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A hemizygous *SCO2* mutation in an early onset rapidly progressive, fatal cardiomyopathy

Scot C. Leary [b,*], Andre Mattman [a,b], Timothy Wai [b], David C. Koehn [c], Lorne A. Clarke [c], Suzanne Chan [a], Brenda Lomax [a], Patrice Eydoux [a], Hilary D. Vallance [a], Eric A. Shoubridge [b]

[a] *Department of Pathology and Laboratory Medicine, Children's & Women's Health Centre of British Columbia, University of British Columbia, 4480 Oak Street, Vancouver, BC, Canada V6H 3V4*
[b] *Montreal Neurological Institute and Department of Human Genetics, McGill University, 3801 University St., Montreal, QC, Canada H3A 2B4*
[c] *Department of Medical Genetics, Children's & Women's Health Centre of British Columbia, University of British Columbia, 4480 Oak Street, Vancouver, BC, Canada V6H 3V4*

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Abstract

Mutations in *SCO2*, a metallochaperone involved in mitochondrial copper delivery, are associated with early onset, fatal hypertrophic cardiomyopathy. All reported patients carry at least one copy of the common 1541G>A (E140K) mutation. Whereas patients with one copy of the E140K allele, in combination with a more deleterious mutation, follow a severe clinical course, patients homozygous for the E140K mutation have a delayed onset of disease and a more prolonged survival. Here, we have investigated a patient who appeared homozygous for the common 1541G>A mutation based on DNA sequencing and restriction enzyme analysis of a PCR product, yet presented with early onset, severe cardiomyopathy. Restriction enzyme analysis of parental DNA revealed that the mother was heterozygous for 1541G>A, while the father was homozygous wild-type. The patient showed biparental inheritance for microsatellite markers spanning the length of chromosome 22, making isodisomy unlikely. Sequencing of several single nucleotide polymorphisms within the 5′-UTR, intron and single exon of the *SCO2* gene was uninformative; however, a 16 bp deletion within the intron was present in the patient and the mother, but not the father. Restriction enzyme analysis confirmed that the mother was heterozygous and that the patient was hemizygous for the deletion. Southern blot, Northern blot, and FISH analyses were consistent with the de novo deletion of one allele of *SCO2* in the patient. This is the first report of hemizygosity in a *SCO2* patient. The patient phenotype underscores the strikingly similar clinical course in all patients with one copy of the E140K allele. Examination of both patient and parental genotypes by thorough molecular analyses can reveal information with important implications for genetic counseling.

Keywords: *SCO2*; Hypertrophic cardiomyopathy; Cytochrome *c* oxidase; Hemizygosity

Introduction

A deficiency in the activity of cytochrome *c* oxidase (COX) is a frequent cause of mitochondrial disease in infants, and in the majority of cases this is inherited as an autosomal recessive trait [1]. Isolated COX deficiency is associated with a variety of clinical phenotypes including Leigh Syndrome, hypertrophic cardiomyopathy, hepatopathy, and fatal infantile lactic acidosis [1,2]. Mutations in six nuclear genes coding for ancillary factors that are necessary for COX assembly have so far been described in these patients [3–8]. Two of these factors, *SCO1* and *SCO2*, are copper metallochaperones that are necessary for copper insertion into the Cu_A site of COX [9–12]. *SCO2* patients exhibit severe reductions in COX activity in the brain, cardiac and skeletal muscle, while other tissues such as the liver are less affected [4,13,14]. *SCO1* patients have a severe COX deficiency in skeletal muscle and liver (the only tissues

* Corresponding author. Fax: +1 514 398 1509.
E-mail address: sleary@po-box.mcgill.ca (S.C. Leary).

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that have been examined) [5]. This still unexplained variation in residual tissue COX activity parallels the clinical severity of organ pathology. All reported cases of *SCO2*-dependent COX deficiency have been associated with a 1541G>A (E140K) mutation, either in compound heterozygous or homozygous form. Compound heterozygotes for E140K develop a severe, early onset hypertrophic cardiomyopathy; delayed onset cases with a slower clinical progression have only been associated with E140K homozygosity [15]. All patients homozygous for the E140K mutation show Leigh-like pathology in the CNS, and predominant involvement of the peripheral nervous system that includes demyelination and denervation [15].

In the present study, we investigated a patient who presented with severe, early onset hypertrophic cardiomyopathy, but who was apparently homozygous for the common E140K mutation based on routine molecular diagnostic testing. Subsequent molecular analyses indicated a de novo deletion of one *SCO2* allele. This first report of hemizygosity in a *SCO2* patient underscores the need for follow up testing in the parents of patients who test homozygous with the simple screening tests.

