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SHORT REPORT

Expanding the phenotypic spectrum of *PORCN* variants in two males with syndromic microphthalmia

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Variants in *PORCN* are a cause of Goltz-Gorlin syndrome or Focal Dermal Hypoplasia, an X-linked dominant disorder affecting heterozygous females and until now considered to be embryonic lethal in males. Exome sequencing was performed in a family in which two male siblings were characterized by microphthalmia and additional congenital anomalies including diaphragmatic hernia, spina bifida and cardiac defects. Surprisingly, we identified a maternally inherited variant in *PORCN* present in both males as well as in two female siblings. This represents the first finding of a *PORCN* variant in non-mosaic males affected with Goltz-Gorlin syndrome. The apparently asymptomatic mother showed extreme skewing of X-inactivation (90%), an asymptomatic female sibling showed skewing of 88%, and the second female sibling affected with cutis aplasia of the scalp showed X-inactivation considered within the normal range.
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INTRODUCTION

Microphthalmia occurs with an incidence of 0.9–2.3 per 10 000,[1] and is estimated to occur in association with additional congenital malformations in up to 90% of those patients, with malformations in the musculoskeletal, cardiovascular and central nervous systems being the most common anomalies.[2] A large number of genes are associated with both isolated and syndromic forms of microphthalmia, with 10 specific OMIM entries for syndromic microphthalmia. Microphthalmia in combination with congenital diaphragmatic hernia (CDH) is infrequent and a clinical diagnosis of Matthew-Wood syndrome (MWS) may be suspected (MCOPS9; OMIM #601186). MWS is caused by variants in the *STRA6* gene (OMIM *610745) and is associated with microphthalmia/anophthalmia, CDH, cardiac anomalies and pulmonary defects.[3] The combination of microphthalmia with CDH has also been reported because of deletions or variants in other genes or genomic loci.[4–6] Here, we investigated a familial case of syndromic microphthalmia in association with CDH, spina bifida and cardiac anomalies. To identify the cause, we performed exome sequencing on two affected male siblings and both parents, which identified a variant in the X-linked *PORCN* gene as the underlying cause.

MATERIALS AND METHODS

Clinical details

From the first relationship of the mother (I.2), with partner (I.3), two healthy daughters were born (II.5 and II.6). From her current relationship there were two affected male fetuses (II.1 and II.2) and two healthy daughters (II.3 and II.4), see pedigree, Figure 2. Patient II.1 was born at term pregnancy with microphthalmia and coloboma of the retina, a left-sided posterolateral diaphragmatic hernia, and an atrial septal defect. Birth parameters were in the normal range: weight 3010 g, length 49 cm and occipitofrontal circumference 34 cm. Aside from the microphthalmia, no other dysmorphic facial features were observed. Further examination showed bilateral simian creases. The skin was normal and no other digital or skeletal abnormalities were observed. He was deceased at day 0, with confirmation of the clinical findings by pathology examination. Patient II.2 had multiple anomalies that were detected at 28 weeks of gestation: bilateral microphthalmia with dense intra-ocular tissue, a large thoraco-lumbar spina bifida and hydronephrosis of the left kidney. He was born at 38 weeks of gestation, with normal birth parameters: weight 3040 g, length 54 cm, and occipitofrontal circumference 35 cm. In addition to the prenatally detected anomalies, hypospadias and absence of the first radial ray of the right hand were detected postnatally. At day 0, the spina bifida was closed and a ventriculo-peritoneal drain was placed. Cerebral imaging showed enlarged ventricles, partial agenesis of the corpus callosum and several intracerebral haemorrhages. No visible pigmentation anomalies were present upon clinical examination. The boy deceased after 10 days due to respiratory insufficiency. No post-mortem examination was performed. A potential diagnosis of Matthew-Wood syndrome (MWS; MCOPS9; OMIM #601186) was made, but mutation analysis of the *STRA6* gene was negative for any causal variants in this patient (performed at the Institute for Human Genetics, Erlangen, Nurnberg, Germany).

Chromosomal microarray analysis

Chromosomal microarray analysis was performed using the CytoSure Syndrome Plus 180k array (Oxford Gene Technology,Oxford, UK) for both affected individuals and both parents to exclude any pathogenic CNV(s) as the cause, as previously described.[7]

Exome sequencing

Written, informed consent was provided by the parents before exome sequencing.

Genomic DNA was sheared by sonication, and whole-genome sequencing libraries were prepared using the TruSeq DNA Library Preparation Kit (Illumina, San Diego, CA, USA) in which platform-specific adaptors and unique DNA indexes were ligated. The gel-free method was performed with no size selection of fragments. DNA sequencing libraries were subsequently enriched with the SeqCap EZ Human Exome Library v2.0 (Roche, NimbleGen, Madison, WI, USA), and 2 × 100-bp paired-end reads were generated on a

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HiSeq2000 (Illumina) with four exome-seq samples pooled in a single lane of a sequencing flow-cell. Sheared DNA, whole-genome libraries and enriched exome-seq libraries were validated using DNA-1000 chips on the BioAnalyser (Agilent, Santa Clara, CA, USA), and library concentrations were determined using the dsDNA Broad Range Assay on the Qubit (Invitrogen, Life Technologies Europe B.V., Gent, Belgium).

