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ORIGINAL ARTICLE

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ABSTRACT

Background Idiopathic dilated cardiomyopathy (DCM) is recognised to be a heritable disorder, yet clinical genetic testing does not produce a diagnosis in >50% of paediatric patients. Identifying a genetic cause is crucial because this knowledge can affect management options, cardiac surveillance in relatives and reproductive decision-making. In this study, we sought to identify the underlying genetic defect in a patient born to consanguineous parents with rapidly progressive DCM that led to death in early infancy.

Methods and results Exome sequencing revealed a potentially pathogenic, homozygous missense variant, c.542G>T, p.(Gly181Val), in *SOD2*. This gene encodes superoxide dismutase 2 (SOD2) or manganese-superoxide dismutase, a mitochondrial matrix protein that scavenges oxygen radicals produced by oxidation-reduction and electron transport reactions occurring in mitochondria via conversion of superoxide anion ($O_2^{-\bullet}$) into H_2O_2. Measurement of hydroethidine oxidation showed a significant increase in $O_2^{-\bullet}$ levels in the patient's skin fibroblasts, as compared with controls, and this was paralleled by reduced catalytic activity of SOD2 in patient fibroblasts and muscle. Lentiviral complementation experiments demonstrated that mitochondrial SOD2 activity could be completely restored on transduction with wild type SOD2.

Conclusion Our results provide evidence that defective SOD2 may lead to toxic increases in the levels of damaging oxygen radicals in the neonatal heart, which can result in rapidly developing heart failure and death. We propose SOD2 as a novel nuclear-encoded mitochondrial protein involved in severe human neonatal cardiomyopathy, thus expanding the wide range of genetic factors involved in paediatric cardiomyopathies.

INTRODUCTION

Dilated cardiomyopathy (DCM) is characterised by left ventricular enlargement and systolic dysfunction that can lead to heart failure and sudden cardiac death. DCM is a major cause of childhood mortality and the most common indication for heart transplantation in children.[1] Annual incidence of DCM in children is approximately 0.57/100 000 and is even higher in children below 12 months of age (8.34/100 000).[1] Recognition of idiopathic DCM as a familial disease[2] has led to the discovery of more than 60, mostly adult-onset, DCM-associated genes that encode transcription factors and cytoskeletal, sarcomeric, ion transport, nuclear membrane and mitochondrial proteins.[3–8] Current next-generation gene panel testing yields a genetic diagnosis in 37%–50% of paediatric cases.[9–11]

Nevertheless, knowledge of the underlying genetic causes of congenital or neonatal DCM remains limited. In these cases, DCM often occurs in the context of a malformation syndrome or disorders of metabolism and/or energy production, which are generally autosomal recessively inherited. Gene panels that target 'adult-onset' DCM genes are therefore less appropriate for severe infantile forms of DCM.[12] In contrast, exome sequencing (ES) (or genome sequencing) provides a powerful platform for novel disease gene discovery, particularly in congenital or neonatal cardiomyopathy. Successful application of ES to identify novel pathogenic variants in paediatric DCM has been recently demonstrated.[13–17]

Here, we report a neonate with lethal DCM born to consanguineous parents. ES identified a homozygous, damaging variant in *SOD2*, which encodes superoxide dismutase 2 (SOD2) or manganese-superoxide dismutase (MnSOD). Consistent with the function of SOD2, the resulting missense variant was shown to affect the catalytic activity of the protein, leading to excess oxygen radical levels that can have strongly damaging effects in the neonatal heart. Lentiviral gene rescue restored superoxide dismutase activity. Our findings suggest a role for *SOD2* in inherited cardiomyopathy and add a novel gene to the still-expanding list of genes implicated in paediatric-onset cardiomyopathy.

PATIENTS AND METHODS

Patient

Medical records of patient X:1 were carefully reviewed. Cardiac evaluation of her parents and siblings included echocardiography and ECG. Diagnostic criteria for DCM were left ventricular end-diastolic and/or systolic short-axis chamber dimension Z-score ≥2.0 and fractional shortening or left ventricular ejection fraction >2 SD below the mean for age in the absence of abnormal loading conditions sufficient to cause global systolic impairment.[18] Genomic DNA of patient X:1 and her

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parents was extracted from peripheral blood. Fibroblast cells were established from a postmortem skin biopsy of patient X:1. Enzymatic activity of the five respiratory chain complexes and citrate synthase were determined in an enriched mitochondrial fraction of fibroblasts by spectrophotometric assays, as described previously.[19 20] The parents provided informed consent for the DNA studies and diagnostic procedures. The UMCG ethical committee approved the study.

Histology

Heart samples obtained at autopsy from left and right ventricular wall and septum were formalin-fixed and paraffin-embedded using standard clinical laboratory protocols. Heart (left ventricle) and skeletal muscle samples were frozen and stored at −80°C.

