Tread Carefully: A functional variant in the human NADPH Oxidase 4 (*NOX4*) is not disease causing.

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All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients included in the study. This project was approved by the Human Research Ethics Committees of the Sydney Children's Hospitals Network (ID number 10/CHW/114) and the University of Queensland (ID number 2012001414).

Synopsis

A genetic diagnosis was attempted using whole exome sequencing and functional studies in a patient, but family cascade testing negated the pathogenic relevance of the functional abnormalities identified.

Conflicts of Interest

Nafisinia Michael, Minal Menezes, Wendy Gold, Lisa Riley, David Coman, and John Christodoulou declare that they have no conflicts of interest.

Details of contributions of individual authors

Michael Nafisinia: design and implementation of structural and functional studies, data analysis and manuscript preparation

Minal Menezes, Wendy Gold, Lisa Riley: contribution to design of structural and functional studies, data analysis and manuscript preparation

Joshua Hatch: initial bioinformatics analysis and manuscript preparation

John Cardinal: initial bioinformatics analysis and manuscript preparation

David Coman: initial bioinformatics analysis, subsequent data analysis and manuscript preparation

John Christodoulou: overall oversight of the research project, data analysis and manuscript preparation

Abstract

In this study, we report a paediatric patient with a lethal phenotype of respiratory distress, failure to thrive, pancreatic insufficiency, liver dysfunction, hypertrophic cardiomyopathy, bone marrow suppression, humoral and cellular immune deficiency. To identify the genetic basis of this unusual clinical phenotype and potentially make available the option of future prenatal testing, whole exome sequencing (WES) was used followed by functional studies in a bid to confirm pathogenicity. The WES we identified a homozygous novel variant, AK298328; c.9_10insGAG; p.[Glu3dup], in *NOX4* in the proband, and parental heterozygosity for the variant (confirmed by Sanger sequencing). NADPH Oxidase 4 *NOX4* (OMIM 605261) encodes an enzyme that functions as the catalytic subunit of the NADPH oxidase complex. NOX4 acts as an oxygen sensor, catalysing the reduction of molecular oxygen, mainly to hydrogen peroxide (H₂O₂).

However, although, our functional data including 60% reduction in NOX4 protein levels and a 75% reduction in the production of H₂O₂ in patient fibroblast extracts compared to controls was initially considered to be the likely cause of the phenotype in our patient, the potential contribution of the *NOX4* variant as the primary cause of the disease was clearly excluded based on following pieces of evidence. First, Sanger sequencing of other family members revealed that two of the grandparents were also homozygous for the *NOX4* variant, one of who has fibromuscular dysplasia. Second, re-evaluation of more recent variant databases revealed a high allele frequency for this variant.

Our case highlights the need to re-interrogate bioinformatics resources as they are constantly evolving, and is reminiscent of the short-chain acyl-CoA dehydrogenase deficiency (SCADD) story, where a functional defect in fatty acid oxidation has doubtful clinical ramifications.

Keywords: *NO4*, H₂O₂, Whole exome sequencing.

Introduction

In recent decades, researchers have successfully identified genes and their variants that contribute to disease, increasing our knowledge about the etiologic basis and biology of these diseases. Here, the discovery of a variant in the NADPH oxidase 4 (*NOX4*) gene in association with a fatal disorder in a female infant is reported. It was initially thought this variant was the primary cause of the phenotype in this child. However, new data about the frequency of this variant, and further cascade testing in the extended family lead us to conclude that this assertion was erroneous.

The human *NOX4* gene belongs to the NADPH Oxidase (NOX) family of oxidoreductases that is comprised of seven homologous proteins (NOX1-5 and DUOX1-2) [1, 2]. The NOX family, in contrast with several enzyme systems that produce reactive oxygen species (ROS) secondary to damage, is the primary source of ROS [3]. NOX4-derived ROS play an important role in intracellular signalling [4], regulation of mitochondrial function and host defences [5], maintenance and differentiation of smooth muscle [6] and cardiovascular embryonic stem cells [7] under both physiological and pathological conditions. Further, NOX4 promotes the activation of autophagy [1] and survival in cardiomyocytes [8].