The paired-end sequence reads were aligned to the human genome (hg19) with the Burrows-Wheeler Aligner (version 0.5.9)[8] using default settings, and the read trimming parameter was set to 15. SAMtools (version 0.1.12a)[9]was used for converting (SAM/BAM), sorting and indexing alignments. The quality metrics for mapping were calculated with Picard tools (version 1.38). Duplicate reads were marked with Picard tools and excluded from downstream analysis. The GATK framework (version 1.0.4974)[10] was used for performing the local realignment, base call recalibration and SNP calling. Indels were called with Dindel (version 1.01)[11] using default parameters. Variants were annotated with ANNOVAR (version 2011),[12] including for dbSNP (dbSNP132), and 1000 Genomes data (release May 2011). Functional predictions for the amino-acid changes according to different models (SIFT, Polyphen2, LRT and MutationTaster) were retrieved from dbNSFP (database of human nonsynonymous SNPs and their functional predictions).[13]

Variant filtering

Variant files annotated by the GATK analysis pipeline were filtered against provided annotations in Excel, and using the web application 'Annotate-it'[14] (http://www.annotate-it.org/). Details of filters applied by each method and the number of variants remaining after filtering are provided as online Supplementary Information Supplementary Tables 1 and 2). Variants of interest were also checked against the Exome Variant Server (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, USA; http://evs.gs.washington.edu/EVS/).

Variant confirmation by classical Sanger sequencing.

Primers were designed using Primer3 software (http://frodo.wi.mit.edu/), PCR products were purified with ExoSAP-IT (GE Healthcare, Little Chalfont, UK) and sequenced using BigDye Terminator v3.1 chemistry (Life Technologies Europe B.V.) on a 3730 DNA Analyzer (Life Technologies Europe B.V.). Sequence traces were aligned to the reference sequence using Sequence Scanner v1.0 software (Applied Biosystems, Life Technologies Europe B.V.) and CLC Main Workbench v6 (CLC Bio, Aarhus, Denmark). Primer sequences used were forward primer 5′-CATGCTGATCTGCTCTCTGC-3′ and reverse primer 5′-CCACTCAGGACCTCACCAGT-3′.

X-inactivation analysis

The methylation status of the X-linked androgen receptor gene was assessed by gene methylation assay, as previously described.[15,16] The resulting products were separated on an ABI 3730 (Applied Biosystems) and peak positions and peak intensity areas were further processed using Excel to calculate the percentage inactivation of both alleles.

Submission of variant details to public database

The *PORCN* variant details and clinical information for individuals I.2, II.1, II.2, II.3, and II.4 have been submitted to the Leiden Open Variation Database v3.0, *PORCN* gene page; http://databases.lovd.nl/shared/genes/PORCN.

RESULTS

Chromosomal microarray analysis of material from both affected fetuses (II.1 and II.2) revealed no pathogenic CNVs. A possible clinical diagnosis of MWS was excluded in the second affected child (II.2) by mutation analysis for the *STRA6* gene by conventional Sanger sequencing, which revealed no pathogenic variants. Given the high likelihood of an autosomal recessive or X-linked mode of inheritance as the cause of the severe phenotypic features, we undertook exome sequencing in the two affected fetuses (II.1 and II.2) and both parents (I.1 and I.2).

Between 52 and 65 million reads were obtained for each of the four samples, with 94.6–96.7% of reads aligned to the reference human genome (hg19). Mean target region coverage of between 43x and 51x was obtained, with 91–92% of target bases having at least 10x coverage.

Variants were filtered on quality parameters, variant type, against the annotated population databases for variants with a MAF $<1\%$, and for functionally damaging predictions. Identification of variants shared by the two affected individuals was aided by the use of 'annotate-it'. Numbers of variants remaining after filtering is provided in Table 1, and the parameters used are provided in Supplementary Tables 1 and 2.