ES, variant filtering and interpretation

ES was performed using a previously described parent–offspring trio approach.[9 13] Briefly, the exome was captured using the Agilent SureSelect XT Human All Exon V6 kit (Agilent, Santa Clara, California, USA). Exome libraries were sequenced on an Illumina NextSeq500 instrument (Illumina, San Diego, California, USA) with 151 bp paired-end reads. Sequence reads were aligned to the GRCh37/hg19 reference genome using BWA V.0.7.5a. Local realignment of insertions/deletions and base quality score recalibration were performed using the Genome Analysis Toolkit Haplotype Caller, V.3.7. Variants were annotated using a custom diagnostic annotation pipeline, then filtered for rarity excluding those with a minor allele frequency (MAF) >1% for heterozygous variants in an autosomal dominant inheritance model and those with MAF >2% for homozygous or compound heterozygous variants in an autosomal recessive inheritance model. After exclusion of variants in known cardiomyopathy-related and nuclear-encoded mitochondrial genes, we selected variants found in homozygous state in the patient and in heterozygous state in both parents.

Variant pathogenicity was assessed using data from the Agilent Alissa clinical informatics platform (Cartagenia, Leuven, Belgium) and Alamut Visual software (Interactive Biosoftware, Rouen, France), a gene browser that integrates missense prediction tools, allele frequencies from different population databases (1000 Genomes Project,[21] Genome of the Netherlands,[22] GnomAD[23] and disease-specific databases (HGMD, ClinVar, LOVD)) and mRNA splicing prediction tools. Literature and allele frequencies from our in-house population database were also used for pathogenicity determinations. Classification of variants was based on American College of Medical Genetics and Genomics guidelines.[24]

Sanger sequencing

Sanger sequencing was performed to confirm the presence/absence of the *SOD2* variant in patient X:1, her parents and her siblings. We also screened all exons and exon/intron junctions of *SOD2* in 79 unrelated patients with childhood-onset cardiomyopathy and 161 adult patients with DCM (primers available on request). PCR was performed by using AmpliTaq Gold PCR Master Mix (Invitrogen Life Science Technologies, Carlsbad, California, USA) following the official protocol. Resulting fragments were sequenced by the Applied Biosystems 96-capillary 3730XL system (Carlsbad, California, USA).

Splicing analysis

RNA was isolated from cultured fibroblasts from patient X:1 and fibroblast cells from a healthy control. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glucose and 1% glutamax in a humidified atmosphere of 5% CO_2 and atmospheric O_2 at 37°C. RNA was extracted using the RNeasy Mini Kit (QIAGEN, Venlo, The Netherlands). cDNA was synthesised from 500 ng of total RNA by RevertAid RNaseH-M-MuLV reverse transcriptase in a total volume of 20 µl according to the supplier's protocol (MBI-Fermentas, St. Leon-Rot, Germany).

To investigate whether the *SOD2* c.542G>T variant affects mRNA splicing, we performed reverse-transcription PCR with gene-specific primers designed to amplify the exon expected to be affected by the variant and flanking sequences (primers available on request). The resulting PCR products were examined by 2% agarose gel electrophoresis and subsequently analysed by Sanger sequencing. To test whether mRNA is degraded by nonsense-mediated mRNA decay (NMD), fibroblasts were treated with the NMD inhibitor cycloheximide for 4.5 hours, followed by RNA analysis using the same procedures used for RNA from untreated cells.

Measurement of hydroethidine-oxidizing reactive oxygen species levels

Fibroblasts, cultured to 70% confluence, were incubated in HEPES-Tris medium containing 10 µM hydroethidine (HEt; Molecular Probes, Leiden, The Netherlands) for 10 min at 37°C. Within the cell, two HEt oxidation products are formed under oxidative conditions: 2-hydroxyethidium (2-OH-Et), the sole reaction product of HEt and $O_2^{-\bullet}$ and ethidium (Et).[25] Both products are positively charged and fluoresce when excited at 490 nm. The reaction was stopped by thoroughly washing the cells with phosphate-buffered saline to remove excess HEt. For quantitative analysis of HEt oxidation products as a measure of HEt oxidising reactive oxygen species (ROS) levels, coverslips were mounted in an incubation chamber placed on the stage of an inverted microscope (Axiovert 200 M; Carl Zeiss, Jena, Germany) equipped with a Zeiss×40/1.3 NA Fluar oil-immersion objective. HEt oxidation products were excited at 490 nm using a monochromator (Polychrome IV; TILL Photonics, Gräfelfing, Germany). Fluorescence emission was directed using a 525DRLP dichroic mirror (Omega Optical, Brattleboro, Vermont, USA) through a 565ALP emission filter (Omega Optical) onto a CoolSNAP HQ monochrome charge-coupled device camera (Roper Scientific, Vianen, The Netherlands). The image-capturing time was 100 ms and 10 fields of view per coverslip were routinely analysed.