NOX4 is predominantly expressed in the kidney, blood vessels, mesangial cells, smooth muscle cells, keratinocytes, osteoclasts, endothelial cells, neurones, and hepatocytes [9-12]. Unlike the other NOXs which localise only to the plasma membrane, NOX4 has been reported to have activity in the endoplasmic reticulum [10], cytoskeleton [13], plasma membrane [14], as well as the mitochondria [10]. Further, while all members of the NOX family produce superoxide ($O_2^{\cdot -}$), *NOX4* is the only family member that generates hydrogen peroxide (H_2O_2) [12]. Variants in

NOX4 have previously been associated with cardiovascular diseases [15], diabetic nephropathy [16], lung fibrosis [17], and cancer [18, 19].

In this study trio, whole exome sequencing using DNA samples from an affected proband and her parents, with a sequence variant in the *NOX4* gene; (c.9_10insGAG; p. [Glu3dup]) identified to be homozygous in the proband and heterozygous in her non-consanguineous parents. This variant was thought to be the potential pathogenic candidate because: (i) *NOX4* function had relevance to the clinical symptoms of the proband, and (ii) it was not reported in the 1000 Genomes Project and dbSNP population databases. This finding was supported by a number of functional studies conducted in our laboratory. However, the possibility of the *NOX4* variant being the primary cause of the disease was eliminated when screening of the maternal and paternal grandparents showed one grandparent from each side of the family was also homozygous for this variant. Subsequently, we found more recent minor allele frequency (MAF) data for this variant to be 0.5821 in the ExAC browser, which was not yet available at the time we conducted our initial studies. Thus, at present, the primary causative gene of this child's phenotype remains elusive. It is possible that the *NOX4* variant may be a disease modifier, but more extensive studies, including identification of the primary causative gene, would be required before this assertion could be corroborated with more confidence.

Materials and Methods

Patient clinical summaries

The patient's mother was G₁P₀ (her first pregnancy did not result in a live birth) The family were non-consanguineous and of Caucasian background with no family history of genetic diseases or birth malformations. The female proband was delivered via forceps-assisting spontaneous

vaginal delivery at 34 weeks gestation, after spontaneous onset of labour following an antepartum haemorrhage. There was foetal distress with meconium stained liquor and a maternal temperature of 37.9°C during the labour. The infant's Apgar scores were 1 and 6 at one and five minutes respectively. Resuscitation took the form of the bag and mask ventilation. The birth weight was 2,020 grams (38th percentile). The infant required continuous positive airways pressure (CPAP) and was transferred to a tertiary facility on day 9 given persistent respiratory distress.

Failure to thrive and persistent loose stools prompted an investigation of pancreatic insufficiency. Evidence of severe exocrine pancreatic insufficiency was demonstrated with a markedly reduced stool pancreatic elastase 1 (< 15 µg/g faeces; reference range > 500), numerous fat globules on faecal microscopic examination, and positive stool reducing substances. Signs of endocrine pancreatic insufficiency ensued late in the clinical course, with the requirement of insulin to manage hyperglycaemia. Liver function tests demonstrated persistent elevations in alanine aminotransferase (ALT), aspartate aminotransferase (AST), a conjugated hyperbilirubinaemia, normal gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) and normal hepatic synthetic function. Abdominal ultrasound demonstrated normal echotexture of the liver, normal bile ducts and a thickened gallbladder with some free ascites. Alpha-1-antitrypsin levels, TORCH serology, and iron studies were all normal.

The infant developed significant cardio-respiratory dysfunction in the neonatal intensive care. Intubation and ventilation were required for respiratory failure. While ventilated the lung fields demonstrated persistent areas of patchy multilobar collapse and consolidation. Given the

concomitant pancreatic insufficiency, investigations for cystic fibrosis ensued with no common pathogenic mutations identified in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Initial echocardiograms demonstrated a patent ductus arteriosus (PDA). However, she developed significant left ventricular myocardial hypertrophy with impaired cardiac contractility requiring inotropic support.

