

## New Splicing-site Mutations in the *SURF1* Gene in Leigh Syndrome Patients\*

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Marie O. Péquignot‡, Isabelle Desguerre§, Runu Dey¶, Marzia Tartari||, Massimo Zeviani||, Alessandro Agostino||, Chantal Benelli\*\*, Françoise Fouque\*\*, Carina Prip-Buus||, Dominique Marchant‡, Marc Abitbol‡, and Cécile Marsac‡¶¶

From the ‡Laboratoire CERTO, Faculté de Médecine Necker, 156 rue de Vaugirard, 75015 Paris, France, §Service de Neuropédiatrie, Hôpital St. Vincent de Paul, 79 avenue Denfert Rochereau, 75014 Paris, France, ¶CNRS UPR1524, 9 rue de Hetzel, 92190 Meudon, France, ||Istituto Nazionale Neurologico “Carlo Besta,” via Celoria 11, 20133 Milano, Italy, and \*\*INSERM U30, Hôpital Necker, 149 rue de Sévres, 75015 Paris, France

The gene *SURF1* encodes a factor involved in the biogenesis of cytochrome *c* oxidase, the last complex in the respiratory chain. Mutations of the *SURF1* gene result in Leigh syndrome and severe cytochrome *c* oxidase deficiency. Analysis of seven unrelated patients with cytochrome *c* oxidase deficiency and typical Leigh syndrome revealed different *SURF1* mutations in four of them. Only these four cases had associated demyelinating neuropathy. Three mutations were novel splicing-site mutations that lead to the excision of exon 6. Two different novel heterozygous mutations were found at the same guanine residue at the donor splice site of intron 6; one was a deletion, whereas the other was a transition [588+1G>A]. The third novel splicing-site mutation was a homozygous [516–2\_516–1delAG] in intron 5. One patient only had a homozygous polymorphism in the middle of the intron 8 [835+25C>T]. Western blot analysis showed that Surf1 protein was absent in all four patients harboring mutations. Our studies confirm that the *SURF1* gene is an important nuclear gene involved in the cytochrome *c* oxidase deficiency. We also show that Surf1 protein is not implicated in the assembly of other respiratory chain complexes or the pyruvate dehydrogenase complex.

Leigh syndrome (LS),<sup>1</sup> or subacute necrotizing encephalomyelopathy (MIM 256000), is a progressive and often fatal neurological disorder in young children, characterized by bilaterally symmetrical necrotic lesions in the brain stem and basal ganglia (1). A common associated biological feature is hyperlactatemia. It is a genetically heterogeneous disease, caused by defects in the enzymes normally involved in the respiratory chain and in mitochondrial energetic metabolism (e.g. pyruvate dehydrogenase). One of the most common enzymatic defects is

the deficiency of cytochrome *c* oxidase (COX), complex IV of the mitochondrial respiratory chain. Recently, mutations in the nuclear *SURF1* gene, which encodes a factor involved in COX biogenesis, have been identified in patients with LS (2, 3). This gene is located on chromosome 9q34, consists of nine exons, and encodes a protein of 300 amino acids. We have studied 58 patients who were thought to have LS based on their clinical features and on magnetic resonance imaging (MRI), which showed lesions on the basal ganglia and the brain stem. 15 of these patients presented cytochrome *c* oxidase deficiency, seven were typical LS, but only four had a severe COX deficiency (LS<sup>cox</sup>). 15 other patients were defective in pyruvate dehydrogenase with mutations in the E1  $\alpha$ -PDH and *Hs-PDX1* genes, 11 were found to have a complex I deficiency, and four had the clinical features of the maternal inheritance Leigh syndrome with a neuropathy ataxia retinitis pigmentosa mutation in the ATPase6 gene of the mitochondrial DNA. We identified six mutations, including three new splicing-site mutations, in the *SURF1* gene in the four patients with typical LS and severe COX deficiency.

### EXPERIMENTAL PROCEDURES

**Patients**—Patient 1 was the first child of non-consanguineous parents. At nine months, she presented delayed growth, difficulties in swallowing, severe hypotonia, trunk ataxia, nystagmus. She had optic atrophy and a demyelinating peripheral neuropathy. Brain MRI showed symmetric lesions of the basal ganglia and brain stem. She had hyperlactatemia (4.9 mmol/liter; normal value is below 2 mmol/liter). She died at 4 years.

