Matters of the heart: genetic and molecular characterisation of cardiomyopathies

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Chapter 3.2

Homozygous SOD2 mutation as a cause of lethal neonatal dilated cardiomyopathy

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ABSTRACT

Although cases are rare, neonatal and paediatric dilated cardiomyopathy (DCM) is a severe and often lethal disease, in which a genetic factor plays an important role in disease development. Identifying this genetic component is of major importance for parents as it enables prenatal diagnostics to be performed in their future pregnancies. Here, we report the results of homozygosity mapping followed by exome sequencing in a DCM-affected neonate in whom autosomal recessive inheritance was anticipated. This approach revealed a potentially pathogenic, homozygous missense mutation, c.542G>T, p.(Gly181Val), in the gene encoding Superoxide dismutase 2 (SOD2). SOD2 is a mitochondrial matrix protein that converts the reactive oxygen species (ROS) superoxide anion (O$_{2}^-$) into H$_2$O$_2$, and is therefore important for preventing cellular damage due to oxidative stress. We measured the oxidation of hydroethidine and detected a significant increase in O$_{2}^-$ levels in the fibroblasts of the patient compared with controls. This indicates that the mutation affects the catalytic activity of SOD2, which could lead to a drastic increase in damaging oxygen radical levels in the neonatal heart and result in rapidly developing heart failure and death. In conclusion, we have identified a novel mitochondrial gene involved in severe neonatal cardiomyopathy, thus expanding the wide range of genetic factors involved in paediatric cardiomyopathies.
INTRODUCTION

Dilated cardiomyopathy (DCM) is characterized by left ventricular enlargement and systolic dysfunction, which can lead to heart failure and sudden cardiac death (Fatkin et al). It is the most common type of cardiomyopathy and the major reason for heart transplantations in children. The incidence of DCM in children is estimated to be 0.57/100,000 per year, and is even higher in children below the age of one year (8.34/100,000) (Towbin et al). Approximately 25-50% of DCM cases are familial, and mutations in more than 50 genes have been reported to be associated with adult-onset familial DCM, some of which are observed in paediatric DCM as well (Somsen et al, Dellefave & McNally, Posafalvi et al). DCM-associated genes encode diverse groups of proteins including cytoskeletal, sarcomeric, ion transport, nuclear membrane and mitochondrial proteins (Somsen et al, Dellefave & McNally, Posafalvi et al). In contrast to adult DCM, knowledge about the underlying genetic causes of paediatric cases is still limited. In familial cases, mutations are regularly found in the known DCM genes (Rampersaud et al). However, these neither explain the majority of pediatric cases in which rare mutations in autosomal recessive inherited genes underlie disease, nor the cases of children whose DCM is part of a syndromic or metabolic disease (Kindel et al). Therefore, Burns et al recently concluded that approaches using gene-panel based applications targeting ‘adult’ DCM disease genes are less appropriate for the severe infantile forms of the disease, and they suggested that gene discovery is likely to proceed more rapidly when exome sequencing (ES) or genome sequencing are applied. Successful application of ES to identify the causal mutations in paediatric DCM has been recently demonstrated (Theis et al 2011, 2014; Louw et al). Here we have used homozygosity mapping followed by ES to identify the genetic cause of lethal DCM in a three-day-old Dutch girl. The homozygous mutation, c.542G>T, p.(Gly181Val), we found in the SOD2 gene (NM_000636.2) most likely affects the catalytic activity of the protein, leading to excess oxygen radical levels with strongly damaging effects in the neonatal heart.

METHODS

Case report

The female patient was born at 39\textsuperscript{w}2 weeks gestation after a caesarean delivery due to breech presentation and meconium staining of the amniotic fluid. The pregnancy was complicated by maternal nephrotic syndrome.
at 19 weeks gestation and treated with prednisone. Her Apgar scores were 2-3 and 9, her 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 with a base excess of -4 mmol/l. The day after birth she presented with apnoeas, poor circulation and mild tachycardia. A chest X-ray was normal. Echocardiography showed a structurally normal heart, but left ventricular function seemed poor. Cardiac troponin and BNP were elevated, 0.28 µg/l (n < 0.16 µg/l) and 2819 pmol/l, respectively.