She developed a persistent transfusion dependent thrombocytopenia and anaemia. Lymphocyte subsets and lymphocyte mitogen studies demonstrated a T-cell based immune deficiency, combined with hypogammaglobulinaemia. The peripheral blood films demonstrated toxic granulation in the neutrophils, thrombocytopenia and occasional spherocytes. A bone marrow aspirate and trephine were performed with non-specific findings including a normocytic marrow with moderate dyserythropoiesis and reduced megakaryocytes. Sideroblasts were not demonstrated in the bone marrow aspirate.

She was noted to have mild dysmorphic features including micrognathia, high arched palate, single palmar creases, and ichthyotic skin. There were no clinical features of hypopigmentation in the skin, iris or hair shafts. Light microscopy and electron microscopy of hair shafts were also normal. Generalised hypotonia was noted with normal deep tendon reflexes. Creatine kinase levels were normal.

Multiple additional investigations were performed all yielding normal results including; very-long-chain fatty acids, karyotype (46XX), CGH array, plasma amino acids, urine amino acids, urine organic acids, urine polyols, mitochondrial DNA common deletion and point mutations. A defect in autophagy was suspected given the nature of the multisystem clinical phenotype and

lack of an alternate diagnosis. A diagnosis of Vici syndrome (OMIM 242840) was considered, however, the lack of hypopigmentation and a normal corpus callosum were atypical.

Death occurred at 38 days following progressive respiratory failure. Consent was provided for a targeted autopsy, not including brain examination. Significant pathology was demonstrated most notably within the haematopoietic and reticuloendothelial systems. White pulp was absent in the spleen. The thymus was markedly hypoplastic in the parenchyma with no cortex. Absent Hassall's corpuscles and plasma cells indicated thymic dysplasia/hypoplasia. Lymph nodes demonstrated abnormal architecture without any primary or secondary follicles. No lymphocytes were observed in the intestinal lamina propria. The lungs demonstrated a saccular phase of development consistent with gestation; the remaining architecture was normal without any histological evidence of pulmonary hypertension. Acute bronchopneumonia changes with diffuse alveolar infiltration were demonstrated.

Histological examination of heart was indicative of both left and right ventricular hypertrophy. Significant atrophy of the exocrine component of the head of the pancreas was noted. There was diffuse intrahepatic cholestasis, with normal intrahepatic bile ducts. There was no giant cell transformation, and foci of extramedullary haematopoiesis were identified. A distal common bile duct could not be identified, and the proximal right and left hepatic ducts were identified but were fibrotic, and the gallbladder wall was thickened and fibrotic. These changes indicated extrahepatic bile duct obstruction. Skeletal muscle examination was abnormal with muscle fibre type disproportion in intercostal muscles, severe atrophy in tongue samples, and an atrophic

diaphragm with extramedullary haematopoiesis and fibrosis. The skin demonstrated hyperkeratosis, parakeratosis and an absent granular layer.

WES and bioinformatics

DNA extracted from the blood of the proband, and her parents were subjected to WES, performed by Otogenetics (Tucker, Georgia, USA). After enrichment of all the coding and flanking regions, sequencing analysis was performed using an Illumina HiSeq platform. Over 97.7% of the targeted regions achieved 100× coverage. Only sequence variations with an allele frequency of <1% were considered pathogenic.

Sanger sequencing

The sequence variant in the *NOX4* gene was confirmed via Sanger sequencing in the proband and her heterozygous parents by Otogenetics (Tucker, Georgia, USA)..

