Confirmation of *TENM3* involvement in Autosomal Recessive Colobomatous Microphthalmia

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ABSTRACT

Anophthalmia and Microphthalmia are the most severe malformations of the eye, corresponding respectively to an absence and a reduced size of the ocular globe. A wide genetic heterogeneity has been reported for these ocular globe defects and more than 20 genes have been demonstrated to be causative of syndromic and non-syndromic forms of anophthalmia-microphthalmia. In a recent study combining autozygome and exome analysis, a homozygous loss of function mutation in the *TENM3* gene (previously named *ODZ3*) was reported in two sibs with isolated bilateral colobomatous microphthalmia from a consanguineous Saudi family. Herein, we report a third patient (not related to the previously reported family) manifesting bilateral colobomatous microphthalmia and mild developmental delay for whom genetic studies identified a homozygous *TENM3* splicing mutation c.2968-2A>T (p.Val990Cysfs*13). This report confirms the involvement of *TENM3* mutations in colobomatous microphthalmia and expends the phenotypic spectrum associated with these mutations.

Key words: coloboma, microphthalmia, *TENM3*, *ODZ3*, targeted sequencing

INTRODUCTION

Anophthalmia and Microphthalmia (AM) are severe ocular developmental defects. Anophthalmia refers to the complete absence of ocular tissue in the orbit (true anophthalmia) or the absence of ocular tissue on clinical examination (clinical anophthalmia). In the latter, remnants of the optic vesicle may be detected by orbital imaging. Microphthalmia corresponds to a globe with a total axial length that is at least two standard deviations (SD) below the mean for age (< 19 mm in a 12 months-old child, < 21 mm in an adult) [Weiss et al., 1989]. The prevalence of AM has been estimated between 3 and 30 per 100,000 births. An estimated 33-55% of AM is associated with extra-ocular abnormalities and around 25% of patients have an underlying genetic syndrome that is recognizable [Slavotinek 2011]. Learning difficulties are described in about one fifth of patients with AM and/or coloboma and may be higher in patients with AM when excluding isolated coloboma phenotype [Morrison et al., 2002].

The aetiology of AM is diverse and includes both environmental and heritable factors. However, genetic causes are thought to account for the majority of AM and the empirical recurrence risk in siblings without a clear aetiology or family history is estimated to 10%. To date, up to 25-30% of children presenting with AM can achieve a diagnosis, either monogenic or chromosomal [Chassaing et al., 2014; Gerth-Kahlert et al., 2013; Shah et al., 2011]. While mutations in the *SOX2* gene are the major cause of these AM disorders, representing around 10-15% of the individuals with severe AM [Chassaing et al., 2014; Fantes et al., 2003], numerous other genes are involved in only a very small percentage of patients with AM. To date, biallelic *TENM3* mutations have been reported only once, in one consanguineous family, with two sibs affected by isolated colobomatous microphthalmia [Aldahmesh et al., 2012].

In this work, we undertook targeted sequencing of 187 known and candidate AM genes in 96 AM individuals for whom molecular screening of the main AM genes was negative. Among these patients, we identified a patient carrying a biallelic mutation in the *TENM3* gene, thus confirming the role of this gene in ocular developmental defects.

MATERIALS AND METHODS

Studied patients

Patients with AM (44 males and 52 females) were recruited as part of a UK national study of developmental eye anomalies and a French cohort of micro-anophthalmic patients. Informed consent was obtained from all individuals in accordance with Ethics Approval obtained for the study from Cambridgeshire 1 Ethics Committee 04/Q0104/129 (UK patients) and approved by the local Ethics Committee (CPP Sud-Ouest et Outre-Mer II) (French patients). These patients were previously screened for mutation in the main AM genes (*SOX2*, *OTX2*, *RAX* and *VSX2*).

TENM3 mutated patient

The patient is a 9-year-old boy, who was born from healthy consanguineous parents (first degree cousins). He had one healthy brother and one healthy sister. He was delivered at 40 weeks of gestation with normal measurements (length of 50 cm [0 SD], weight of 3.620 kg [+ 0.25 SD], and OFC of 36 cm [+0.75 SD]). At birth, bilateral colobomatous microphthalmia was evident. He had pendular nystagmus and esotropia. Ophthalmic examination showed transparent lenses, bilateral microcornea and coloboma of the iris (Fig.1) and of the optic disc involving the macula. The eyes were myopic (-6 diopters of spherical equivalent on both eyes). He developed a retinal detachment on the left eye and after 8 years of age a retinal detachment of the right eye. He always had poor visual acuity. At 9 years of age, his visual acuity was estimated at hand motion on both eyes. His developmental milestones were delayed. He sat at 8 months and walked at 24

months. His language was delayed. His auditory evoked potentials were normal. He entered a specialized institution at the age of 8 because of his apparent intellectual delay. At 8 years of age, he had a normal head circumference (-0.5 SD) height (+0.5 SD) and weight (+0.3 SD). He had moderately low-set ears, but no other facial anomalies. Cerebral Magnetic Resonance Imaging with spectroscopy, renal ultrasound and transthoracic echocardiography were normal. Direct sequencing of *OTX2*, *PAX6*, *RAX*, *SOX2* and *VSX2* failed to identify any causative mutation.