On the third day after birth she developed cardiogenic shock with frequent ventricular extrasystoles and tachycardia. Both ventricles showed dilatation and she died three days postpartum. Biochemical studies showed a high level of lactate in the blood, possibly due to poor circulation; increased amino acids, including proline and alanine; and increased organic acids, including 3-methylglutaconic acid. Pompe disease was excluded by normal plasma alpha-glycosidase levels. Disorders of N-glycosylation and peroxisomal metabolism were also excluded. Viral serology showed no abnormality. At autopsy, macroscopic examination of the heart revealed severe dilatation of both ventricles, without any histological abnormalities. The cardiac weight was consistent with 41 weeks gestation, while the weights of other organs were consistent with 32-33 weeks gestation. Skeletal muscle showed no abnormalities and there were no indications of disorders of fatty acid oxidation or of mitochondrial disease, although these could not be fully excluded by histological examination. Actin, dystrophin, sarcoglycan, dystroglycan, dysferlin, caveolin-3, merosin, myosin and spectrin-1 staining were normal, and respiratory chain complexes were measured and also normal. Intracranial examination showed small cerebral subependymal cysts.

Genomic DNA of the child and her parents was extracted from peripheral blood using standard protocols. The parents provided informed consent for DNA studies, and for diagnostic procedures. The UMCG ethical committee approved this study.

**Homozygosity mapping**

Genome-wide genotyping with the HumanCytoSNP-12 BeadChip® 300K SNP array (Illumina, San Diego, CA, USA) was performed according to the manufacturer’s protocols. Data from the arrays were converted to genotypes using the GenomeStudio® data analysis software (Illumina). The genotype data was subject to homozygosity mapping using Microsoft® Office Excel
2010 (Version 14.0; Microsoft, Redmond, WA, USA) software by searching for homozygous regions in the patient’s DNA, allowing for a 1% genotyping error margin. The size of the homozygous regions was calculated in megabases (Mb) and in centiMorgans (cM), based on the deCODE genetic map (Kong et al).

**Exome sequencing**

ES on the patient’s DNA was performed using the SureSelect 50Mb exome capture kit (Agilent, Santa Clara, CA, USA) following the manufacturer’s protocol. The enriched fragments captured were sequenced using the Illumina HiSeq platform in paired-end mode, with a read length of 100 bp following the manufacturer’s protocol. The raw Fastq files were aligned by using bwa-0.5.9 to the human reference genome (hg 19, NCBI build 37) (Li et al, 2009a), SAM/BAM files were manipulated by Samtools-0.1.10, and Picard-1.57 (Li et al, 2009b). Then the Genome Analysis Toolkit (GATK) was used to perform base quality score recalibration, duplicate removal and INDEL realignment (McKenna et al). The output vcf files were annotated by our in-house Bioinformatics pipeline and Seattleseq (http://gvs.gs.washington.edu/).

**Subsequent mutation analysis**

Sanger sequencing was used to confirm the presence/absence of the SOD2 mutation in the patient and her family members. In addition, screening of all exons and exon/intron junctions of the SOD2 gene was performed in other patients. PCR was performed by using AmpliTaq Gold PCR Master Mix (Invitrogen Life Science Technologies, Carlsbad, CA, USA) following the official protocol and resulting fragments were sequenced by Applied Biosystems’ 96-capillary 3730XL system (Carlsbad, CA, USA).