***In silico* analyses**

In silico analyses of the *NOX4* variant were performed using online databases and prediction programs. dbSNP (<https://www.ncbi.nlm.nih.gov/SNP/>), the 1000 Genomes Browser (<https://www.1000genomes.org/>) and ExAC (<http://exac.broadinstitute.org/transcript/ENST00000413594>) were used to determine the minor allele frequency of the identified variant. To predict the pathogenicity of the variant, MutationTaster (<http://www.mutationtaster.org/>) was used. PhyloP (<http://ccg.vital-it.ch/mga/hg19/phylop>) and PhastCons (<http://compugen.bscb.cornell.edu>) were both used to determine the grade of conservation of the identified variant.

Immunoblotting

Patient and control fibroblast cells were lysed, and protein prepared as previously described [20]. Thirty micrograms of each protein sample were resolved on a 4–12% Bis-Tris gel (Life Technologies) and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were probed with 1:1000 anti-NOX4 (ab109225; Cambridge, UK). Protein loading was normalised to GAPDH (1:5000) (Cat#MAB374; Millipore, Billerica, USA) for 3 hours at room temperature. All blots were then probed with either 1:2,500 anti-rabbit or anti-mouse IgG horseradish peroxidase (HRP; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 2 h at room temperature. Membranes were then incubated with enhanced chemiluminescence (ECL) reagents and exposed to Hyperfilm ECL (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Films were scanned using a SRX-101A (Konica Minolta Holdings, Inc., Tokyo, Japan) scanner and analysed with ImageJ version 1.49 [21].

Measurement of ROS in fibroblasts

As NOX4 produces 90% hydrogen peroxide (H_2O_2) and 10% superoxide [22], both H_2O_2 and mitochondrial superoxide levels were measured.

Measurement of H_2O_2

Hydrogen peroxide (H_2O_2) levels were measured in fibroblast cell lines using a Colorimetric Hydrogen Peroxide Kit (ab102500, Massachusetts, USA) according to the manufacturer's protocol. Briefly, 2×10^6 fibroblast cells were harvested and washed with cold PBS. Cells were resuspended in 4 volumes of the assay buffer on ice. The mixture then homogenised (25 passes) using a 1ml syringe and 23 gauge needle (Becton Dickinson®, North Ryde, Australia) on ice.

Samples were centrifuged, and the supernatant was collected and placed on ice. Ice cold perchloric acid (PCA) with a final concentration of 1 M was added to the supernatant, mixed and incubated on ice for 5 minutes. Samples were centrifuged at 13,000 g for 2 minutes at 4°C, and the supernatant was transferred to a fresh tube. Ice-cold 2M KOH then added to the supernatant, equal to 34% of the supernatant. The pH of the supernatant was adjusted to 6.5 using 0.1 M KOH or PCA. Samples were centrifuged at 13,000 x g for 15 minutes at 4°C, and the supernatant was collected. Two to fifty microlitres of the supernatant (adjusted to 50 µL/well with Assay Buffer) were added to a 96 well plate. Fifty microlitres of each standard dilution were used to create a standard curve (0, 10, 20, 30, 40, 50 µl of the 0.1 mM H₂O₂ standard). Fifty microlitres of the reaction mix containing assay buffer, OxiRed Probe, and Horse Radish Peroxidase (HRP) were added to each well. The plate was incubated at room temperature for 10 minutes protected from light and measured at 570 nm using a Beckman Coulter DU 650 Spectrophotometer (Beckman Coulter, Fullerton, CA).

Flow cytometric assessment of mitochondrial superoxide levels in fibroblasts using MitoSOX Red

To evaluate the effect of the *NOX4* variant on superoxide production, superoxide levels in fibroblasts was measured using flow cytometry following staining with MitoSOX™ Red (details). MitoSOX Red is a selective dye for mitochondrial superoxide in live cells that efficiently competes with superoxide dismutase (an enzyme that helps break down potentially harmful reactive oxygen species in cells) for superoxide. MitoSOX is a derivative of ethidium bromide, thus, oxidised MitoSOX red becomes highly fluorescent upon binding to nucleic acids [23]. Flow cytometric analyses were performed as previously described [24]. Briefly,