Patient 2 was born to non-consanguineous parents. She developed a mild psychomotor delay with progressive ataxia, ophthalmoplegia and retinopathy, and a demyelinating neuropathy. Recurrent vomiting attacks are associated with increasing ptosis and ataxia. At 2 years, brain MRI showed lesions in the brain stem and thalamus and cerebellar atrophy. High levels of lactate were found in blood (4.14 mmol/liter). She is still alive at 14 years of age.

Patient 3 was the third child of non-consanguineous parents. The mother had two miscarriages. A sister died at 2 months of age with the diagnosis of sudden death. At 1 year of age the patient had a severe growth delay, a trunk ataxia, pyramidal signs, dystonia, and abnormal ocular movements. She had difficulties in swallowing and presented recurrent respiratory distress. She had optic atrophy, demyelinating neuropathy, and hyperlactatemia (4.75 mmol/liter). MRI showed bilateral, symmetrical lesions of the basal ganglia and the brain stem. She died at the age of 2 years.

Patient 4 was born to consanguineous parents and had a cousin affected by the same condition. She had a metabolic encephalopathy, which was revealed at 3 months of age by severe hypotonia, hyperlactatemia, and nystagmus. The MRI exhibited bilateral lesions in the caudate nuclei.

Patient 5 was a Libyan Arab male baby born from first cousin parents. Persistent vomiting, profound developmental delay, and severe hypotonia were the main clinical features leading to his death at 18

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¶¶ To whom correspondence should be addressed: Laboratoire CERTO, Faculté de Médecine Necker, 156 rue de Vaugirard, 75015 Paris, France. Tel.: 1 40 61 53 55; Fax: 1 40 61 54 74; E-mail: marsac@necker.fr.

<sup>1</sup> The abbreviations used are: LS, Leigh syndrome; COX, cytochrome *c* oxidase; LS<sup>cox</sup>, Leigh syndrome associated with highly reduced COX activity; MRI, magnetic resonance imaging; PDH, pyruvate dehydrogenase; PDHC, pyruvate dehydrogenase complex; RT-PCR, reverse transcriptase polymerase chain reaction; bp, base pair.

months. The MRI showed lesions typical of LS.

Patient 6 was the only child of consanguineous parents (common grandmother). Growth retardation and hypotonia were observed at 4 months. At the age of 6 years he presented a progressive encephalomyelopathy, with cortical atrophy and necrosis of the basal ganglia and brain stem. The lactate level in the blood was 3.2 mM and 5.4 mM in the cerebrospinal fluid.

Patient 7, a girl, was born to consanguineous parents (first cousin parents) and had a brother affected by the same disorder (developmental delay and hyperlactatemia). Both showed typical Leigh syndrome features confirmed by cerebral tomography scan examination. MRI detected lesions in the basal ganglia. They are still alive at the age of 10 and 7 years, respectively.

**Biochemical Studies**—Enzymatic assays were carried out on mitochondria isolated from muscle or lymphoblasts (4) of all patients except patient 5, for whom assays were performed in muscle homogenate. The activities of the respiratory chain complexes were measured spectrophotometrically as described previously (5). Pyruvate dehydrogenase complex (PDHC) activity was measured in lymphoblastoid cell lines by the release of [ $^{14}$ C]CO $_2$  from [1- $^{14}$ C]pyruvic acid (6).

For Western blot analysis, proteins derived from lymphoblastoid cell lines (patients 1, 2, 3, 4, and 7) or fibroblasts (patient 5) were separated by 10% SDS polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membrane (7). The blots were probed with polyclonal antibodies directed against the PDH holoenzyme or against the C terminus of the Surf1 protein (peptide-Y-13-V) (3).

**DNA Genomic Studies from Cultured Lymphoblasts**—Genomic DNA was purified by use of the DNAzol Reagent (Life Technologies, Inc.) as described by the manufacturer. The nine exons of the *SURF1* gene were amplified as described by Tiranti *et al.* (3) and sequenced in a commercially available, automated sequencer (Genome Express, Grenoble, France).