**RNA extraction and Reverse Transcriptase-PCR (RT-PCR) product analysis**

RNA was isolated from cultured fibroblasts from the patient. Cells were cultured in standard medium for human fibroblasts (Dulbecco’s modified Eagle’s medium with 10% FBS, 1% penicillin/streptomycin, 1% glucose, 1% glutamax) with 5% CO₂ at 37°C. RNA was extracted with the RNeasy Mini Kit (QIAGEN, Venlo, the Netherlands) following the manufacturer’s protocol. cDNA was synthesized from 500 ng of total RNA by RevertAid RNaseH- M-MuLV reverse transcriptase in a total volume of 20 μl according to the
protocol provided by the supplier (MBI-Fermentas, St Leon-Rot, Germany). To investigate whether the c.542G>T mutation could have an effect on mRNA splicing, we performed RT-PCR with primers specific for SOD2 and designed to amplify the exon that was expected to be affected by the mutation and flanking sequences (primers are available upon request). Target regions were amplified by PCR and the PCR products were examined by 2% agarose gel and analysed by Sanger sequencing. To test for effects of nonsense-mediated decay, fibroblasts were treated with cycloheximide for 4.5 hr, followed by RNA analysis using the same procedures as those for RNA from untreated cells.

**Measurement of superoxide substrate levels**

Fibroblasts, cultured to 70% confluence, were incubated in HEPES-Tris medium containing 10 μM hydroethidine (HEt) for 10 min at 37°C. Within the cell, HEt reacts with O$_2^-$ to form the fluorescent and positively charged product ethidium (Et) or oxyethidium. The reaction was stopped by thoroughly washing the cells with PBS to remove excess HEt. For quantitative analysis of Et emission signals, 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 fluor lens objective. Et was 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, VT) 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. Routinely, 10 fields of view per coverslip were analysed.

**SOD2 protein’s 3D structure**

As the 3D-structure of the SOD2 protein is known, HOPE software was applied to predict the potential effect of the p.(Gly181Val) missense mutation on the 3D structure of the protein (Venselaar et al). Additionally, the Uniprot protein database (www.uniprot.org) was used 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

Case report

Genealogical analysis found a distant relationship between the parents 6 to 8 generations previously, suggesting an autosomal recessive inheritance (figure 1). Array-CGH showed no pathogenic copy number variations. Diagnostic Sanger sequencing results of mitochondrial DNA, isolated from fibroblasts, and of the POLG, MYL2, MYH7, LMNA, DES, SUCLA2 and RYR2 genes were normal. Respiratory chain complexes were found to function normally. Echocardiography revealed no abnormalities in the mother or father (aged 27 and 29, respectively) or in the patient’s younger brother (cardiologically evaluated aged 1 week).

Figure 1. Pedigree of a Dutch family with a child with severe, lethal DCM, in whom autosomal recessive inheritance was expected due to the pedigree composition. The patient is marked with a black symbol.
Homozygosity mapping

Homozygosity mapping in the patient (figure 1; X:1) revealed the longest homozygous region was on the X chromosome (figure 2). The longest autosomal region of homozygosity was located on chromosome 6, between rs378512 and rs9458499 (159,949,340-162,713,427 bp; UCSC Genome Browser, build hg19), spanning 268 SNPs and 4.26 cM. This homozygous region contains 26 genes, including the SOD2 gene.

Exome sequencing

ES was performed to target all exons and exon/intron junction sequences of the known genes in the human genome to identify potentially pathogenic, disease-causing mutations. Using the sequence analysis pipeline from GATK, we identified 41,621 different variants in the patient’s exome data. Data filtering was performed to exclude all known variants with a high frequency (> 1%) in the dbSNP129, the 1000 Genomes Project, GoNL, ESP6500 databases and in our in-house database. We then selected for coding variants in the remaining 325 variants and subsequently for nonsense, missense, splice site, and frame shift variants in concordance with autosomal recessive inheritance.
(i.e. homozygous or compound heterozygous variants in one gene). This resulted in the identification of a homozygous mutation, c.542G>T; p.(Gly181Val) (NM_000636.2), in the SOD2 gene located in the second longest homozygous region on chromosome 6 (figure 2). This mutation was absent from known control populations (ESP6500, GoNL, and 1000 Genomes). Our ES data was also analysed for potential causal mutations in known cardiomyopathy genes, relevant metabolic and syndromic genes, and nuclear encoded mitochondrial genes, but no putative pathogenic mutations were identified.