Methods

Patient

The patient was the product of a dizygotic twin pregnancy born at 35 weeks gestational age via Cesarean section for fetal distress. In the weeks preceding delivery, his growth had been retarded. His parents, of Northern European descent, were not related, and had no family history of cardiac or neurologic disease. At birth, he weighed 1843 g and his Apgar scores were 4 at 1 min and 9 at 5 min. He was intubated and given intermittent positive pressure ventilation for 2 min. He was discharged at three weeks of age. He was noted to have a mild ptosis, weak grasp and poor feeding. His twin was a healthy female.

At four weeks of age he had progressive feeding difficulty and increased work of breathing, culminating in a respiratory arrest requiring intubation and ventilatory support. He developed arching episodes. Echocardiography revealed a hypertrophic left ventricle and ECG showed elevated ST segments. At five weeks of age, he had normal head CT and MRI scans. Biochemical abnormalities were noted including an elevated venous lactate. An EMG showed peripheral neuropathy due to demyelination. A brain MRI scan was normal. After failed attempts to wean the child off of his ventilator, he was extubated and allowed to die at eight weeks of age.

RNA and DNA analyses

Detection of *SCO2* mutations and single nucleotide polymorphisms (SNPs) was performed using DNA extracted from whole blood. Standard techniques were used to amplify and sequence the *SCO2* cDNA. In addition, the patient's *SCO2* E140K genotype was confirmed via PCR mismatch amplification and restriction endonuclease digestion with *Bsr*B1 as previously described [16]. Analysis of SNPs contained in the last third of the 5′-UTR, the entire intron and the first third of the *SCO2* gene was done by sequencing a 1564 bp PCR fragment (forward primer, 5′-ggccgtctcgatgaacac-3′, and reverse primer, 5′-acaaaagccaggacctcaga-3′). The same forward primer was subsequently used with the *Bsr*B1 mismatch primer to generate an amplicon of 1733 bp for restriction fragment length polymorphism (RFLP) analysis with *Bsg*I.

Southern blot analysis was performed using 10 μg of genomic DNA that was digested overnight with *Bam*HI and *Xba*I prior to electrophoresis. The *SCO2* amplicon used for the *Bsr*B1 digest was used as a probe to detect the *SCO2* gene, while the full-length *SURF1* cDNA was used to detect the *SURF1* gene. Poly A^+ RNA was isolated using standard techniques, and 2.5 μg was electrophoresed on formaldehyde agarose gels and subsequently transferred to a nylon membrane (Duralon, Stratagene). *SCO2* and *GADPH* cDNAs were used as probes. Radiolabelled probes for both Southern and Northern blots were prepared by adding 50 ng of cDNA and 50 μCi [^{32}P]dCTP to Ready-to-Go labeling beads (Pharmacia). Blots were analyzed on a Phosphorimager and relative signal strength was quantified using ImageQuant software (Molecular Dynamics).

Tissue culture and fluorescence in situ hybridization (FISH)

Primary cell lines from control and patient skin fibroblasts were immortalized as previously described [11], and grown in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2.

For FISH analysis, patient fibroblasts were thawed and cultured. A sample of parental peripheral blood was cultured for 72 h in RPMI-1640 medium. Cytogenetic cell pellets were prepared for FISH experiments according to standard procedures. Dual color FISH was performed using pre-labeled commercial probes, one mapping to the ARSA locus (green) and a control probe encompassing the HIRA locus (red).

BAC and fosmid probes were selected using the May 2004 University of California at Santa Cruz (UCSC) freeze of the human genome. BAC probes were obtained from the Michael Smith Genome Sciences Centre of British Columbia, Vancouver (Courtesy J. Schein and M. Marra); fosmids were obtained from the CHORI facility. BAC and fosmid DNA was isolated by small-scale (mini-prep) preparation, and labeled with Spectrum Red or Green using a Vysis nick translation reagent kit (Vysis Inc., Downers Grove, Illinois, USA). The labeled product was mixed with 3 μg of human Cot-1 DNA (Invitrogen, Burlington, Ont., Canada) and isolated using a standard DNA precipitation method. Denaturation of probes and slides, hybridization and post-hybridization were performed according to standard procedures. Chromosomes and nuclei were visualised by DAPI counterstaining. Slides were read using a Zeiss microscope and a Metasystems® image analysis software. For each probe, a minimum of 10 metaphases and 30 interphase nuclei were analyzed.

Enzyme analysis

NADH Ubiquinone Reductase, Succinate Dehydrogenase, COX enzyme activities were determined from frozen muscle specimens as described [17,18].