Of 11 compound heterozygote variants shared by both affected individuals, none were predicted to be functionally damaging, and no shared *de novo* variants were identified excluding germline mosaicism as a possible cause. Further examination of rare variants shared by both affected individuals identified a likely pathogenic variant; a hemizygous X-linked non-synonymous variant in the *PORCN* gene (NM_203475.1: ENST00000326194: c.470G>A:p.(Gly157Asp)). Annotate-it provides text-based genotype–phenotype associations (according to HPO and LDDB terms) from 'A Gene Apart' and respective *P*-values for this association. Both anophthalmia (*P*-value = 1.348 E-05) and congenital hernia of diaphragm (*P*-value = 7.655 E-03) are listed in association with the candidate variant in *PORCN*, thus aiding causal variant identification. This novel variant has thus far not been reported in association with Focal Dermal Hypoplasia (FDH)/Goltz-Gorlin syndrome, and has also not been observed in population databases of normal individuals (dbSNP, 1000 genomes or the Exome Variant Server). The *PORCN* p.(Gly157Asp) variant is predicted to be functionally damaging by four algorithms (SIFT, PolyPhen2, LRT and MutationTaster).

Extended familial *PORCN* mutation analysis and X-inactivation studies

Variants in *PORCN* are associated with Goltz-Gorlin syndrome or FDH (OMIM #305600), an X-linked dominant disorder in which heterozygous females are affected. Conventional Sanger sequencing of the *PORCN* gene confirmed the presence of the *PORCN* variant in the mother (I.2) and both affected males (II.1 and II.2). Analysis of the four daughters revealed the same variant in II.3 and II.4, but not in II.5 and II.6. We therefore investigated the X-inactivation status in genomic DNA from the mother (I.2) and the four daughters from

Table 1 Numbers of shared rare variants remaining after filtering

	II.1	*II.2*
% Captured regions, coverage >10x; *Q*>30	92	91
Average coverage of captured region (*x*)	51	43
Total number of SNPs	91 412	82 221
Total number of indels	6851	5937
Rare homozygous variants	0	
Rare compound heterozygous variants	11 (0)	
Rare X-linked variants	4 (2)	
Rare *de novo* variants	0	
Rare inherited variants (maternal)	56 (5)	
Rare inherited variants (paternal)	49 (5)	

Abbreviation: SNP, single-nucleotide polymorphism.
Provides the numbers of rare variants (MAF <1%) remaining after filtering against population databases (1000 genomes and the Exome Variant Server), with the number predicted functionally damaging by all four prediction algorithms within brackets, and listed by potential mechanisms of inheritance.

both relationships (II.3 and II.4; and II.5 and II.6). This revealed extreme skewing of X-inactivation (90%/10%) in the heterozygous mother; and levels of 88%/12% and 70%/30% in the two heterozygous daughters (II.3 and II.4, respectively) with the same maternally inherited X chromosome. All daughters were examined by a clinical geneticist upon these results. One of the heterozygous female carriers, initially considered unaffected (II.4), displayed aplasia cutis on the scalp, shown in Figure 1. Interestingly, the skewing of X-inactivation of 70%/30% observed in this girl is considered within the normal range. No additional skin, ocular or skeletal abnormalities were seen in either of the heterozygous female carriers. The two daughters who do not carry the variant (II.5 and II.6) showed random X-inactivation with levels of 54%/46% and 67%/33%, respectively, and inheritance of the alternative maternal X chromosome. The pedigree is shown in Figure 2, including the results of the X-inactivation analysis.

PORCN sequencing in additional unrelated individuals

All coding exons of the *PORCN* gene were investigated by conventional Sanger sequencing in seven additional unrelated patients with overlapping phenotypic features of eye anomalies in combination with diaphragm or lung defects, or neural tube defects (online Supplementary Information, Supplementary Table 3). However, this did not reveal any pathogenic variants.

DISCUSSION

We describe the first case of non-mosaic males affected with syndromic microphthalmia because of a non-synonymous variant in the *PORCN* gene. The two fetuses were affected with multiple congenital anomalies including microphthalmia, CDH and an atrial septal defect (II.1); and bilateral microphthalmia and spina bifida (II.2). To our knowledge, there are four previous cases reported of *PORCN* variants in association with syndromic CDH: a female fetus with multiple congenital anomalies including CDH, limb anomalies, microphthalmia and lung anomalies; a female with phenotypic features consistent with FDH and Pentalogy of Cantrell, including an anterior diaphragmatic hernia; and two unrelated female fetuses born to affected mothers who displayed ectopia cordis, diaphragmatic hernia and abdominal wall defects.[5,17,18] Our finding thus adds further support for *PORCN* variants as a cause of syndromic CDH and not a coincidental association. The novel variant that we report affects the fourth transmembrane domain of the PORCN (porcupine) protein and the putative amino-acid change is predicted to be functionally damaging by four algorithms (SIFT, PolyPhen2, LRT and MutationTaster). Pathogenic *PORCN* variants have previously been reported that affect the same transmembrane domain, which further supports the pathogenicity of the p.(Gly157Asp) variant.[5,19–21]