Superoxide dismutase assay

Superoxide dismutase was measured using the Sigma SOD assay kit (19160-KT-F) following the manufacturer's procedures. This assay is based on the reduction of the tetrazolium salt WST-1 by superoxide into a formazan dye that can be monitored spectrophotometrically at 440 nm. Measurements were performed in mitochondria-enriched fractions prepared from cultured skin fibroblasts. SOD activity assays were performed in two independent experiments, each performed in duplicate (four measurements per sample in total). To confirm specific SOD2 residual activity in muscle, superoxide dismutase activity was measured in patient-derived muscle cells using the ENZO SOD assay kit (ADI-900–157) according to the manufacturer's procedures. Cu/ZnSOD (SOD1) activity was inhibited by 20 mM potassium cyanide (KCN). MnSOD (SOD2) activity is not inhibited at these levels of KCN. Measurements were performed in triplicate in a

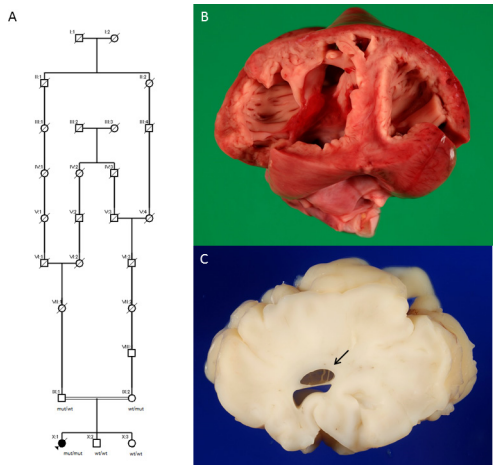

Figure 1 Pedigree, heart and brain imaging of proband. (A) Pedigree of Dutch family with a child with severe, lethal DCM in whom autosomal recessive inheritance was expected due to consanguinity. Squares=males; circles=females; solid symbol=affected; mut=mutant allele; wt=wild type allele. (B) Macroscopic section of the heart, showing severe LV and RV chamber dilation. (C) Macroscopic cross-section of the brain showing SECs (arrowhead). LV, left ventricle; RV, right ventricle; SECs, subependymal cysts.

single-cell suspension prepared from patient and control muscle tissue.

Proteomics analysis

To quantify the amount of SOD2 protein in skin fibroblasts, we used QconCAT technology in combination with mass spectrometry according to Wolters *et al*.[26]

Lentiviral complementation

A plasmid containing full length hSOD2 cDNA (NM_001024466.1) was purchased from PlasmID Repository, Harvard, USA (clone HsCD00042604). The open reading frame was recombined into the pLenti6.2V5-DEST destination vector (Invitrogen) using the Gateway LR clonase II enzyme mix (Invitrogen). The production of lentiviral particles containing the SOD2 cDNA and transduction of patient-derived fibroblasts was performed as described previously.[27] As a negative control, cells were transduced by the gene encoding green fluorescent protein.[27]

SOD2 3D structure

We used HOPE software[28] to predict the effect of the SOD2 missense variant on 3D protein structure and the Uniprot protein database (www.uniprot.org) to search for known functional features within the mitochondrial superoxide dismutase (Mn) protein (accession number: P04179) in the region affected by the genetic variation.

RESULTS

Clinical findings

The female proband (patient X:1) was delivered by secondary caesarean section for breech presentation, prolonged labour and meconium-stained amniotic fluid at 39 weeks of gestation. Prenatal ultrasound at gestational age 19+4 weeks was normal. Apgar scores were 2, 3 and 9 at 1, 5 and 10 min, respectively. Birth weight was 2240 g (<p2.3), length at birth was 49 cm (p25) and head circumference was 33.0 cm (p5). Umbilical artery pH was 7.14 (n=7.37–7.45) with a base excess of −4 mmol/L indicative of metabolic acidosis. Cerebral function monitoring was normal, but cerebral echography showed intraventricular septa. The day after birth, she presented with apnoeas, poor circulation and mild tachycardia. Mean arterial pH was 7.38 (n=4), haemoglobin 8.6 mmol/L (n=8.3–12.4 mmol/L) and blood lactate 10.4 mmol/L (n=0.0–2.2 mmol/L). A chest X-ray was normal. Echocardiography showed a structurally normal heart, but left ventricular function seemed poor. Cardiac troponin and B-natriuretic peptide were elevated at 0.28 µg/L (n<0.16 µg/L) and 2819 pmol/L (n<35 pmol/L), respectively.

