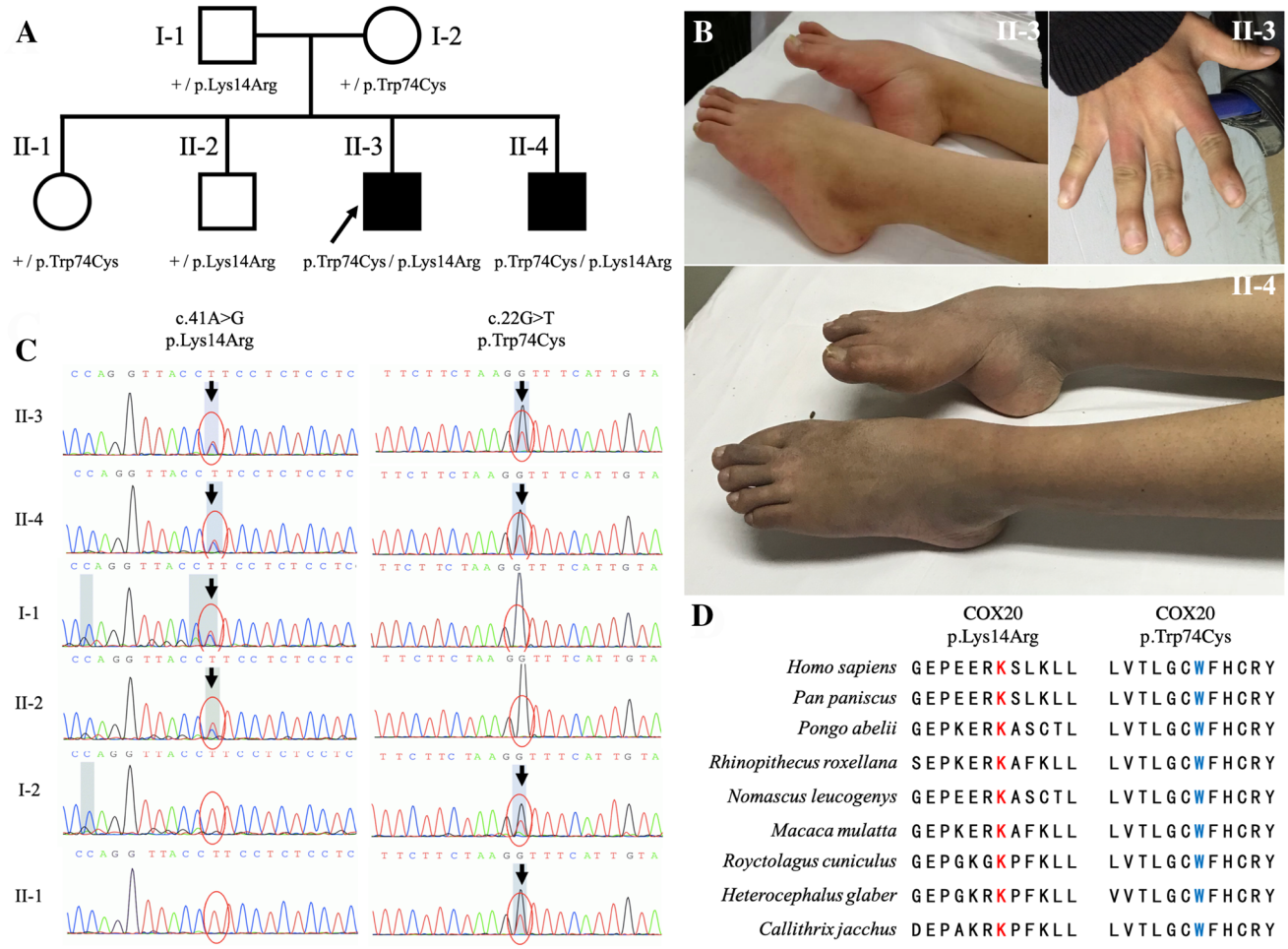

Fig. 1 Identification of COX20 variants in the family. **a** Pedigree of the family with segregation analysis. The pedigree is shown with squares representing males, circles representing females. Filled and open symbols represent affected and unaffected individuals, respectively. The genotypes are indicated under each symbol. Proband is indicated with an arrow. **b** Pictures from the patients show claw hands and pes cavus. **c** Representative sequence chromatograms are shown for the identified variants (protein and DNA annotations are provided at the top) in the indicated individuals. Arrows indicate the position of each variant. **d** The sequences of the COX20 protein from a range of divergent species, compared with multiple sequence alignment tool. The mutated regions of the lysine amino acid at position 14 (highlighted with red color) and the tryptophan amino acid at position 74 (highlighted with blue color) are highly conserved

to the knees bilaterally, with normal Romberg. No cerebellar abnormality was observed. Blood tests were normal, including consistently normal creatine kinase (CK), lactic acid and pyruvic acid levels. Electrocardiogram and echocardiography were normal. Cranial magnetic resonance imaging (MRI) showed an arachnoid cyst, whereas whole-spinal MRI revealed no abnormalities. Cognitive examinations, performed at age 19, revealed noteworthy cognitive impairment, with 15/30 and 6/30 on the mini-mental state examination (MMSE) and Montreal Cognitive Assessment (MoCA) score, respectively. NCS were consistent with axonal loss affecting predominantly sensory nerves, whereas the motor nerves were relatively preserved (Table 1). The needle EMG revealed increased duration and amplitude of motor unit action potentials (MUAP) but no fibrillation potentials or positive sharp waves (Table 1) in the detected muscles of four extremities, which indicated a chronic motor neuropathy.