**RNA Extraction and RT-PCR**—Total RNA was extracted from cultured lymphoblasts with an RNA-plus™ kit (Quantum Bioprobe, Quebec, Canada). Each RNA sample (5  $\mu$ g) was reverse-transcribed using d(T)18 primers and a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The coding sequence from exon 4 to exon 9 was amplified by PCR as described by Poyau *et al.* (8). We also used three new primer pairs; the first pair was chosen to study patient 3. For161 (5'-CAAAAGCGGAAGATGACTCC-3') and Rev558 (5'-GGTTCGTTCCAGGAAGAAA-3') amplified the cDNA region from base 181 to base 538. For RT-PCR of patient 5, a first PCR was done using the primers For-9 (5'-CCGGGTGCGATGGCG-GCGGTGGCTGCGTTGCAG-3') and Rev955 (5'-CAGTAGCAGATGATCCAGCATAAAG-3'). A "nested" PCR was then carried out on this first product with For435 (5'-GGCCGGGAGGGCGGCTC-3') and Rev927 (5'-CAGTCTTGAAATACTGCATTATCCAGGG-3'). The PCR products were analyzed by agarose gel electrophoresis (2%), stained with ethidium bromide (0.5  $\mu$ g/ml), and visualized by UV transillumination.

TABLE I

COX activity in muscle mitochondria (from controls and patients 1, 2, 3, and 4) and muscle homogenate (controls and patient 5), expressed in milliUnits/mg of protein and in COX/citrate synthase ratio

	milliunits/mg of protein	COX/citrate synthase ratio
<b>Muscle mitochondria</b>		
Control values (N = 24)	3151 $\pm$ 760	1.3 $\pm$ 0.3
Patient 1	880 – 825	0.2
Patient 2	1335 – 1440	0.28
Patient 3	764 – 689	0.17
Patient 4	1760	0.68
Patient 6	1505 – 1577	0.50
Patient 7	1461 – 1455	0.60
<b>Muscle homogenate</b>		
Control range	80 – 180	0.85 – 1
Patient 5	15	0.10

nation. cDNA fragments containing putative deletions in the gene were purified from PCR products by use of a Qiaquick PCR extraction kit (Qiagen, Courtaboeuf, France) and sequenced as above.

## RESULTS

We studied seven unrelated patients with typical LS. Four of them (patients 1, 2, 3, and 5) had dramatically reduced COX activity. This decrease was obvious in mitochondria purified from fresh muscle biopsy (Table I) and lymphoblastoid cell lines (Table II). Patients 4, 6, and 7 presented only a slight decrease of COX activity in muscle (Table I).

No band corresponding to the Surf1 protein was found by Western blot analysis of the four typically LS<sup>cox</sup> patients 1, 2, 3, and 5 (Fig. 1). Conversely, a Surf1-specific band was detected in patients 4 and 7. The intensity of the band was similar to that found in the controls. A nonspecific band just below the Surf1 protein was equally intense in all samples, suggesting that the quantity of total proteins was approximately the same.

These results prompted us to look for mutations in the *SURF1* gene by directly sequencing PCR fragments obtained with primers described previously (3). Six different mutations were found in the four patients who lacked the Surf1 protein (patients 1, 2, 3, and 5) (Fig. 2). Three of these mutations have already been described. The most frequent mutation reported in *SURF1* gene, the [312\_321del311\_312insAT], was found in patients 1 and 2 (2, 3); the [737T>C] mutation was detected in patient 2 (8), and a mutation in the splicing donor site of intron 3 [240+1G>T] was found in patient 3 (9).

Three new mutations were found, two of which involved the first base of intron 6: a deletion of the G in position 588+1 was detected in patient 1, and a transition [588+1G>A] was found in patient 3. Patient 5 harbored an AG deletion in the acceptor site of intron 5, [516–2\_516–1delAG]. Patients 1, 2, and 3 were compound heterozygotes, whereas patient 5 was homozygous for the mutation.

We carried out RT-PCR to study the consequences of these mutations on the RNA sequence on patients 1, 2, 3, and 5 (Fig.