On the third day after birth, patient X:1 developed acute cardiogenic shock with oliguria, metabolic acidosis with increased blood lactate of 18 mmol/L and poor body circulation. ECG showed signs of myocardial ischaemia, and QTc was slightly prolonged (QTc 442–474 ms). Echocardiography revealed DCM with severe biventricular dilation and decreased left ventricular ejection fraction. Frequent ventricular extrasystoles and tachycardia were observed. At day 4, she developed ventricular fibrillation and was successfully defibrillated four times. However, there was no improvement of ventricular function despite intensive treatment with dopamine, milrinone and adrenalin, and she died at age 4 days. Biochemical studies at day 3 showed increased amino acids in blood, including proline and alanine, and increased organic acids in urine, including 3-methylglutaconic acid. No underlying cause for her DCM was identified despite extensive premortem and postmortem diagnostic workup that included viral serology in blood, sputum and faeces, metabolic testing (including for Pompe disease) and copy number variant analysis (Agilent 180K oligo-array). As recommended in children with 3-methylglutaconic aciduria type IV, sequencing of mitochondrial DNA and the nuclear genes *POLG*, *RYR1* and *SUCLA2* was performed as previously described.[29] Additionally, the *MYL2*, *MYH7*, *LMNA* and *DES* genes were assessed for (biallelic or de novo) variants because these genes were at that time known to putatively carry variants associated with early-onset DCM. However, no pathogenic variants were found.

The parents were of Dutch origin. Genealogical evaluation revealed a distant relationship between the parents 6–8 generations ago (figure 1A). Three-generation family history was negative for heart failure. The paternal grandfather underwent coronary bypass surgery. Cardiac evaluations in the parents (aged 27 and 29 years) and siblings, both evaluated at age 1 week, were normal.

Histopathological examination and enzymatic activity

Examination of the heart at autopsy revealed severe dilatation of both ventricles compared with an age-matched control (figure 1B), without fibroelastosis, interstitial fibrosis, cardiomyocyte hypertrophy or disarray. Electron microscopy was not performed. Cardiac weight (20.6 g) was consistent with 41 weeks gestation (and relatively heavy as expected with DCM developing in the neonatal period[30]), while the weights of other organs were low and consistent with 32–33 weeks gestation. There were no signs of glycogen storage or intracellular lipid vacuoles in hepatocytes, cardiomyocytes, skeletal muscle or tubular cells of the kidney. Skeletal muscle showed no abnormalities, and there were no indications of disorders of fatty acid oxidation or mitochondrial disease although these could not be fully excluded by histological examination.

Intracranial examination showed small cerebral subependymal cysts, some with small septa surrounded by macrophages, suggestive of hypoxic/ischaemic damage that occurred in utero (figure 1C). Neurons in the brainstem, cerebellum, basal ganglia, thalamus, hippocampus and cerebral cortex showed signs of

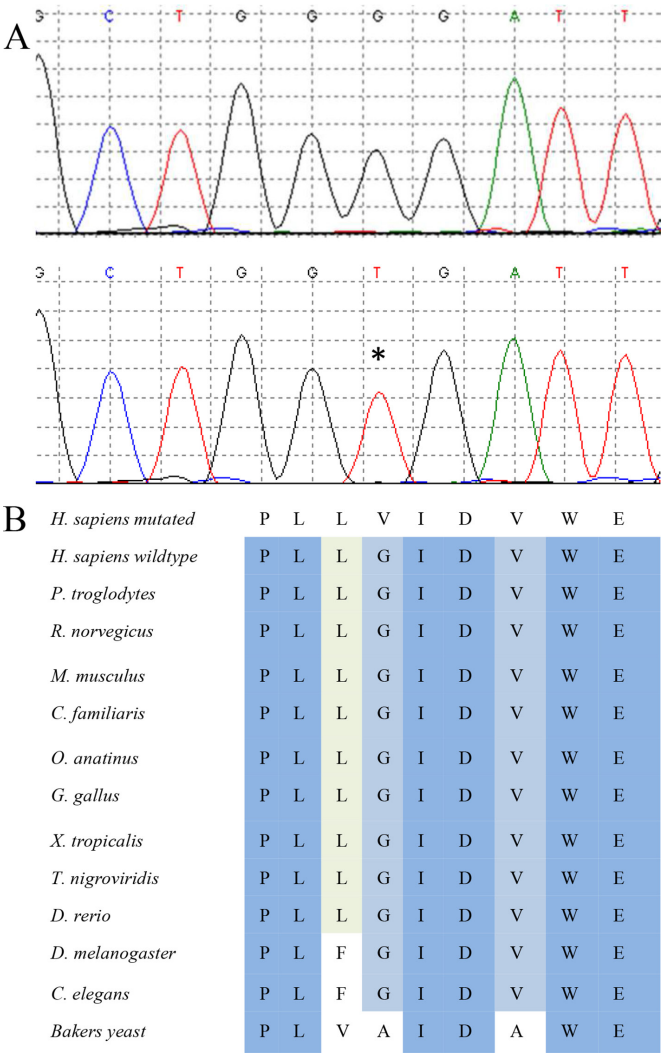

Figure 2 Confirmation and properties of *SOD2* variant c.542G>T, p.(Gly181Val). (A) Sanger sequencing verified the presence of the *SOD2* variant c.542G>T, p.(Gly181Val) in the affected patient (bottom) compared with control (top) and in heterozygous form in her parents (not shown). (B) Conservation of Gly181 and surrounding residues.

recent hypoxia. Actin, dystrophin, sarcoglycan, dystroglycan, dysferlin, caveolin-3, merosin, myosin and spectrin-1 staining were normal in skeletal muscle, and stains for the respiratory chain complexes, including succinate dehydrogenase and cytochrome c oxidase, in frozen material from heart and skeletal muscle were normal (data not shown).