Results and discussion

The patient's *SCO2* E140K genotype was confirmed by sequence analysis on DNA extracted from whole blood, and by PCR amplification and restriction endonuclease digest with *Bsr*B1 as previously described [13], both of which suggested a homozygous mutation. A severe isolated COX deficiency was present in skeletal muscle (less than 10% of the lowest control value), and COX activity was reduced to 60% of control in fibroblast cultures. The clinical course and the extent of the biochemical deficiency were much more severe than all previously reported patients homozygous for this mutation, prompting us to carry out further investigations of the parents.

Restriction enzyme analysis of DNA from the parents showed that the mother was heterozygous for the 1541 G>A mutation, while the father carried only the wild-type allele (Fig. 1A). As non-paternity was ruled out, we considered that this was either a case of maternal segmen-

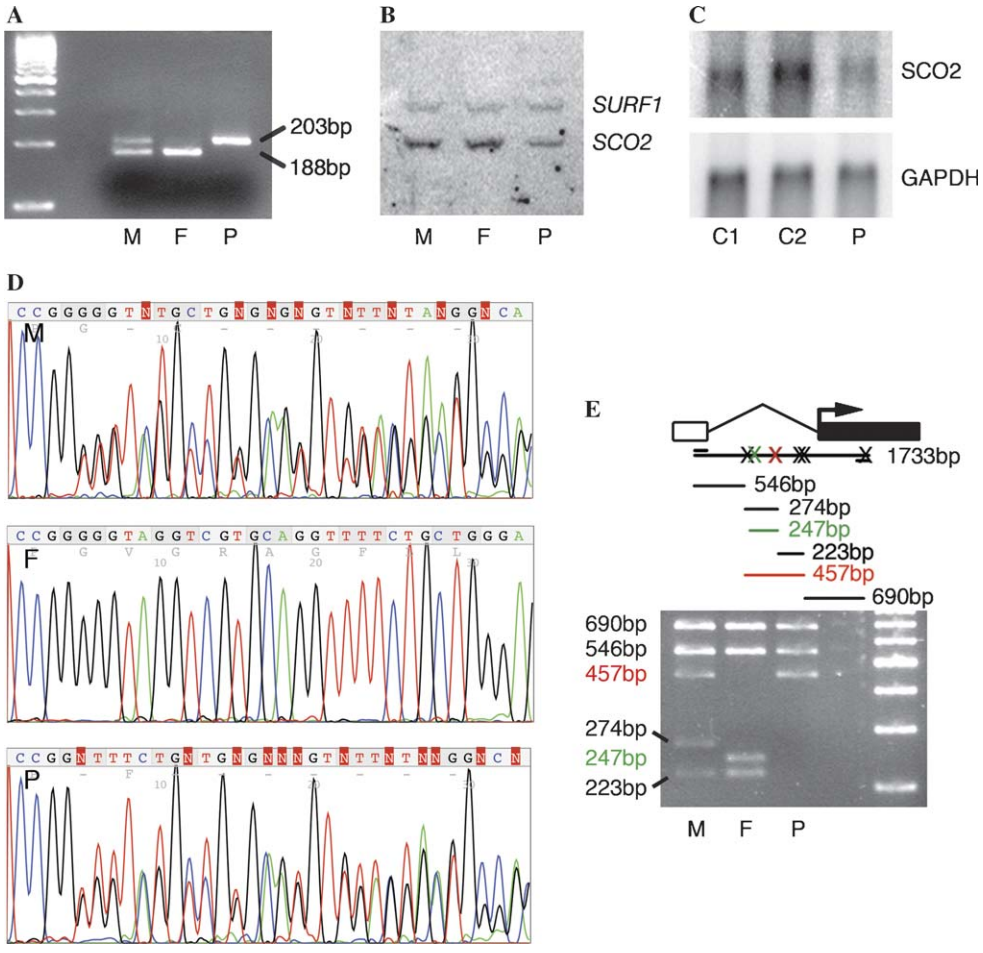

Fig. 1. Molecular characterization of the genetic defect in patient C. (A) Restriction enzyme analysis of a PCR product from the *SCO2* gene. The patient (P) has a single band corresponding to the E140K mutation, the mother (M) is heterozygous for the same mutation, while the father (F) is wild-type. (B) Southern blot analysis of the affected patient (P), mother (M) and father (F). *SURF1* was used as an internal loading control. (C) Northern blot analysis of *SCO2* mRNA in fibroblasts cultured from the affected patient (P) and two controls (C1, C2). (D) Representative chromatograms showing the 16 bp deletion in the intron of the *SCO2* gene in the patient (P) and mother (M), but not the father (F). (E) Restriction enzyme analysis of a PCR product spanning the last third of the 5′-UTR through the first third of the coding sequence of the *SCO2* gene (see schematic). Consensus *Bsg*I sites are denoted by black crosses. The red cross represents the *Bsg*I site that is abolished by the 16 bp deletion, while the green cross reflects the G>C polymorphism that creates a novel *Bsg*I site. PCR products from the mother (M), father (F) and patient (P) were digested with *Bsg*I and *Bsr*B1 and run on a 1.9% Metaphor agarose gel. Differential digestion of the 690 bp product, which is dependent on the presence or absence of the E140K mutation, could not be resolved, even using a 5% polyacrylamide-TBE gel (data not shown).