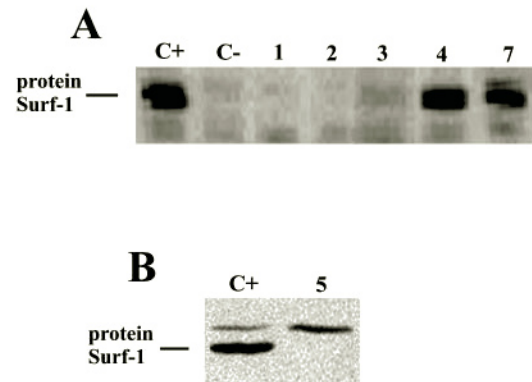


FIG. 1. Western blot with antibodies directed against the Surf1 protein. A, C+, control; C-, patient with LS<sup>cox</sup> and *SURF1* mutation; lanes 1, 2, 3, 4, and 7, correspond to patients 1, 2, 3, 4, and 7, respectively. B, C+, control; lane 5, corresponds to patient 5. Patients 1, 2, 3, and 5 and the control, C-, have no band corresponding to the Surf1 protein, whereas patients 4 and 7 have a band of the same intensity and size as the control, C+.

TABLE II  
COX activity of mitochondria purified from lymphoblastoid cell lines (control, 1, 2, and 3)

	COX (milliunits/mg of protein)	CII (milliunits/mg of protein)	COX/CSase	CII/CSase	PDH (milliunits/mg of protein)
Control	252	84	0.45	0.15	552
Patient 1	60	199	0.07	0.21	543
Patient 2	120	187	0.14	0.21	856
Patient 3	56	128	0.06	0.15	641

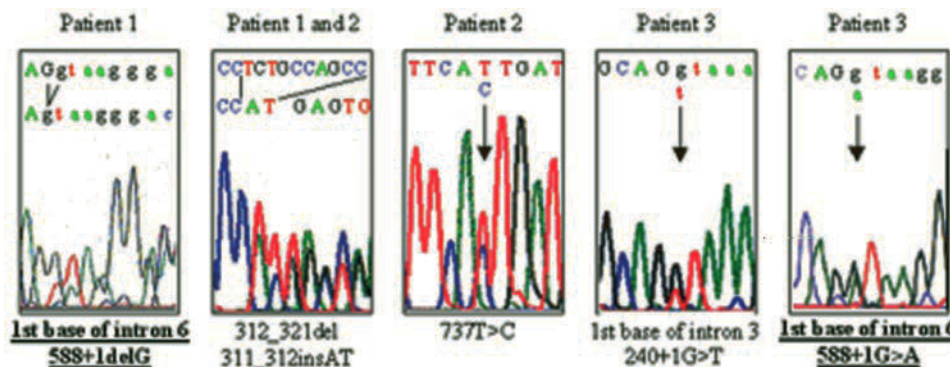


FIG. 2. **Sequence analysis of the SURF1 gene of patients 1, 2, and 3.** For patient 5, SURF1 was sequenced in the laboratory of M. Zeviani; the DNA sequence is not shown here. The DNA sequence shows the five mutations found in patients 1, 2, and 3. Mutations in **bold** are novel.

3A). A single band of 730 bp was obtained in samples from patient 2 and a control. However, using the same primers we obtained two bands from the samples of patients 1 and 3. The additional band was ~60 bp smaller than the control band in both cases (660 bp).

The sequencing of these RT-PCR products of patients 1 and 3 permitted us to conclude that the two mutations in the first base of the donor splice site of intron 6 (point mutation or deletion) led to the complete excision of exon 6. The second mutation [240+1G>T] in patient 3 has been described before (9), but its consequence on the RNA sequence was unknown. RT-PCR with the forward primer used to amplify exons 4 to 9 could not detect this mutation, because the mutation was within the primer 4 region. Thus, we used two new primers (For261 and Rev558) that annealed on either side of intron 3. The PCR product (460 bp) obtained with the new primers was 80 bp longer than in the control (380 bp) (Fig. 3B), and the additional sequence corresponded with that of intron 3. For patient 5 we performed nested PCR with two other primer pairs that normally give a 520-bp fragment, and we obtained a unique band of ~450 bp (Fig. 3C). The sequencing of this RT product revealed that the [516-2\_516-1delAG] mutation at the acceptor site of intron 5 led to the complete excision of the 73 bp of exon 6, as in patients 1 and 3.

We sequenced the genomic DNA PCR products of patients 4, 6, and 7. We found no mutations in DNAs from patients 6 or 7, but we could detect a homozygous polymorphism in intron 8, [835+25C>T] in the DNA of patient 4.

The common mitochondrial DNA mutations (myoclonic epilepsy and ragged red fibers, mitochondrial encephalopathy lactic acidosis stroke-like episodes, neuropathy ataxia retinitis pigmentosa, and deletions) associated with LS were ruled out in all patients by restriction fragment length polymorphisms and Southern blot analysis.