The proband's 16-year-old brother (II-4, Fig. 1a) was later referred to our clinic with concerns of hereditary sensorimotor neuropathy because of similar symptoms. Pregnancy and birth at term were uneventful. He became aware of numbness in the four extremities after an accidental burn to lower limbs at age 3. Similar to his brother, he experienced distal limb weakness at age 6, and recalled difficulty running and keeping up with peers. He noted intellectual disability and dropped out of primary school at age 7. He could not read or write words. He became wheelchair-bound after a few years due to aggravated limb weakness, and exhibited progressive dysarthria and dysphagia. He denied autonomic symptoms. Neurological examination at 16 years of age revealed dysarthria,

Table 1 Electrophysiological studies in the family

Motor nerves	Proband (19/M)		Younger brother (16/M)	
	MNCV (m/s)	CMAP (mV)	MNCV (m/s)	CMAP (mV)
L median	52 (≥48)	9.5 (≥8)	49	6.1
R median	52	7.5	60	9.1
L ulnar	55 (≥48)	4.7 (≥8)	55	5.2
R ulnar	59	10.1	64	5.8
L deep peroneal	44 (≥40)	6.2 (≥6)	38	1.1
R deep peroneal	44	4.3	37	2.6
L tibial	43 (≥40)	20.6 (≥6)	38	11.3
R tibial	42	13.5	39	9.3
Sensory nerves	SNCV (m/s)	SNAP (μV)	SNCV (m/s)	SNAP (μV)
L median	NE	NE	NE	NE
R median	NE	NE	NE	NE
L ulnar	NE	NE	NE	NE
R ulnar	NE	NE	NE	NE
L superficial peroneal	NE	NE	NE	NE
R superficial peroneal	NE	NE	NE	NE
L sural	NE	NE	NE	NE
R sural	NE	NE	NE	NE
Mean values for MUAP	Dur. (ms)	Amp. (μV)	Dur. (ms)	Amp. (μV)
L opponens pollicis	11.1 (8–12)	1289 (300–1000)	12.1	2280
R opponens pollicis	11.3	1251	10.0	1178
L extensor digitorum communis	11.7 (8–12)	4050 (300–1000)	ND	ND
R extensor digitorum communis	ND	ND	15.3	5087
L biceps brachii	ND	ND	12.9	1185
R biceps brachii	12.1 (8–12)	1929 (300–1000)	ND	ND
L rectus femoris	14.1 (8–12)	2449 (300–1000)	ND	ND
R rectus femoris	16.8	2742	15.9	2163
L tibialis anterior	12.7 (8–12)	1355 (300–1000)	ND	ND
R tibialis anterior	12.4	1824	ND	ND

