The utility of massively parallel sequencing for posterior polymorphous corneal dystrophy type 3 molecular diagnosis

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ABSTRACT

The aim of this study was to identify the molecular genetic cause of disease in posterior polymorphous corneal dystrophy (PPCD) probands of diverse origin and to assess the utility of massively parallel sequencing in the detection of ZEB1 mutations. We investigated a total of 12 families (five British, four Czech, one Slovak and two Swiss). Ten novel and two recurrent disease-causing mutations in ZEB1, were identified in probands by Sanger (n=5), exome (n=4) and genome (n=3) sequencing. Sanger sequencing was used to confirm the mutations detected by massively parallel sequencing, and to perform segregation analysis. Genome sequencing revealed that one proband harboured a novel ~0.34 Mb heterozygous de novo deletion spanning exons 1-7 and part of exon 8. Transcript analysis confirmed that the ZEB1 transcript is detectable in blood-derived RNA samples and that the disease-associated variant c.482-2A>G leads to aberrant pre-mRNA splicing. De novo mutations, which are a feature of PPCD3, were found in the current study with an incidence rate of at least 16.6%. In general, massively parallel sequencing is a time-efficient way to detect PPCD3-associated mutations and, importantly, genome sequencing enables the identification of full or partial heterozygous ZEB1 deletions that can evade detection by both Sanger and exome sequencing. These findings contribute to our understanding of PPCD3, for which currently, 49 pathogenic variants have been identified, all of which are predicted to be null alleles.

Key words: ZEB1; posterior polymorphous corneal dystrophy type 3; massively parallel sequencing; exome; genome; aberrant splicing; breakpoint mapping
1. INTRODUCTION

Posterior polymorphous corneal dystrophy (PPCD) is a genetically heterogeneous, autosomal dominant disorder, characterised by vesicular lesions, bands and opacities at the level of Descemet membrane and the corneal endothelium (Cibis et al., 1977). Decreased visual acuity can result from amblyopia, irregular astigmatism from corneal steepening, secondary glaucoma and/or corneal edema (Krachmer, 1985; Liskova et al., 2010; Liskova et al., 2013). To date, variants within three genes **ZEB1**, **OVOL2** and **GRHL2** have been identified to be disease-causing (Krafchak et al., 2005; Davidson et al., 2016; Le et al., 2016; Liskova et al., 2018).

Mutations in the zinc finger E-box binding homeobox 1 (**ZEB1**) cause PPCD type 3 (PPCD3; OMIM #609141) (Krafchak et al., 2005). In addition to ocular features, it has been reported that patients with PPCD3 have an increased incidence of hernia and hydrocele (Krafchak et al., 2005; Liskova et al., 2013). Three cases with agenesis or hypoplasia of the corpus callosum have also been documented (Jang et al., 2014; Chaudhry et al., 2017). Individuals with PPCD3 can be asymptomatic, and there are rare reports of incomplete penetrance (Krafchak et al., 2005; Liskova et al., 2013).

**ZEB1** encodes a zinc finger transcription factor. The protein plays a role in epithelial-mesenchymal transition (EMT) by activating the transcription of mesenchymal genes while repressing epithelial genes (Park et al., 2008). EMT is important for embryonic development, wound healing, fibrosis and cancer progression (Chen et al., 2017). Importantly, it is hypothesised that dysregulation of this pathway underpins the pathophysiology of PPCD (Davidson et al., 2016; Liskova et al., 2018). PPCD3 is attributed to ZEB1 haploinsufficiency, (Liskova et al., 2016; Chung et al., 2017) and the reduced levels of ZEB1 induce an altered cellular response to apoptotic stimuli and cell barrier function (Zakharevich et al., 2017).

In this study, we demonstrate the utility of massively parallel sequencing for the identification of **ZEB1** mutations. Furthermore, we establish that it is possible to determine the effects of **ZEB1** variants on pre-mRNA splicing using blood-derived RNA. Finally, we present a
comprehensive summary of \textit{ZEB1} mutations that have been reported to cause PPCD3 to date.