Sanger Sequencing, gene-panel-based resequencing and RT-PCR product analysis

Using Sanger sequencing, the homozygous mutation c.542G>T; p.(Gly181Val) was confirmed in the affected child (figure 3) and in heterozygous form in her parents, but it was absent in her brother (data not shown). Furthermore, Sanger sequencing of the SOD2 gene in an additional DCM cohort of 27 different paediatric patients and 161 adult patients, and gene-panel-based resequencing of the gene in more than 1,000 adult cardiomyopathy patients revealed no pathogenic SOD2 mutations. RT-PCR product analysis of RNA isolated from patient fibroblasts, and cultured both with and without cycloheximide, showed only a transcript of wild type size, indicating that this mutation has no effect on splicing.

Superoxide (O$_2$•⁻) substrate levels

For superoxide substrate level measurements, hydroethidine was used as an intracellular probe to measure the levels of superoxide (O$_2$•⁻) in the patient fibroblasts. Notably, hydroethidine is not sensitive to H$_2$O$_2$. Hydroethidine is a cell-permeable compound that interacts with O$_2$•⁻ to form ethidium or oxyethidium. The oxidation levels of hydroethidine measured in our in vitro assay indicated a significant increase of superoxide (O$_2$•⁻) levels in the fibroblasts of the patient comparable to the order of magnitude seen in complex I deficient fibroblasts (figure 4). What we could not directly determine from this data was whether the significant increase of O$_2$•⁻ levels resulted from a complex I deficiency or from abnormal SOD2 enzyme activity. However, mitochondrial respiratory chain enzyme activities (complexes I, II, III, IV, and V) were also measured and revealed no differences in the activity, suggesting SOD2 activity as the likely mechanism.
Figure 3. Sanger sequencing confirmed the presence of the homozygous SOD2 variant c.542G>T, p.(Gly181Val) in the affected patient (bottom) compared to control (top) and in heterozygous form in her parents (not shown).
**SOD2 3D structure: predicting the effect of the p.(Gly181Val) mutation**

Using the HOPE software we retrieved the 3D structure information of the SOD2 protein through the WHAT IF Web services, the Uniprot database and a series of DAS-servers, in order to predict the effect of the p.(Gly181Val) mutation on the protein structure. The Gly181 residue is part of a manganese/iron superoxide dismutase domain, which is important for the main activity of the protein. The domain has a function in superoxide dismutase activity (oxidoreductase activity) and metal ion binding. According to the Uniprot database, four important amino acid residues are involved in the formation of the Mn-binding pocket that binds the manganese co-factor of the enzyme (accession number: P04179). These residues are His50, His98, Asp183 and His187. 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 manganese/iron superoxide dismutase domain and, as a consequence, the catalytic activity of the enzyme (figure 5).

**DISCUSSION**

Using a combination of homozygosity mapping and ES in the patient, we detected a novel homozygous missense mutation, c.542G>T; p.(Gly181Val), in an evolutionarily highly conserved domain of the SOD2 gene located in the...
second longest homozygous region on chromosome 6. To our knowledge, this is the first report of a major role for mutated SOD2 in human disease. Two facts support the potential pathogenicity of this mutation. The first is that the mutation is located in the functionally important C-terminal manganese/iron superoxide dismutase region of the respective protein. The second is that drastic differences between the size and the physicochemical characteristics of the wild-type glycine (which is the smallest of all residues and its presence is known to often provide flexibility to protein structures) and the mutant valine residues are predicted to disturb the core structure in this crucially important domain. Furthermore, according to the Uniprot database, the mutation is localized only two amino acids away from one of the four Histidine/Aspartic acid residues that are involved in the binding of the manganese co-factor.