Targeted sequencing of 187 genes

We have designed a targeted panel of 187 genes known or candidate genes to be involved in ocular development. This panel comprises genes that have already been associated with ocular developmental defects (*ALDH1A3, ATOH7, BCOR, BMP4, BMP7, c12orf57, CHD7, COX7B, FNBP4, FOXC1, FOXE3, GDF6, GJA8, HCCS, MFRP, OTX2, PAX2, PAX6, PITX2, PITX3, RARB, RAX, SIX6, SMOC1, SOX2, STRA6, TENM3, VAX1,* and *VSX2*) as other candidate genes either part of the retinoic acid or SHH pathways or identified through our previous exome sequencing experiments.

600ng of genomic DNA was captured using Agilent SureSelect Target Enrichment System kit designed to capture the 2310 coding exons. Sequence capture, enrichment and elution were performed according to manufacturer's instruction and protocols (SureSelect, Agilent) without modification except for library preparation performed with NEBNext® Ultra kit (New England Biolabs®). Libraries were pooled and sequenced on an Illumina HiSeq 2000 as paired-end 75b reads. The bioinformatics analysis of sequencing data was based on the Illumina pipeline (CASAVA1.8.2). CASAVA performed alignment of a sequencing run to a reference genome (hg19), called the SNPs based on the allele calls and read depth and detected variants (SNPs & Indels). Genotypes were called at all positions with high-quality sequence bases and filtered to

retain SNPs and insertion-deletions with Phred-like quality scores of at least 20. We focused on indel, non-synonymous and canonical splice-site variants with minor allele frequency less than 0.5 % from public datasets (including dbSNP135, HapMap and the 1000Genomes Project) and in-house exome and full-genome data. We used PolyPhen-2 and SIFT software tools to predict the functional effects of mutations.

Sanger confirmation of variants

All variants retained after filtering from targeted sequencing data were confirmed by Sanger sequencing. Parental studies were performed to determine whether these variants were inherited or *de novo*.

Reverse Transcription-PCR (RT-PCR)

Total RNA was extracted from lymphoblastoid cell cultures obtained from the patient and control using QIAmp RNA Blood MiniKit (QIAGEN, Germany) according to the manufacturer's instructions. Then, cDNA was synthesized with the Omniscript RT Kit (QIAGEN, Germany) according to the manufacturer's protocol and subsequently amplified by PCR using primers designed from sequences in exons 16 and 18 of the *TENM3* gene (ENST00000511685). To evaluate splicing of exon 17 of the *TENM3* gene, PCR fragments from agarose gel were purified and subsequently sequenced.

RESULTS

Using a targeted high throughput sequencing of known and AM candidate genes, we identified a new patient bearing a homozygous predicted loss of function mutation in the *TENM3* gene. This patient harbored the *TENM3* homozygous c.2968-2A>T (RefSeq: NM_001080477). This variant was absent from the Exome Variant Server and ExAC databases. Familial segregation studies showed that both parents and his sister were heterozygous for the c.2968-2A>T mutation, while

his brother had two wild-type alleles. RT-PCR analysis demonstrated the deleterious effect of this substitution on splicing, leading to the skipping of the exon 17 of the *TENM3* gene (r.2968_3235del), resulting in a frameshift and the introduction of a premature stop codon at the protein level (p.Val990Cysfs*13) (Fig.2, and Supplementary Fig. S1).

In addition, causative mutations were found in 9 of the remaining 95 patients (2 patients with *BCOR* mutation, two patients with a *PITX3* mutation, and one patient for each of the following genes: *PAX6*, *FOXE3*, *CHD7*, *GJA8*, and *MFRP*). Thus this targeted sequencing allows increasing mutation detection rate by ~10 % (10/96) to the targeted Sanger sequencing strategy previously used for these patients. In addition, some candidate variants are still under investigation.

DISCUSSION

Recent advances in genetics, especially in DNA sequencing, now allow identification and testing of many new genes that contribute to human congenital eye diseases, especially in patients with AM. Such advances in the knowledge of these genetic bases are important as they improve the quality of care, in terms of diagnosis, prognosis and genetic counseling delivered to the patients and their families. However, when a mutation in a new AM gene is reported in only one family, it is challenging to provide the definitive evidence that links the identified genetic mutation with the ocular defect, even if *in vitro* and *in vivo* functional studies are performed. As such, Aldahmesh AM *et al*. identified a homozygous loss of function mutation in the *TENM3* gene in one consanguineous family. Two sibs were homozygous for the c.2083dup (p.Thr695Asnfs*5) [Aldahmesh et al., 2012]. Since only one family was reported before the present report, this gene was classified with an “uncertain likelihood of pathogenic significance” in a recent review [Williamson and FitzPatrick 2014].