Exome sequencing

To look for potential disease-causing variants, ES targeted all exons and exon/intron junction sequences of known genes in the human genome. Using the filtering pipeline discussed earlier, we excluded likely pathogenic or pathogenic variants in 312 genes related to (childhood-onset) cardiomyopathy and 355 nuclear-encoded mitochondrial genes (online supplemental table 1). Assuming homozygosity by descent of an autosomal-recessive variant to be the likely cause of DCM in patient X:1, we selected for homozygous variants in concordance with autosomal-recessive inheritance. This identified eight homozygous variants with allele frequencies<2% in the public databases (online supplemental table 2). Only one, c.542G>T in *SOD2*, was classified as likely pathogenic, and it was predicted to cause a substitution of a highly evolutionarily conserved glycine at position 181 with valine (p.(Gly181Val), *SOD2*, NM_000636.3) at the protein level (figure 2). The p.(Gly181Val) missense variant was present in 1 of 248 906 control alleles in GnomADv2.2.1 (absent homozygously), absent in control individuals from GoNL and predicted to be damaging (eg, phyloP 6.18, Grantham-score 109). Notably, this variant is located in the longest autosomal homozygous region on chromosome 6, between rs378512 and rs9458499 (2.76 Mb; UCSC Genome Browser, build hg19) (data not shown). We validated this result by Sanger sequencing and verified segregation of the variant in all available family members. Both parents were heterozygous for the variant. DNA testing in saliva of the unaffected brother (X:2) excluded the *SOD2* variant in homozygous state and chorionic villous biopsy performed in the third pregnancy showed absence of the variant in the fetus (X:3).

Reverse transcription polymerase chain reaction (RT-PCR) and SOD2 protein quantification

Two of five mRNA splicing prediction tools in the Alamut software indicated a possible new donor site. To further assess the pathogenicity of this variant at RNA level, we performed RT-PCR analysis. RT-PCR product analysis of RNA isolated from patient-derived fibroblasts cultured both with and without cycloheximide showed a transcript of only wild-type size, indicating that this variant did not affect splicing. Quantitative protein analysis with targeted quantitative Liquid Chromatography-Mass Spectrometry-Selected Reaction Monitoring (LC-MS-SRM) using stable isotope-labelled concatemers[26] confirmed the presence of the SOD2 protein (figure 3A). We therefore concluded that the *SOD2* missense variant probably results in a conformational change that affects protein folding or flexibility, protein-protein or protein-DNA interaction or the activity of the Manganese (Mn)-cofactor binding domain, rather than resulting in absent or decreased expression of SOD2.

SOD2 mutational screening

Sanger sequencing of *SOD2* in a cohort of 79 unrelated patients with childhood-onset cardiomyopathy and 161 adult patients with DCM and NGS-gene-panel–based sequencing of this gene in 1150 mostly adult cardiomyopathy patients revealed no pathogenic or likely pathogenic *SOD2* variants.

Effect of the SOD2 variant on intracellular superoxide ($O_2^{-\bullet}$) levels

HEt was used as a probe to measure the intracellular levels of HEt-oxidising ROS levels, including $O_2^{-\bullet}$, in patient fibroblasts. The oxidation levels of HEt measured in our in vitro assay indicated a significant increase of $O_2^{-\bullet}$ levels in patient fibroblasts comparable in order of magnitude to that seen in complex I–deficient fibroblasts (figure 3B). Because mitochondrial respiratory chain enzyme activities (complexes I–V) were normal in mitochondria-enriched fractions from skin fibroblasts, the significant increase in $O_2^{-\bullet}$ levels we observe probably resulted from impaired SOD2 enzyme activity.

Effect of identified variant on SOD2 activity

MnSOD (SOD2) enzyme activity in patient-derived muscle cells was investigated in the cell pellet fraction. Total SOD activity was shown to be reduced in this cell pellet fraction when compared with a control (49 U/mg protein vs 58 U/mg protein, respectively). These data suggest that enzymatic SOD2 activity was decreased in the patient, despite higher levels of SOD2 protein.