Nerve conduction studies data were acquired using conventional methods for all family members, with limb temperature maintained at 36 °C. The nerve conductions of healthy subjects in the family are all in normal range, which are not shown in the table. Normal values of age 15–20 years are given in brackets

Amp. amplitude, *CMAP* compound muscle action potential, *Dur*. duration, *M* male, *MNCV* motor nerve conduction velocity, *MUAP* motor unit action potentials, *ND* no data, *NE* not evoked, *SNCV* sensory nerve conduction velocity, *SNAP* sensory nerve action potential

high arches, curled toes, champagne bottle-shaped legs (Fig. 1b). Obvious atrophy of the distal muscles of lower limbs and mild wasting of interosseous muscles of the hands were observed. Manual muscle test revealed mild weakness of upper limbs (4/5), whereas severe weakness of proximal (2/5) and distal (0/5) lower limbs, respectively. Deep tendon reflexes cannot be elicited. Length-dependent sensory loss was found affecting all modalities. Electrocardiogram, echocardiography and brain MRI revealed no abnormalities. Cognitive examinations revealed severe cognitive impairment, with 6/30 and 2/30 on the MMSE and MoCA score, respectively. The needle EMG and NCS revealed similar findings to the proband, indicating a sensory-dominant axonal neuropathy (Table 1) with relatively mild motor nerve involvement.

The rest of the family members, including the proband's parents (I-1 and I-2, Fig. 1a) and two other siblings (II-1 and II-2, Fig. 1a), denied any symptoms of limb weakness or numbness. They underwent detailed physical examinations and neurological evaluations including NCS, which turned out normal.

WES analysis identifies compound heterozygous mutations of COX20 shared by both patients

To identify candidate variants for the unexplained phenotype, whole-exome sequencing analysis was performed on genomic DNA isolated from the whole family. We identified two variants in the gene encoding COX20 protein that were co-segregated perfectly with the disease state and met the Mendel's law of inheritance. The COX20 variants included: (1) c.41A>G, which was predicted to cause a missense variant p.Lys14Arg; and (2) c.222G>T, which was predicted to cause a missense variant p.Trp74Cys. Both affected individuals were compound heterozygous for the COX20 variants, while their unaffected mother and father carried p.Trp74Cys and p.Lys14Arg in the heterozygous state, respectively (Fig. 1c). Both missense variants were located at evolutionarily highly conserved amino acid residues (Fig. 1d), and neither variant was found in 200 healthy Chinese controls or 1000 genomes database. The ACMG standards and guidelines were used to classify the pathogenicity of these two variants (Richards et al. 2015). The p.Trp74Cys variant was regarded as pathogenic (one piece of strong pathogenic evidence, two pieces of moderate pathogenic evidence and two pieces of supporting evidence), and the p.Lys14Arg variant was classified as likely pathogenic (one piece of strong pathogenic evidence, two pieces of moderate pathogenic evidence and one piece of supporting pathogenic evidence).

Nerve biopsy confirms an axonal neuropathy with mitochondrial impairment

We performed a sural nerve biopsy of the right leg for the proband at age 18. The pathological changes of the sural nerve were uniform in different nerve fascicles without significant inflammatory cell infiltration and edema (Fig. 2a). Semi-thin sections (Fig. 2b, c) showed a pronounced depletion of large and middle-size myelinated fibers (MF). We did not observe any regenerative clusters or onion bulbs. No amyloid deposit was detected on Congo red staining (not shown). As observed in light microscopy, the ultrastructural analysis confirmed the predominant loss of large and medium MF, reflecting a severe axonal neuropathy (not shown). Results of EM analyses (Fig. 2e, f) revealed that numerous mitochondria had vacuoles, compared to a healthy control (Fig. 2d). We also observed the separation of the axons from their associated myelin sheaths in some myelinated nerves (Fig. 2g).