We report herein a third patient bearing a homozygous splicing mutation in the *TENM3* gene. Interestingly, there are no homozygotes with nonsense or canonical splice mutations in the ExAC database, thus arguing for the deleterious effect of the homozygous mutation in this gene. The mutation identified in our study leads to exon skipping of exon 17 of the gene and subsequently to a premature stop codon. This stop codon is located more than 50 nucleotides from the last exon-exon junction and is thus predicted to undergo nonsense mediated decay. However, this mechanism should be partial as RT-PCR allows amplification of the mutated mRNA. If the protein is translated, the mutant protein would be lacking a large part of its extracellular domain (located downstream of the EGF-like domain and including the large globular C-terminal domain), presumably affecting its functions, especially in cell to cell adhesion.

Tenm/Odz is a family of four distinct type II transmembrane molecules. These proteins can be divided into at least five functional units: a cytoplasmic part, a transmembrane part, a linker region, a dimerization (EGF) unit, and a large globular COOH terminal domain [Feng et al., 2002]. Genetic studies of the fly Tenm/Odz revealed a crucial role during segmentation. *In vitro* and *in vivo* studies support roles for teneurins in promoting neurite outgrowth and cell adhesion [Young and Leamey 2009]. Furthermore, the intracellular domains of at least two teneurins can undergo proteolytic cleavage and translocate to the nucleus where they regulate transcriptional activity [Young and Leamey 2009]. *Tenm3* is expressed in the developing vertebrate eye, particularly in the optic stalk [Ben-Zur et al., 2000], supporting its role in ocular development. *Tenm3* knockout mice are described as having significantly impaired binocular vision due to an abnormality in mapping ipsilateral projections in the optic pathway [Leamey et al., 2007]. *tenm3* knockdown in zebrafish induces stratification and targeting errors of both dendrites and axons in a subset of Retinal Ganglion Cells (RGCs) suggesting that *tenm3* is involved in wiring subsets of

functionally defined visual circuits [Antinucci et al., 2013]. However, no ocular malformation is observed in both animal models.

In human, the ocular phenotype associated with *TENM3* mutations seems to be consistent, even if only three patients have been described to date. The previously reported affected siblings had isolated bilateral colobomatous microphthalmia with microcornea. The patient reported herein had similar ocular involvement without any other anomalies. However, unlike the two reported sibs, this patient had apparent intellectual disability. This intellectual disability may thus be an inconstant feature associated with *TENM3* mutation. It also may be due to mutations in other gene, hypothesis that is reinforced by parental consanguinity.

In conclusion, we report here a second biallelic *TENM3* mutation leading to colobomatous microphthalmia, thus confirming the role of this gene in ocular defects. Our information regarding *TENM3* gene action during ocular development in human is limited, and further studies are needed to understand the mechanism(s) leading to these ocular developmental defects.

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FIGURE LEGENDS

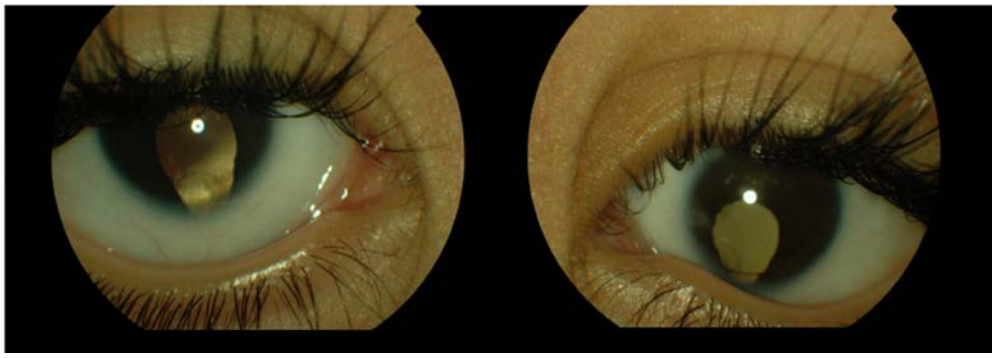


Figure 1: Ocular phenotype of the patient reported in this study. Note the presence of bilateral ocular coloboma affecting the iris. [Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833.]