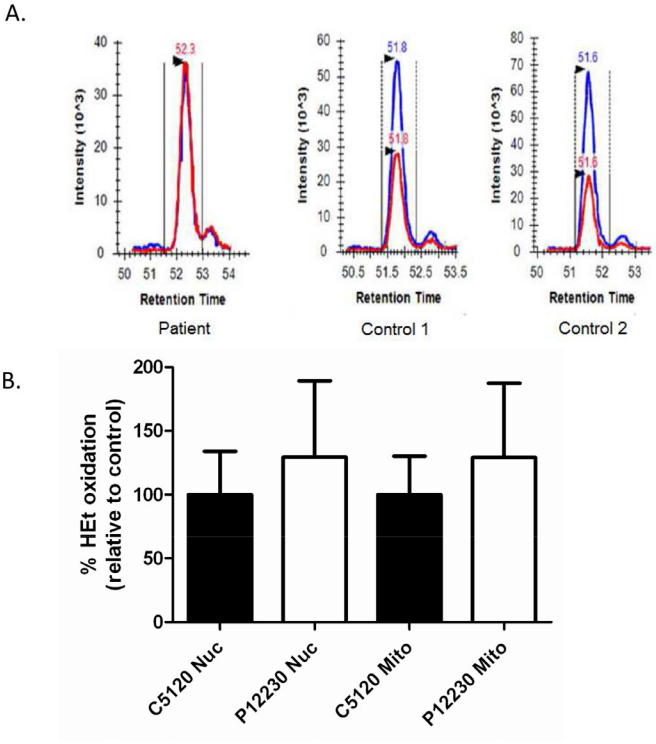

Figure 3 (A) Quantitative protein analysis in mitochondria-enriched fractions of patient-derived skin fibroblasts by targeted quantitative LC-MS-SRM using stable isotope-labelled concatemer. Blue peak represents the isotope-labelled standard. Red peak represents the same endogenous peptide. A known amount of the isotope-labelled standard was added to the protein sample, allowing the amount of the endogenous peptide (and therefore protein) to be calculated. The SOD2 protein is at least two-times higher compared with controls (patient 27.6 fmol/µg compared with two different controls 11.7±1.3 fmol/µg total mito protein). (B) The oxidation of hydroethidine analysis shows a significant increase of ROS OR ($O_2^{\bullet -}$) level in the fibroblasts of the patient compared with control fibroblasts. LC-MS-SRM, Liquid Chromatography-Mass Spectrometry-Selected Reaction Monitoring; MITO, mitochondria; Nuc, nucleus; SOD2, superoxide dismutase 2.

To confirm this, the same experiment was performed after inhibition of SOD1 and SOD3 by KCN (20 mM). Our data indicated a residual SOD2 activity of 40% in the patient; however, our data also suggested different inhibition levels between the patient and control; therefore, we could not draw firm conclusions from this experiment.

Rescue of superoxide dismutase activity by lentiviral complementation

To further investigate whether the genetic defect in *SOD2* was responsible for the increased superoxide levels, we stably transfected patient-derived fibroblasts with wild-type *SOD2* cDNA and monitored the superoxide activity in whole-cell lysates and a mitochondria-enriched fraction. Our results confirmed that the observed superoxide dismutase activity was clearly reduced (0.46±0.04 U/mg in mitochondria-enriched fraction from the patient vs 0.80–1.80 U/mg in controls; table 1). In comparison, the total superoxide dismutase activity in whole-cell lysates was very similar (results not shown). On transduction of the patient cells with wild-type *SOD2* cDNA, mitochondrial superoxide dismutase activity was completely restored (table 1), providing strong evidence for the pathogenicity of the SOD2 variant in this patient.

Table 1 Rescue of superoxide dismutase 2 activity in patient fibroblasts by lentiviral transduction of wild-type SOD2

	SOD2 activity (U/mg protein)
Patient X:1	**0.46**±0.04
Patient X:1+GFP	**0.37**±0.15
Patient X:1+SOD2	1.74±0.06
Control range (n=6)	0.80–1.80*

Values in bold are below the control range.
*Average 1.23.
GFP, green fluorescent protein; SOD2, superoxide dismutase 2.

SOD2 3D structure: the effect of the p.(Gly181Val) variant

Using the HOPE software, we retrieved 3D-structure information for the SOD2 protein through the WHAT IF Web services, the Uniprot database and a series of DAS-servers to predict the effect of the p.(Gly181Val) variant on the protein structure. The Gly181 residue is part of a Mn/iron superoxide dismutase domain important for superoxide dismutase activity (oxidoreductase activity) and metal ion binding. According to the Uniprot database, four important amino acid residues (His50, His98, Asp183 and His187) are involved in the formation of the Mn-binding pocket that binds the Mn cofactor of the enzyme (accession number: P04179). Interestingly, the aspartic acid residue of key importance (Asp183) is only two amino acids away from the Gly181 residue that was mutated in our patient. The increased size of the mutant residue is predicted to disturb the core structure of the Mn/iron superoxide dismutase domain and, in consequence, the catalytic activity of the enzyme (figure 4A,B).