**The role of the mutation**

Hydroethidinium oxidation measurements indicated a significant increase in the levels of O$_2^-$ (one of the major ROS which are the physiological substrate of the SOD2 enzyme) in the fibroblasts of the patient; this substrate level was comparable in order of magnitude to the levels seen in complex-I-deficient fibroblasts. Since no deficiency in any of the mitochondrial respiratory chain
complexes I-V was seen, this significant increase in $O_2^{-}$ could probably be explained by the pathogenic effect of the c.542G>T; p.(Gly181Val) SOD2 mutation on the function of the encoded enzyme, leading to malfunctioning and accumulation of damaging oxygen radicals in the cells and increased oxidative stress.

The role of superoxide dismutase in disease

SOD2 belongs to the manganese/iron superoxide dismutase family which is 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: Cu/ZnSOD (SOD1), Mn/FeSOD (manganese superoxide dismutase 2; SOD2) and extracellular SOD3. SOD2 is a mitochondrial matrix protein which converts superoxide anion ($O_2^-$) to $H_2O_2$ which is then metabolized by glutathione peroxidase into $H_2O$ (Alscher et al). Oxidative stress is a deleterious process mediated by ROS, and it can lead to severe damage of cellular structures and their building blocks, including proteins, DNA and lipids (Valko et al). ROS are naturally formed during mitochondrial metabolism, and cells self-regulate their ROS levels by producing antioxidant enzymes (Starkov, 2008). Deficiency of one the antioxidant enzymes, such as SOD2, may affect any organ at any age, but most often affect organs with a high energy demand, such as the heart and brain, as is commonly observed in mitochondrial disorders (Meyers et al). Furthermore, it has been reported that oxidative stress and mutations in the SOD2 gene are involved in the pathogenesis of several diseases such as mitochondrial dysfunction, cancer, neurological disorders, diabetes, and many cardiovascular diseases including hypertension, atherosclerosis, and restenosis (Hedskog et al, Jenner, 2003, Louzao & van Hest, Cai & Harrison, Griendling & FitzGerald). There have also been reports of the involvement of other nuclear genes, such as TAZ (D’Adamo et al), TXNRD2 (Conrad et al, Sibbing et al), DNAJC19 (Davey et al, Ojala et al), and SDHA (Levitas et al), in mitochondrial cardiomyopathy, and this also seems applicable to the current case.

Superoxide dismutase in cardiomyopathy

Oxidative stress and disturbed mitochondrial respiratory function are known to play a substantial role in the development of heart failure (Huss & Kelly) and the role of the SOD2 protein in cardiomyopathy has previously been demonstrated in mice. Homozygous Sod2 knockout mice showed
neonatal lethality due to neurodegeneration and cardiomyopathy (Li et al 1995). In addition, the intake of antioxidants improved their phenotypes of dilated cardiomyopathy and muscle fatigue and had beneficial effects on electrophysiological disturbances in heart and muscle (Koyama et al, Sunagawa et al). Interestingly, heterozygous SOD2+/- mice showed reduced SOD2 enzyme activity, yet did not exhibit any disease phenotype at 9 months of age (Li et al 1995). Likewise, the parents of the severely affected child described here, who are heterozygous carriers of the SOD2 mutation, did not show any cardiac abnormalities. Finally, chemotherapeutic (anthracyclin-induced) cardiomyopathy and heart failure is believed to be a side effect of superoxide radical accumulation leading to the induction of mitochondrial dysfunction in the heart (Thayer, 1988). In fact, this phenotype was successfully rescued in transgenic mice by the overexpression of SOD2 (Yen et al), underscoring the cardioprotective role of this enzyme in healthy individuals.

**CONCLUSIONS**

Here we have reported the successful use of a combined approach using homozygosity mapping and exome sequencing to identify the causal mutation in the mitochondrial protein, SOD2, in a child with severe neonatal cardiomyopathy. Protein conformation predictions and functional evaluation support the role of SOD2 deficiency in the abnormally elevated levels of oxidative stress found in our patient. Oxidative stress itself is known to be involved in the development of various diseases, including cardiomyopathies. The result from our patient adds a novel, nuclear-encoded disease gene to the list of genes involved in severe mitochondrial cardiomyopathies.

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