To determine whether the Surf1 protein is implicated in the stability of other mitochondrial complexes, we carried out enzymatic assays on the respiratory chain complexes and the pyruvate dehydrogenase complex (PDHC) in patients 1, 2, and 3 (who lack the Surf1 protein). Complex II and PDHC activity (Table II), as well as the activities of complexes I and III (data not shown), were comparable with those of controls. Western blot analysis with an antibody directed against PDHC showed subunit-specific bands of similar size and intensity in samples from patients and controls (data not shown).

#### DISCUSSION

In this study we found that LS<sup>cox</sup> was associated with SURF1 mutations in four of the seven patients (patients 1, 2, 3, and 5). Their clinical presentations were similar to those previously reported, but the incidence of mutations in our study (57%) was between that found by the studies done by Tiranti *et*

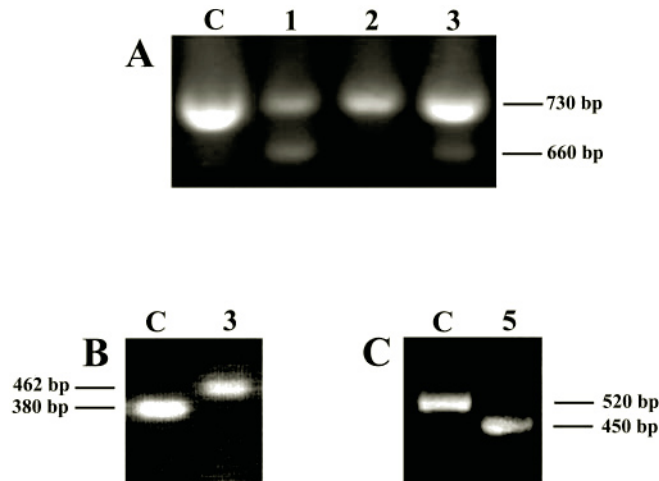


FIG. 3. **RT-PCR analysis of SURF1 expression.** A, C, control; lanes 1, 2, and 3, patients. Patients 1 and 3 have the expected 730-bp band and an additional 670-bp band, which is the result of the deletion of exon 6 in one allele. B, C, control; 3, patient 3. The mature mRNA from patient 3 contains the entire intron 3; the corresponding band is 82 bp larger than the control. C, C, control; lane 5, patient 5. The corresponding band in patient 5 is 80 bp smaller because of the deletion of exon 6 in one allele.

*al.* (9) (75%) and Sue *et al.* (10) (25%). These differences can be explained by the biochemical and genetic heterogeneity of LS. Of the 58 cases of suspected LS that we studied, 15 had a deficiency in COX activity, 15 had pyruvate dehydrogenase deficiency, 11 were suspected of having complex I defects, and four had neuropathy ataxia retinitis pigmentosa mutations. However, we can confirm that the four cases of typical Leigh syndrome were associated with severe cytochrome oxidase deficiency and have SURF1 mutations (patients 1, 2, 3, and 5).

Patients 4, 6, and 7 were also considered to be typical LS cases based on the stringent inclusion criteria, which include the presence of a progressive neurological disease, elevated levels of cerebrospinal lactic acid, and typical MRI abnormalities involving the basal ganglia and brain stem. However, they only had a partial decrease of COX activity, and patients 6 and 7 had no mutations in the SURF1 gene. Patient 4 was homozygous for the [835+25C>T] mutation. Interestingly, we found a partial COX defect in the muscles of her cousin; this cousin had the same symptoms and was heterozygous for the same mutation. This change is probably a polymorphism with no pathological significance.

We did not notice any phenotypic differences between the three patients (1, 3, and 5) with splicing-site mutations in the SURF1 gene. They had early onset severely delayed growth, optic atrophy, neuropathy, and died very early (between 18



TABLE III

Summary of the clinical features and sequence variations of the SURF1 gene in all patients studied

The nomenclature was according to Péquignot *et al.* (13).