DISCUSSION

We have demonstrated, for the first time, an association between a damaging variant in *SOD2* and DCM in humans. ES in a patient with neonatal DCM born to consanguineous parents revealed a novel homozygous missense variant, c.542G>T; p.(Gly181Val), in *SOD2*. This gene encodes a mitochondrial superoxide scavenger enzyme that protects cells from ROS damage by detoxifying oxygen radicals such as $O_2^{-\bullet}$ to yield O_2 and hydrogen peroxide, which is then metabolised into H_2O by glutathione peroxidase[31] (figure 4C). The *SOD2* variant in our patient was not identified as homozygous in any individual in the GnomAD database and was predicted to affect protein function. It is located in the functionally important C-terminal Mn/iron superoxide dismutase region of the protein, very close to one of the four histidine/aspartic acid residues involved in binding the Mn cofactor. Significant differences between the size and physicochemical characteristics of the wild-type glycine, which is the smallest amino acid residue and provides flexibility for enzyme active sites, and the mutant valine residue are predicted to disturb the core structure in this crucially important domain. This suggests that the identified variant would significantly affect the protein's stability and/or activity.

We performed several analyses to evaluate the predicted effect on SOD2 stability/activity. RNA studies showed a stable SOD2 transcript not subject to nonsense-mediated decay. Protein quantification by LC-MS-SRM showed increased SOD2 levels, which can be explained by the upregulation of SOD2 expression in response to oxidative stress.[32] HEt oxidation measurements in patient fibroblasts indicated a significant increase in

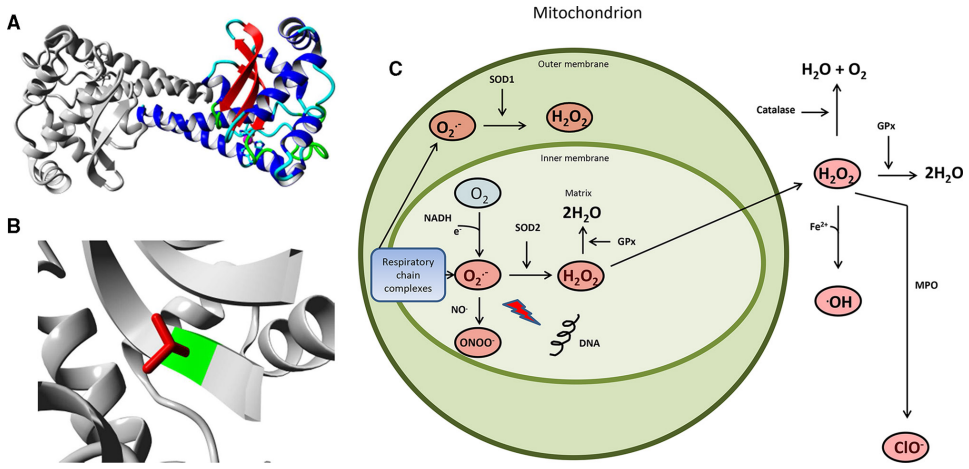

Figure 4 (A) Overview of the SOD2 protein in ribbon-presentation. (B) Zoom-in on the part of the manganese/iron superoxide dismutase domain where the mutated residue is located. The protein backbone is shown in grey, the side chains of both the wild-type in green and the mutant residue in red. The mutant residue is larger than the wild-type residue, which might disturb the core structure of this domain and affect the catalytic activity of the enzyme. (C) Mitochondrial free radical generation and its catabolism. Highly reactive superoxide anions are mainly produced in mitochondria and by the cytosolic xanthine oxidase, plasma membrane-associated NOX and the cytochrome p450 monooxygenases, which are present mainly in the endoplasmatic reticulum. Superoxide anions can either react with nitric oxide to generate the strong oxidant $ONOO^-$ or be degraded by SOD into the less reactive species H_2O_2. H_2O_2 can then be catabolised by GPx or catalase, react with Fe^{2+} to form hydroxyl radicals via the Fenton reaction or be degraded by MPO into ClO^-, another source of hydroxyl radicals (adapted from Ref. 57). ClO^-, hypochlorite; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; NOX, NADPH-oxidase complex; $ONOO^-$, peroxynitrite; SOD, superoxide dismutase.

levels of $O_2^{-\bullet}$, one of the major ROS substrates of SOD, comparable in order of magnitude to the levels seen in complex-I-deficient fibroblasts.[25 33] Since no deficiency was seen in any of the mitochondrial respiratory chain complexes I-V, the significant increase in $O_2^{-\bullet}$ is likely explained by the pathogenic effect of the c.542G>T; p.(Gly181Val) *SOD2* variant on the function of the encoded enzyme, leading to increased oxidative stress and accumulation of damaging oxygen radicals in the cells. Despite increased *SOD2* expression, decreased SOD2 activity was measured in patient-derived skin fibroblasts and muscle. Together with our successful rescue experiments, where patient fibroblasts were complemented with wild-type SOD2 cDNA, these data support a role for mutated SOD2 in the pathogenesis of DCM in this patient.

SOD2 belongs to the Mn/iron superoxide dismutase family, one of the primary families of antioxidant enzymes in mammalian cells. These antioxidant enzymes protect cells from the damage caused by ROS. In eukaryotic cells, there are three SOD homologs: cytosolic Cu/ZnSOD (SOD1), Mn/FeSOD (manganese superoxide dismutase 2; SOD2) and extracellular SOD3. SOD2 is a mitochondrial matrix protein that forms a homotetramer and binds one Mn ion per subunit.