mitochondria-associated ROS levels were measured by staining cultured fibroblasts using the MitoSOX kit (M36008, Invitrogen) at 2.5 mM for 30 minutes at room temperature. Cells were then washed with PBS solution and resuspended in cold PBS solution containing 1% FBS for flow cytometric analysis. Flow cytometric measurements of the patient samples and two different control fibroblast cell lines were carried out using FACSCalibur™ (BD Bioscience). MitoSOX Red was excited by laser at 448 nm to measure oxidised MitoSOX Red in the FL2 channel. At least 5000 events were collected in each sample. To validate mitochondrial superoxide generation with MitoSOX Red (positive control), both patient and control fibroblast cell lines were treated with Antimycin A as described previously [24]. As a negative control, PBS without MitoSOX was used. The flow cytometry experiment was performed twice, each time, in three replicates with two different control cell lines.

Results

WES analysis and Sanger sequencing

WES revealed a novel homozygous variant (AK298328; c.9_10insGAG; p. [Glu3dup]) in the gene *NOX4* in the proband. Other candidate genes with homozygous, compound heterozygous or *de novo* dominant deleterious variants were not evident from the SNV and Indel variant calls, thereby making *NOX4* gene the lead candidate gene for the observed phenotype. The parents were heterozygous for the identified variant. Results were confirmed by Sanger sequencing in the School of Medicine, Griffith University.

***In silico* analysis**

In silico analysis was performed to identify the minor allele frequency of the identified variant. This variant was not seen in the 1000 Genomes Project or dbSNP datasets when our initial analysis was undertaken at the beginning of 2014. This prompted us to undertake a series of functional studies (see below). However, at the end of 2014, ExAC browser revealed an average minor allele frequency (MAF) of 0.5821 for this variant among Europeans, Latinos, South and East Asians, and Africans (<http://exac.broadinstitute.org/variant/11-89224131-T-TCTC>). Of note, the p.Glu3dup variant is only expressed in a non-canonical isoform of NOX4. Variants with a MAF >0.1% are considered common [25]. The *in silico* review of the identified variant predicted it to be polymorphism by Mutation-Taster (Table 1). Of note, the other publicly available pathogenicity prediction programs such as PolyPhen-2 and SIFT have not been developed for duplication detection. PhastCons and PhyloP revealed that the identified variant is not conserved among species (Table 1).

Immunoblotting

To evaluate the effect of the *NOX4* variant on protein expression, immunoblotting was performed using a NOX4 antibody raised against the amino acids 500 to the C-terminus. This antibody is expected to detect all seven isoforms of the NOX protein family. Immunoblotting in patient fibroblast extracts showed a 60% reduction in NOX4 protein levels compared to the control (Fig. 1a).

Measurement of ROS in fibroblasts

H₂O₂ measurements

H₂O₂ is a reactive oxygen metabolic by-product with a key regulatory role for a number of oxidative stress-related states. Here, the production of H₂O₂ was measured in patient fibroblasts using a colourimetric assay. The results showed a 75% reduction in the production of H₂O₂ compared to that in controls (Fig. 1b).

Flow cytometric assessment of fibroblasts using MitoSOX Red

As the NOX4 protein is localised to mitochondria [26], we measured the level of mitochondrial superoxide. Using flow cytometry, the results showed equivalent levels of mitochondrial superoxide in the proband's fibroblasts compared to the controls in both untreated cells and cells treated with antimycin A (positive control), suggesting the *NOX4* variant does not affect mitochondrial superoxide levels. The untreated controls (C1 and C2), and proband fibroblasts are shown in red, orange, and grey respectively (Fig. 1c). Fibroblasts treated with Antimycin (C1-A and C2-A), and the proband (P-A) are shown in aqua, light green, and dark green respectively (Fig. 1c).