tal disomy for chromosome 22, or alternatively that the patient had inherited a null allele from the father. To test these hypotheses, we first genotyped the patient with 10 microsatellite markers covering the entire chromosome. This analysis showed unequivocal maternal and paternal contributions along the whole of chromosome 22, making isodisomy unlikely (data not shown). To test whether the patient had inherited a null allele from the father, we analyzed the *SCO2* gene and mRNA by Southern and Northern blot analysis, respectively (Figs. 1B,C). Southern blot analysis showed an approximate twofold reduction in the ratio of *SCO2* to *SURF1* in the patient relative to the parents, suggesting that the patient had a single copy of the *SCO2* gene while both parents had two copies. The steady-state level of *SCO2* mRNA was similarly reduced in the patient to about 30% of control.

To confirm these findings, we performed FISH on patient fibroblasts and samples of peripheral blood cultured from the parents. Using the ARSA and HIRA probes, no large scale rearrangements were seen. Using 2 BAC probes (RP11-825H3 and RP11-636O19, respectively, 184 and 158 kb) encompassing the *SCO2* locus (location 49,252,141–49,254,116) we were not able to see a difference in signal size or intensity between the 2 chromosome 22 homologues. We then used smaller probes, fosmids G248P87145H7 (location 49,225,062–49,265,250; 40.2 kb) and G248P89139B6 (location 49,237,402– 49,282,211; 44.8 kb). Signals generated by fosmid G248P89139B6 were of equal size; however, a smaller signal was consistently seen on one of the chromosome 22 homologues in the patient with fosmid G248P87145H7. There was no difference in either signal intensity or size when the same fosmid was used on parental blood samples (Fig. 2). These findings