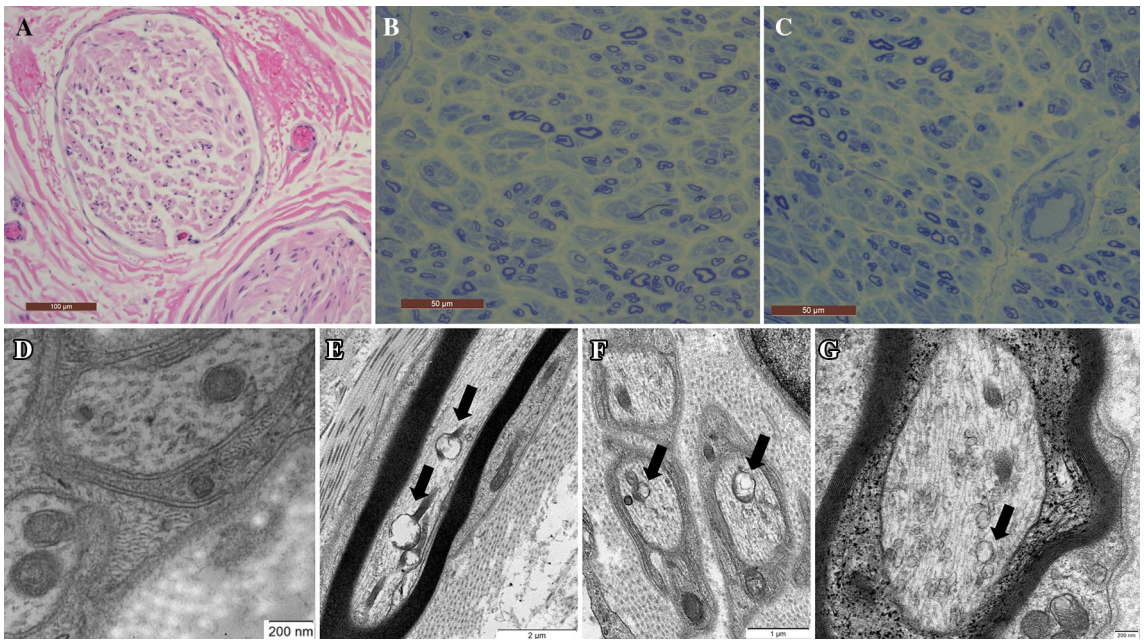

Fig. 2 Sural nerve pathological findings of the proband. The pathological changes are uniform in different nerve fascicles without inflammatory cell infiltration and edema at HE staining (**a**). Semi-thin sections show a pronounced depletion of large and middle-size myelinated fibers (**b**, **c**). Electron micrographs of the proband (**e** longitudinal section, and **f**, **g** transverse section) show that numerous mitochondria have vacuoles (arrows), compared to a healthy control (**d**). The control mitochondria are derived from a 28-year-old woman with numbness in four extremities and who was finally diagnosed with the anxiety state. The separation of the axons from their associated myelin sheaths can also be observed in some myelinated fibers, with the cytoplasmic periaxonal collar ranging from 31 to 393 nm (**g**)

COX20 mutations lead to decreased level of COX20 protein

To further evaluate the pathogenic role of the COX20 mutations, we tested whether the mutations aberrantly affected the expression of COX20 protein. We performed Western blot analysis using PBL isolated from proband (II-3), his affected brother (II-4) and a normal control. Our results revealed an up to 90% reduction in the COX20 protein levels of the patient PBL cells compared with that of the control cell line (Fig. 3).