Discussion

In this study involving a female infant with multisystem disease, affecting heart, lung, liver and bone marrow, who died at 38 days following progressive respiratory failure, we found what was initially believed to be an apparently novel variant in the *NOX4* gene. This c.9_10insGAG; p.[Glu3dup] variant, which is only expressed in non-canonical isoform five of NOX4, was

homozygous in the proband and heterozygous in her parents. We initially found no reported MAF in dbSNP and the 1000 Genome Project database. At autopsy, evidence of acute lung injury, skin ichthyosis, and the significant immune deficiency was found. Primary defects in *NOX4* are postulated to contribute to all the autopsy findings [27-31]. Moreover, *NOX4* is constitutively expressed and active in a variety of cells [32, 33]. With regard to these findings, we hypothesised that the *NOX4* variant was responsible for the proband's clinical presentation, prompting us to undertake functional studies.

We found that both NOX4 protein levels and H₂O₂ levels were significantly decreased in the proband fibroblasts. These findings suggested that although the variant is in a rare isoform of NOX4, in the patient this rare isoform may have regulated the other NOX4 isoforms. The normal levels of the mitochondrial superoxide in the proband were in agreement with the prediction that there would have been little impact of NOX4 in generating mitochondrial superoxide.

However, subsequently, the allele frequency of this *NOX4* variant changed from 'not reported' to having a MAF of 0.5821, with 2042 homozygous cases reported in the ExAC database. Further, on the screening of the proband's grandparents, the same homozygous variant was subsequently identified in one of the maternal grandparents and one of the paternal grandparents, with one of these grandparents also having a clinical diagnosis of fibromuscular dysplasia (FMD). FMD causes abnormal cell development in the endothelium, resulting in stenosis of the arteries connected to the neck, heart, brain, and kidney [34]. The endothelium contributes to the maintenance of cardiovascular homeostasis, mainly through the activity of endothelium-derived nitric oxide (NO). Elevation of ROS has been proposed to result in NO breakdown, causing FMD [35]. The possibility that homozygosity for this variant may have caused the development

of FMD in this individual is tempting, but the lack of any significant health problems in the other homozygous grandparent or the apparently healthy 2042 homozygotes in the ExAC database, argue strongly against this notion, and clearly rules it out as the primary cause of the severe phenotype in the proband. Moreover, FMD is a relatively common disorder [36], so it is most likely that FMD and homozygosity of the *NOX4* variant are purely coincidental.

With regard to the uncertain pathogenicity of the *NOX4* variant, there is a slim possibility that the interplay of *NOX4* (as the primary disease causing gene) with disease modifiers including other genes or environmental factors may exert a range of phenotypic effects varying from negligible to lethal in close family members. For example, the same hypertrophic cardiomyopathy (HCM) gene variant in family members may result in marked clinical variability, ranging from asymptomatic left ventricular hypertrophy (LVH) to progressive heart failure to sudden cardiac death in members of the same family[37]. However, the high allele frequency of this variant of the non-canonical *NOX4* isoform is very likely to give rise to a possibility of this variant being a disease modifier. This suggestion is strengthened by the recent report that variants in the *NOX4* gene may act as modifiers for a rare autosomal recessive neurodegenerative disorder, Ataxia Telangiectasia (AT), by increasing DNA damage and apoptosis in the cerebellum of AT patients [38]. AT is primarily caused by mutations in the Ataxia Telangiectasia Mutated (*ATM*) gene [38].

The modifier gene concept, introduced in 1941 by Haldane, refers to a gene that alters the expression of another disease-causing gene [39]. Our findings are analogous to the Short-Chain Acyl-CoA Dehydrogenase (SCADD) Deficiency (OMIM # 201470) story. SCADD is an

autosomal recessive genetic disorder of fatty acid catabolism caused by mutations in the acyl-coenzyme A dehydrogenase, C-2 to C-3 short chain (*ACADS*) gene (OMIM 606885) [40]. The *ACADS* protein catalyses the first step in the mitochondrial oxidation of fatty acids four to six carbons in length [41]. The SCADD clinical phenotype is quite variable ranging from no symptoms to neuromuscular symptoms such as myopathy and hypotonia, neonatal acidosis, failure to thrive and developmental delay [41, 42].