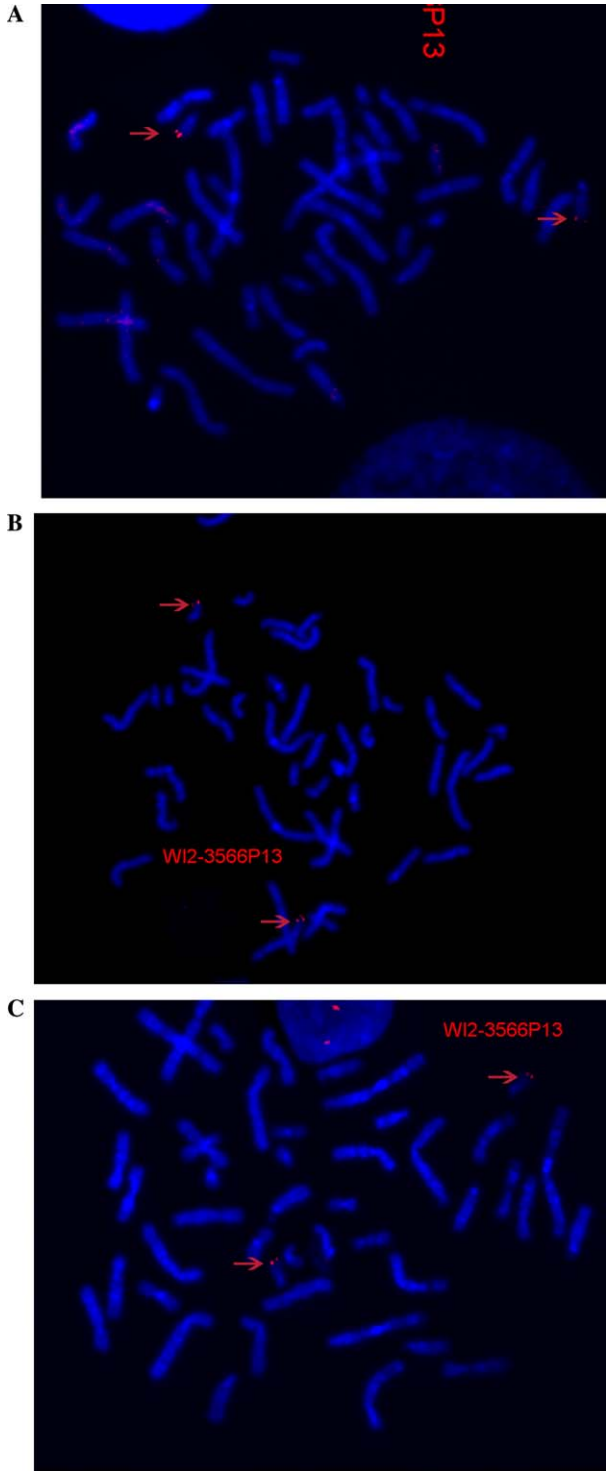

Fig. 2. FISH characterization of the deletion. FISH was performed with fosmid G248P87145H7 in the patient and the parents. BAC and fosmid DNA probes were labeled with Spectrum Red or Green by nick translation, mixed with human Cot-1 DNA, and isolated using a standard DNA precipitation method. Denaturation of probes and slides, hybridization and post-hybridization were performed according to standard procedures. Chromosomes and nuclei were visualised by DAPI counterstaining. Slides were read using a Zeiss microscope and a Metasystems® image analysis software. For each probe, a minimum of 10 metaphases and 30 interphase nuclei were analyzed. One FISH signal was consistently barely visible on one chromosome 22 in the patient (A). Both signals had the same size in the father (B) and the mother (C).

are consistent with the molecular biology results and further suggest that the patient is hemizygous for the E140K *SCO2* mutation.

Several SNPs within both the coding sequence and non-coding regions surrounding the *SCO2* locus have been identified, and Ensembl indicates at least 14 SNPs are contained within the genomic sequence spanning the last third of the 5′-UTR to the stop codon at the end of the *SCO2* gene. To further confirm hemizygosity in the patient, we analyzed the entire length of this sequence in both the patient and parents. None of the SNPs were informative; however, a 16 bp deletion was found in the intronic sequence of the mother and patient, but not the father (Fig. 1D). This deletion abolishes a *Bsg*I restriction site. An additional polymorphism (C>G) 5′ of the deletion, that introduces a *Bsg*I site, was also observed in all three samples; however, the father and patient were homozygous for this base change, while the mother was heterozygous. We exploited these sequence variants to verify that the patient has only a single, maternally inherited *SCO2* allele. RFLP analysis confirmed the absence of the deletion in the father and the homozygosity for the C>G polymorphism that introduced an additional *Bsg*I site in the 274 bp fragment, further truncating it to 247 bp (Fig. 1E). The mother was clearly heterozygous for this allele, with the C>G polymorphism being carried on the same allele as the deletion, resulting in the unique digestion product of 457 bp. The identical product was observed in the patient; however, there was a complete absence of the smaller molecular weight products that would be expected if a second locus were present (Fig. 1E). Collectively, these data demonstrate that the patient is hemizygous for the E140K *SCO2* mutation, the other allele being a de novo deletion of all or part of the *SCO2* locus.

Hemizygosity for E140K has not been previously reported, and it is unlikely that we would have uncovered it in our patient had it not been for the severe clinical phenotype that prompted us to investigate the parents. We estimate that for the deletion to be visible by FISH, it has to cover half to two thirds of the probe length. The deletion in this patient is therefore probably in the range of 15–30 kb. As the patient's phenotype is clinically and biochemically indistinguishable from that observed in compound heterozygous cases (E140K and a null allele due to a nonsense mutation), a contiguous gene syndrome resulting from the deletion is unlikely.

There is still no satisfactory explanation for the observation of a common allele in all reported *SCO2* patients. It does not appear to be an ancient mutation that arose on a common haplotype (unpublished observations), and although it appears to be transmitted more often by males than females, males may transmit alternate *SCO2* mutant alleles. The mutation is thought to disrupt the function of the SCO2 copper-binding site, but interestingly the homologous mutation in yeast Sco1p does not have a respiratory phenotype [10]. This suggests that it is a comparatively mild mutation, and this is supported by the fact that patients

homozygous for this mutation have a later onset and more prolonged course of disease. However, the mutation still results in a profound reduction in protein content in fibroblasts [11; data not shown] and this may be more important than the effect of the mutation on copper binding. It may be that more severe mutations are embryonic lethal and are therefore never seen, or that they are associated with other clinical phenotypes that have not yet been linked to COX deficiency.

There are obvious and important implications for genetic counseling in the case presented here, as the father of the patient is homozygous wild-type for *SCO2*. The results from the simple restriction enzyme test for the common E140K mutation should be verified whenever homozygosity is predicted.

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