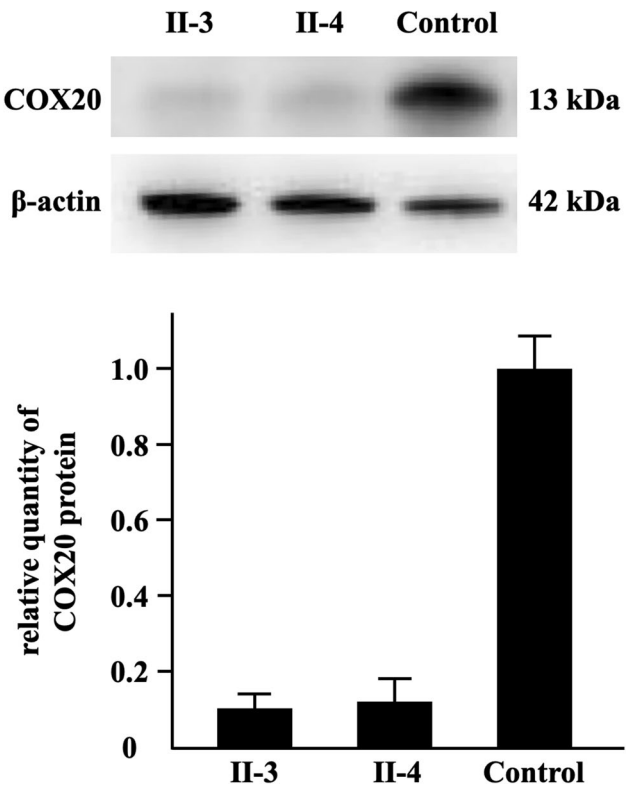

Fig. 3 Western blotting of COX20 protein from peripheral blood leucocytes isolated from the patients and a normal control. Western blot analysis is performed in the peripheral blood leucocytes derived from the patients and a normal control. The normal control is a healthy 20-year-old man who is unrelated to this family. Lower-molecular-weight bands are observed in both patients (**a**). Quantitative analysis shows that there is up to 90% decrease in COX20 protein in patients compared with control (**b**). Patient numbers are shown beneath each bar. β-Actin is included as a loading control. Western blot finding is replicated in a separate set of experiments. The bars represent the average, and the error bars represent the standard deviation of three repetitions

Discussion

We describe novel autosomal recessive mutations of the COX20 gene possibly related to a family presenting an axonal sensory-dominant neuropathy and static encephalopathy. Indeed, the spectrum of clinical involvement in this COX20-related disease is different from previously appreciated, in which muscle hypotonia and ataxia are the clinical hallmarks. The reason for that variability in phenotype remains unclear; however, it might be because of the different mutation sites (Doss et al. 2014; Szklarczyk et al. 2013). The findings broaden the genetic and clinical spectrum of COX20-related disease.

COX20 might be the culprit of the complex inherited disease in view of its crucial role in the mitochondrial respiratory chain (MRC). In human mitochondria, the MRC is composed of four multi-subunit enzyme complexes, with the COX as the terminal enzyme (Fernandez-Vizarra et al. 2009; Herrmann and Funes 2005). Human COX is formed by three catalytic core subunits (COX1, COX2, and COX3) and 11 additional subunits (Ott et al. 2016; Richter-Dennerlein et al. 2015). The enzymatic activity of the oxidase resides in the highly conserved central subunits COX1 and COX2 (Soto et al. 2012). COX biogenesis follows a linear pathway with the different subunits being added in an ordered manner around a seed formed by COX1. The current concept suggests that COX2 and COX3 associate with a specific set of assembly factors first and subsequently associate to the COX1-containing module (Dennerlein and Rehling 2015; Mick et al. 2011). Among COX2-specific assembly factors, the inner membrane protein COX20 is critical for its maturation. COX20 acts as a chaperone that binds, stabilizes newly synthesized COX2 and presents COX2 to its metallochaperone module, which in turn facilitates the incorporation of mature COX2 into the COX assembly line (Bourens et al. 2014; Szklarczyk et al. 2013). In this regard, we hypothesize that the mutations of COX20 may be associated with the disease by causing dysfunction of the MRC, and the underline mechanisms may be affecting the assembly and maturation of COX by altering the structural stability of COX20 protein. This assumption is supported by the following evidences: (1) theoretically, the variants might affect the folding and stability of COX20 protein, promoting the proteasomal degradation of misfolded COX20. COX20 is a transmembrane protein, with two transmembrane domains (Dennerlein and Rehling 2015). The variant p.Trp74Cys locates in the transmembrane domain, and the change from the tryptophane, a nonpolar amino acid, to the cysteine, a polar amino acid, is likely to cause an instability of transmembrane structure due to polarity changes; (2) Western blot analysis confirmed a decreased level of COX20

protein in the affected individuals; (3) EM evaluations revealed morphological changes of mitochondria to some extent. Additional studies are definitely needed to gain more insights into this mitochondrial dysfunction.