Variants in *PORCN* were first reported as a cause of FDH in 2007.[19,22] Froyen *et al* later identified *PORCN* variants and gene deletions in a cohort of patients with a clinical diagnosis of FDH.[23] FDH is characterized by phenotypic features, including longitudinal striation of the long bones, the combination of split hand with syndactyly and absence of rays (also termed 'lobster-claw hand'), as well as atrophy and linear pigmentation of the skin, herniation of fat through dermal defects and multiple papillomas of the mucous membranes or skin. Oral anomalies, in addition to lip papillomas, include hypoplastic teeth. Ocular anomalies are another characteristic feature of FDH, including microphthalmia, coloboma of the iris and choroid, and strabismus. Intellectual disability has also been reported, although in a minority of patients. Recently, a 'mutation update' for *PORCN* reported a number of new cases and reviewed those reported to date.[24]

Interestingly, FDH is mainly observed in heterozygous females, whereas the few reports of *PORCN* variants in affected males are limited to cases of somatic mosaicism. Variants in *PORCN* thus cause an X-linked dominant condition, which, until now, was considered to be embryonic lethal in males. Our finding thus represents the first report of affected non-mosaic males, adding evidence for *PORCN* variants in males as a cause of phenotypic features, including eye anomalies, diaphragm defects, spina bifida, cardiac defects, kidney defects and structural brain anomalies. This is in contrast to some of the characteristic phenotypic features more commonly seen in females affected with Goltz-Gorlin syndrome or FDH, especially the skin anomalies. Of particular, interest is the difference in phenotypic severity seen between the male patients we report and the female sibling with cutis aplasia for whom skewing of X-inactivation of 70%/30% would be considered in the normal range. Possible explanations for this disparity are differing degrees of X-inactivation in different tissues in this female, which although not investigated, but have been reported to occur,[25] combined with the likelihood that the non-synonymous mutation observed encodes a hypomorphic allele with reduced function only and not complete loss of protein function. A possible clinical diagnosis of MWS/Microphthalmia, Syndromic 9

Figure 1 Cutis aplasia of the scalp in female II.4.

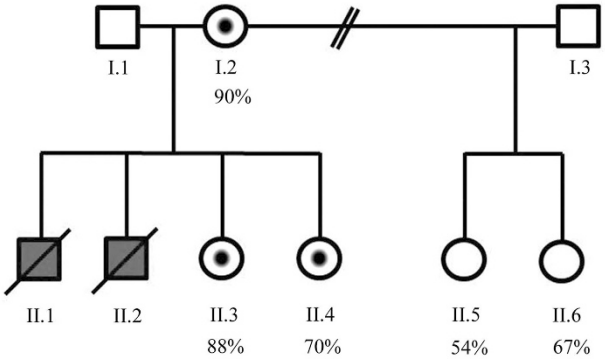

Figure 2 Family pedigree. Individuals I.1, II.2, II.1 and II.2 underwent exome sequencing, which revealed the p.(Gly157Asp) variant in the *PORCN* gene in both affected individuals (II.1 and II.2) as well the asymptomatic mother (I.2) who was shown to have 90% skewing of X-inactivation. Further analysis of additional family members revealed that the two female siblings II.3 and II.4 also carry the variant, with 88% and 70% skewing of X-inactivation, respectively. The two females from the previous relationship (II.5 and II.6) have not inherited the variant and demonstrate 54% and 67% skewing of X-inactivation, respectively. Individual I.3 was not tested.

(MCOPS9; OMIM #601186) in one of our patients because of the phenotypic features of CDH and microphthalmia may suggest that some male fetuses without a genetic diagnosis of MWS owing to variants in *STRA6* may actually harbour variants in *PORCN*. However, we did not identify any damaging *PORCN* variants in a screen of seven additional unrelated patients with eye anomalies in combination with diaphragm or lung abnormalities, or neural tube defects.

PORCN is involved in the trafficking of Wnt proteins between the endoplasmic reticulum and Golgi (reviewed in Clements[26]). Defective PORCN impairs transfer of Wnt proteins through the cell leading to reduced Wnt secretion affecting downstream pathways reliant on Wnt signalling. Wnt signalling is essential for many aspects of embryonic development and this explains the constellation of congenital anomalies in multiple organs. A conditional mouse model of PORCN loss of function was used to study its requirement in Wnt signalling and embryonic development.[27] Consistent with the female-specific inheritance pattern of FDH, *Porcn* hemizygous male murine embryos arrest during early embryogenesis and fail to generate mesoderm, a phenotype previously associated with loss of Wnt activity. Heterozygous *Porcn* mutant murine females exhibit a spectrum of limb, skin and body patterning abnormalities resembling those observed in human patients with FDH. In a conditional mouse model of PORCN, loss of function defects were observed in ectodermal- and mesenchymal-derived structures.[28] It has been proposed that CDH originates from a defect in the mesenchymal cells of the developing pleuroperitoneal folds, and aberrant Wnt signalling provides a plausible mechanism for the association with CDH.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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