To date, more than 60 different mutations in the *ACADS* gene have been linked to SCADD. Two common variants (polymorphisms): c.511C>T, p. [Arg171Trp] (minor allele frequency = 0.03132 with 97 cases reported homozygous in the ExAC browser), and c.625G>A, p. [Gly209Ser] (minor allele frequency = 0.2592 with 4537 reported homozygous in the ExAC browser) have also been found in the *ACADS* gene [43]. Most people carrying these polymorphic variants are clinically well. There is considerable doubt as to whether SCADD causes a primary disease phenotype. However, developmental delay and hypotonia have been observed in some cases, raising the possibility that SCADD is a disease modifier rather than a consistent primary disorder in its own right [44].

This paper reflects the fast-moving pace in the acquisition of new genomic knowledge and the global challenges in the diagnosis of patients with genetic conditions. This study also highlights the importance of sequencing browsers such as the ExAC browser for providing professionals with aggregate exome sequencing data from a variety of large-scale sequencing projects. For the specific *NOX4* variant in question, new knowledge with regard to its MAF prompted further cascade testing in the proband's grandparents. Although, the patient phenotype can be due to

sporadic form, resulting from complex gene-gene and gene-environment interplay with *NOX4*, given the presence of the homozygous variant in one of each of the grandparents and the high allele frequency of the variant in the apparently healthy population, we must conclude that this variant is unlikely to be of primary pathogenic significance.

However, our functional analyses suggest that the variant in *NOX4* may act as a disease modifier in concert with an as yet unidentified primary disease gene to cause the very severe phenotype in our patient. However, at this stage, the role of *NOX4* as a modifier is not definitive. The variant is not in the canonical isoform, demanding more experiments, including a specific enzyme assay for *NOX4* to examine whether the *NOX4* enzyme activity is compromised. Moreover, as little is known about the function of isoform five, further investigation at the mRNA level would be important to understand the role of this isoform. Last, but not least, in the absence of the primary disease-causing gene in our patient, further bioinformatics re-analysis of the family trio's WES data or implementation of whole genome sequencing and/or RNA-seq is required to identify the causative gene.

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Figure Legends

Figure 1: **a)** Evaluation of protein expression of NOX4 by immunoblotting. Immunoblot showing reduced NOX4 expression in cultured fibroblasts from patient (P) compared to a control (C) in duplicate with a total of 30 µg of protein in each lane. GAPDH was used as a loading control. **b)** Hydrogen peroxide (H₂O₂) levels in fibroblasts. Bars represent the average H₂O₂ level in the patient (P) relative to the control (C) average (mean ± SEM values) (C). Each plot was produced from two independent experiments in triplicate (n=6). Statistical significance of **p = 0.004 is indicated. **c)** Determination of mitochondrial superoxide production measured by flow cytometry using MitoSOX Red. Control 1 (aqua), control 2 (light green) and patient (dark green) fibroblasts treated with Antimycin A. Flow cytometric analysis was performed in triplicate.

Tables

Table 1: *In silico* analyses of the *NOX4* variants identified in this study. MutationTaster indicated that the variant is a polymorphism; phastCons and PhyloP showed low level of conservation. PhastCons values vary between 0 and 1 and reflect the probability that each nucleotide belongs to a conserved element. The closer the value is to 1, the more probable the nucleotide is conserved. In PhyloP, sites predicted to be conserved are assigned positive scores (values between -14 and +6).

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Gene	Exon	cDNA	Protein	MutationTaster	PhyloP	PhastCons	Preliminary MAF dbSNP 1000 Genome	Secondary MAF ExAC	Number of Homozygotes in ExAC	Genotype	Inheritance
NOX4	1	c.7_9ins GAG	p.Glu3dup	Polymorphism	-1.383	0	NA NA	0.5821	2042	Heterozygous	Paternal & Maternal

nucleotide is conserved. In PhyloP, sites predicted to be conserved are assigned positive scores (values between -14 and +6).

- NA: Not Available.

Fig. 1

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Tread carefully: A functional variant in the human NADPH oxidase 4 (NOX4) is not disease causing

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