The association between COX20 and axonal neuropathy is not very surprising, as peripheral nerves have peculiar energetic requirements because of considerable length of axons, and thus correct mitochondria function and distribution along nerves are fundamental. So far, quite a few genes related to mitochondria have been reported to cause axonal neuropathies. For example, a mutation of COX6A1, encoding a component of COX, is reported to cause a recessive axonal or mixed form of CMT disease by reducing COX activity (Tamiya et al. 2014). Recessive mutations of SCO2, encoding an assembly protein of COX, have been proven to cause early-onset axonal CMT by resulting in the failure of SCO2 to bind and deliver copper to COX within the MRC, which will hamper the ability of rapidly respiring cells to meet their energy needs (Rebelo et al. 2018). Abnormalities of mitochondrial dynamics produced by mutations in proteins involved in mitochondrial fusion (mitofusion-2, MFN2), fission (ganglioside-induced differentiation-associated protein-1, GDAP1), and mitochondrial axonal transport (kinesin family member-5A, KIF5A) can also present with axonal neuropathies (Baxter et al. 2002; Cassereau et al. 2014; Crimella et al. 2012; Cuesta et al. 2002; Liu et al. 2014; Zuchner et al. 2004). Both patients developed dysarthria in the second decade of life, reflecting that there is a possibility of glossopharyngeal and vagus nerve involvement. We assume that dysarthria might be due to the oropharyngeal weakness, which could be part of the underlying axonal neuropathy. However, it is also possible that a soft tissue or cartilage defect is playing a role in this problem (Hamamoto et al. 2009).

Static encephalopathy is another hallmark of the patients, although neither of them show significant abnormalities on brain MRI. Central nervous system involvement is a common feature of COX-related diseases (Pecina et al. 2004; Rak et al. 2016). The dysfunctions of specific COX assembly proteins (e.g., SURF1, SCO1 and COX10) have been reported to cause Leigh's syndrome, leukodystrophy or encephalopathy (Antonicka et al. 2003; Bruno et al. 2002; Valnot et al. 2000). However, the combination of encephalopathy and sensory-dominant axonal neuropathy is relatively rare, and has been reported in several cases of mutations in CLTCL1 (congenital insensitivity to pain), TECPR2 (familial dysautonomia, hereditary, sensory AR autonomic neuropathy, with intellectual disability) and C10ORF2 (mitochondrial DNA depletion syndrome 7) (Rossor et al. 2017). The phenomenon that the COX20 variants result in symptoms of simultaneous central and peripheral involvement suggests that the pathogenesis likely involves defective energy-dependent processes in neurons, similar to other mitochondrial disorders (Del Bo et al. 2008).

In conclusion, we present clinical, genetic, and pathological data that possibly relate novel COX20 variants to a complex inherited disease with features including sensory-dominant axonal neuropathy and static encephalopathy. This observation expands the phenotypic heterogeneity associated with COX20 variants. However, due to the limitation of a single-family study, the association is not very conclusive, which would need further similar cases and mechanistic analyses.

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Author contributions XHL and JT wrote the paper. XHL, JT and LYJ conceived the idea and evaluated the patients. XHL and JT analyzed the genetic results. XHL and LS performed the sural nerve biopsy. XHL, JT, YYH and DXB evaluated the pathological findings. CX, WSY and XHL performed the Western blotting. LYJ supervised the diagnosis and treatment. All authors approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interests.

Informed consent Written informed consent was obtained from the patients and/or their guardians prior to sample collection and open sural nerve biopsy.

Ethical approval This study was approved by the ethics committee of the Zhengzhou University First Hospital and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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