ROS are formed during normal mitochondrial metabolism as byproducts of oxidative phosphorylation, and cells self-regulate their ROS levels by producing antioxidant enzymes.[34] Deficiency of one of the antioxidant enzymes, such as SOD2, may lead to accumulation of ROS, particularly during times of environmental stress. Excessive amounts of ROS can damage cellular proteins, DNA and lipids.[35] As is commonly observed in mitochondrial disorders, ROS-mediated oxidative stress can affect any organ at any age, but it particularly affects organs with high energy demand such as the heart and brain.[36]

Oxidative stress and *SOD2* gene polymorphisms have been associated with premature ageing,[37] cancer,[38] neurological disorders,[39 40] diabetes[41 42] and cardiovascular diseases including hypertension and atherosclerosis.[43–45] Oxidative stress and disturbed mitochondrial respiratory function are also known to play a substantial role in the development of heart failure.[46] Hiroi and colleagues found an increased frequency of the *SOD2*-16VV genotype in Japanese patients with non-familial idiopathic cardiomyopathy and suggested that this polymorphism contributes to the susceptibility to non-familial idiopathic cardiomyopathy.[47] In addition, anthracyclin-induced cardiomyopathy is believed to be a side effect of superoxide radical accumulation that leads to the induction of mitochondrial dysfunction in the heart.[48] This phenotype was successfully rescued in transgenic mice by overexpression of SOD2,[49] further underscoring the cardioprotective role of this enzyme.

The role of defective SOD2 protein in the pathophysiology of DCM has previously been demonstrated in mice. Homozygous *Sod2* knockout (*Sod2*-/-) mice (outbred background, CD1) lacking exon 3, which results in the complete loss of SOD2 enzyme activity, display cardiomyopathy at age 5 days. Similar to our case, these SOD2-deficient mice had enlarged hearts with a dilated left ventricular cavity.[50] Given our patient's early death at age 3 days, it is perhaps not surprising that we did not observe the endocardial fibrosis or cardiomyocyte hypertrophy seen in mutated mice, which usually die at age 10 days.[50] Another strain of *Sod2*-/- mice ($SOD2^{m1BCM}/SOD2^{m1BCM}$, hybrid background) was shown to be able to survive for up to 18 days, with only 10% developing severe DCM.[51] In these (older) mice, electron microscopy of the brain and spinal cord at postnatal day 10 revealed degenerative injury to large CNS neurons, particularly in the basal ganglia and brainstem. Both SOD2-deficient mice strains develop lipid depositions in the liver, as shown at autopsy. Neither degenerative abnormalities nor fatty accumulation in the liver were observed in our patient, although she may have died too early to develop these features. Examination of her brain did reveal extensive white matter damage, most likely due to hypoxic/ischaemic-induced changes in utero. We hypothesise that this resulted from severe oxidative stress due to SOD2 deficiency, but cannot exclude birth asphyxia and/or poor circulation as contributing factors. Both SOD2-deficient mouse strains were severely growth-restricted compared with

their littermates and showed metabolic acidosis, resembling our patient. Interestingly, further characterisation of the metabolic defects of the *Sod2*-/- mice showed large amounts of 3-methylglutaconic, 2-hydroxyglutaric, 3-hydroxy-3 methylglutaric and 3-hydroxyisovaleric acids in urine,[52] as also seen in our patient. Respiratory chain deficiencies were not detected in our patient, a finding which is compatible with the animal model where histological and ultrastructural evidence of mitochondrial injury has been detected predominantly in older mice (P8-P18).[50 51]

Using QconCAT proteomics, we observed increased SOD2 protein levels in patient fibroblasts compared with controls. This mutated protein showed approximately 40% residual enzyme activity in skin fibroblasts (table 1). In contrast, no SOD2 activity was detected in the homozygous mutant mice.[50] This residual enzyme activity may explain the phenotypic differences between the mouse models and our patient. Increased labour-induced stress response[53 54] may have contributed to extreme metabolic disruption in our patient, although the exact disease mechanism leading to her dramatic disease course and early death remains unknown.

CONCLUSION

This study is the first to demonstrate a homozygous damaging variant in SOD2 as a cause of human DCM. Our results highlight a potential role for reduced SOD2 activity and abnormally elevated levels of oxidative stress in the pathogenesis of DCM. Together with results from previously published SOD2 animal studies, our findings suggest a model for mitochondrial oxidative damage in which superoxide-induced injury compromises mitochondrial function and accelerates damage to cells and tissues with high energy demand, including cardiac myocytes. The beneficial effects of antioxidants on heart function and muscle fatigue seen in SOD2-deficient mice[55 56] may lead to new therapeutic strategies, but further studies are needed to unravel the role of this enigmatic kinase in cardiac function and pathological remodelling.

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