Patients	Onset (mo)	Evolution	Clinical features	MRI	Mutation or polymorphism	Protein
1	9	Died 4 yr	Typical LS	+	[312_321del311_312insAT] + [588+1delG]	[P104fsX105] + [V172fsX183]
2	36	Alive 12 yr	Typical LS	+	[312_321del311_312insAT] + [737T>C]	[P104fsX105] + [I246T]
3	12	Died 3 yr	Typical LS	+	[240+1G>T] + [588+1G>A]	[Q80fsX90] + [V172fsX183]
4	4	Alive 15 mo	Typical LS; familial case, consanguinity	+	[835+25C>T] + [835+25C>T]	
5		Died 18 mo	Typical LS consanguinity	+	[516-2_516-1delAG] + [516-2_516-1delAG]	[V172fsX183] + [V172fsX183]

months and 4 years). Residual COX activity was always less than 25% of normal. This can be explained by the nature of the mutations and their effect on the protein; patient 1 had both the [312\_321del311\_312insAT] in exon 4 (2, 3), which leads to a truncated protein (with a stop codon at position 105 in the protein), and a deletion in the first base of intron 6, [588+1delG], which results in the production of an RNA in which the skipping of exon 6 predicts the synthesis of a truncated protein (with a stop codon at position 183). Likewise, patient 3 had two splicing mutations, a mutation in the first base of intron 3, [240+1G>T], which results in an mRNA that contains the entire intron 3, with a stop codon after amino acid 89. The second mutation is located in the first base of intron 6; this mutation [588+1G>A] has the same result on the RNA and on the protein as the [588+1delG] mutation described for patient 1. The amplification of only one abnormal band, corresponding to an mRNA retaining intron 3 (Fig. 3B), may be because of the extreme instability of the abnormal RNA that lacks exon 6. Indeed, we obtained a very faint band corresponding to this allele when the first primer pair was used for RT-PCR analysis (Fig. 3A).

As patient 5 was homozygous for the [516-2\_516-1delAG] mutation in intron 5, he had only one species of RNA in which exon 6 was excised, giving a truncated protein with a stop codon at position 183.

Three of the six splicing-site mutations have never been described before, two of them are in intron 6 of patients 1 and 3, and the third is the homozygous mutation in intron 5 of patient 5. All of these mutations lead to the excision of exon 6 in mRNA.

Paradoxically although the first base of the 5' splice site is mutated in both the splicing-site mutations in patient 3, the consequences seem to be different. One leads to the retention of the mutated intron 3, whereas the other leads to the excision of the preceding exon, exon 6. The most common effect of a 5' splice-site mutation is the excision of the preceding exon as observed in the latter case. However, inclusion of the mutant intron is rare. We can explain the retention of intron 3, because intron 3 is generally the last intron to be excised. As a result, the spliceosome machinery does not find any adjacent normal 5' splicing site, and no splicing occurs (11).

Patient 2, with no splicing-site mutation, had a different phenotype to the other patients (patients 1, 3, and 5). Even though she had typical features of Leigh symptom, she was still alive at 14 years of age. This is probably because of the fact that she had a less severe COX deficiency. We found about 40% residual COX activity in both muscle and lymphoblastoid mitochondria. Patient 2 had the [312\_321del311\_312insAT] mutation, which results in a truncated protein, and also carried the missense [737T>C] mutation, which transforms Ile<sup>246</sup> (ATT) to Thr (ACT). The protein is probably abnormal and too unstable to be detected by Western blot. It is an interesting

case, because it is the first described in the literature with a phenotype of a late Leigh syndrome associated with two SURF1 mutations.

Our results suggest that respiratory chain complexes (other than complex IV) and PDHC, are normally assembled in patients in which the Surf1 protein is either lacking or abnormal. The Surf1 protein is currently only known to be involved in complex IV assembly.

Patients 4, 6, and 7 had all the clinical features of LS<sup>cox</sup>, but no mutation in the SURF1 gene was found. This suggests that although most cases of LS<sup>cox</sup> can be explained by homozygous or compound heterozygous mutations in the SURF1 gene, mutations in other genes may lead to LS<sup>cox</sup>.

In this paper, we revealed new splicing-site mutations that are well correlated with the severity of the illness and the dramatic decrease in COX activity. Further studies are necessary to characterize the COX deficiency affecting patients 4, 6, and 7. However, we have confirmed that SURF1 mutations are responsible for Leigh syndrome associated with severe COX deficiency (12) and found a new phenotype of late Leigh syndrome associated with SURF1 mutations never described previously (2, 3, 8, 13).

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