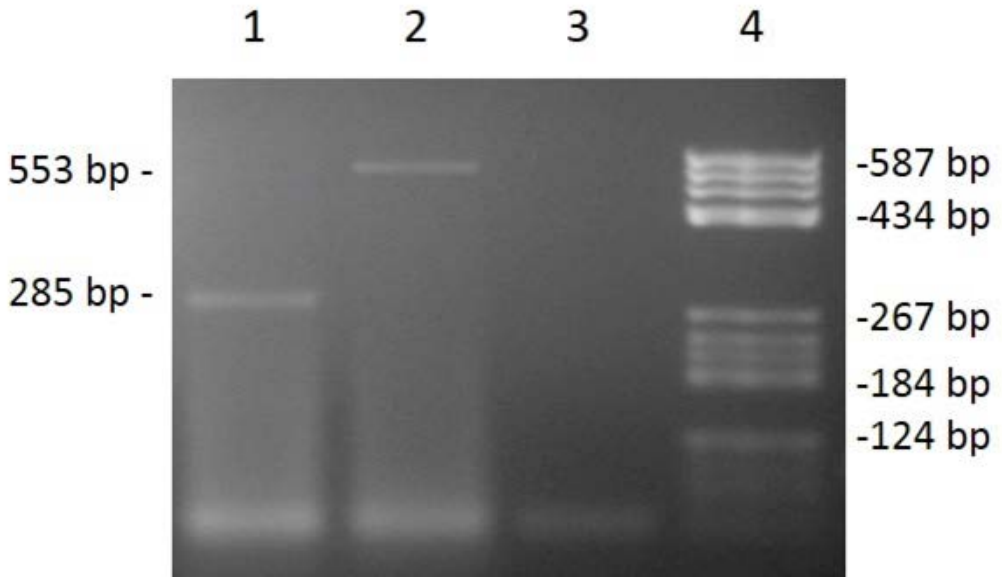
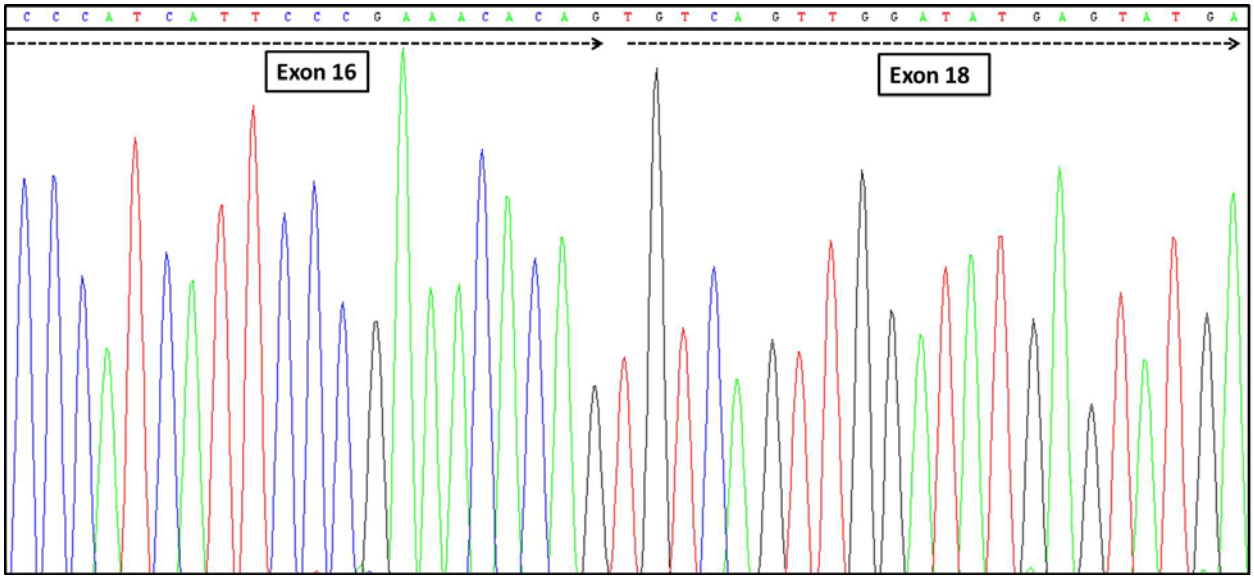


Figure 2: 2% agarose gel picture showing the intronic mutation c.2968-2A>T effect on the *TENM3* pre-mRNA splicing. **Lane 1**: the affected homozygous proband. Note the 285bp amplicon (exon 17 skipping, not in phase) versus the 553bp amplicon (normal splicing) in the healthy control (**Lane 2**). **Lane 3**: negative control. **Lane 4**: pBR322 DNA/BsuRI (HaeIII) DNA ladder (ThermoFisher scientific).



Supplementary Figure S1: Electropherogram of the *TENM3* cDNA sequence in the proband, showing complete deletion of exon 17 from mRNA as a result of disrupted splicing caused by the c.2968-2A>T mutation. [Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4833.]