2. METHODS

2.1. Participants, clinical examination and samples

The study was approved by the Ethics committee of the General University Hospital in Prague (reference no. 151/11 S-IV) or Moorfields Eye Hospital (REC references 13/LO/1084 and 09/H0724/25) and adhered to the tenets of the Helsinki Declaration. All participants signed informed consent. Twelve families were investigated; four white Czech (C31, C32, C34, C36), four white British (B9, B10, B11 and B12), one South Asian British (B13), one white Slovak (SK1) and two white Swiss (SW1, SW2). A diagnosis of PPCD was based on established clinical criteria (Cibis et al., 1977; Liskova et al., 2010; Liskova et al., 2013). Best corrected visual acuity (BCVA) was measured using Snellen charts and converted to decimal values. Participating individuals were asked to provide information on their general health status. Genomic DNA from probands and any additional available family members was extracted from venous blood using a Gentra Puregene blood kit (Qiagen, Hilden, Germany) or from saliva using an Oragene kit (Oragene OG-300, DNA Genotek, Canada). In one affected individual total RNA was isolated from venous blood using a QIAamp RNA Blood Mini Kit (Qiagen), transcribed into cDNA with the SuperScript III Reverse Transcriptase (Thermo Fisher, Waltham, Massachusetts).

2.2. Sanger sequencing

We first excluded the presence of pathogenic variants in regulatory regions of \textit{OVOL2} and \textit{GRHL2} in all probands by conventional Sanger sequencing (Davidson et al., 2016; Liskova et al., 2018). In probands from families C31, C32, C34 and B9-B11 (Figure 1) the entire \textit{ZEB1} coding region and flanking intronic sequences (NM_030751.5, NG_017048.1) were
amplified by PCR and directly sequenced (Evans et al., 2015). Sequence variants were described according to the Human Genome Variation Society guidelines (http://varnomen.hgvs.org/) (den Dunnen et al., 2016). Direct sequencing was also used to confirm the likely disease-causing variants found by massively parallel sequencing which included a large deletion. Furthermore, direct sequencing was also used for segregation analysis within the families.

2.3. Massively parallel sequencing

DNA from the probands from families C31, B12, B13, SW1 and SW2 (Figure 1) were subjected to exome sequencing. Sequencing libraries were generated using a SureSelect Human All Exome V6 capture kit (Agilent, Santa Clara, California). Libraries were sequenced on HiSeq4000 sequencer (Illumina). Probands from families C31, C36 and SK1 (Figure 1-3) were analysed by genome sequencing using a TruSeq Nano DNA library preparation kit and a HiSeq X Ten sequencer (Illumina, San Diego, California). Reads were aligned to the GRCh37/hg19 human reference sequence with NovoAlign V3.02.08 (Novocraft, Malaysia). Variant calling was performed with Genome Analysis Tool Kit (GATK) HaplotypeCaller (version 4.0.1.2) (McKenna et al., 2010). Variants were annotated using the Variant Effect Predictor (McLaren et al., 2016).

Both coding and non-coding rare variants with a minor allele frequency (MAF) ≤ 0.005 as per gnomAD (Lek et al., 2016) in genes known to be implicated in the pathogenesis of corneal endothelial dystrophies (ZEB1, GRHL2, OVOL2, COL8A2, SLC4A11, TCF4) (Biswas et al., 2001; Krafchak et al., 2005; Vithana et al., 2006; Baratz et a., 2010; Davidson et al., 2016; Le et al., 2016; Liskova et al., 2018) were further investigated for potential pathogenicity. The frequency of detected variants was also determined in 2,430 exomes generated from Czech individuals available through projects of the National Centre for Medical Genomics (http://ncmg.cz/en). Larger structural variations were compared to entries in the Database of Genomic Variants (MacDonald et al., 2014). The exome and genome sequencing data,
aligned to the human reference GRCh37/hg19, were visualized with the Integrated Genomics Viewer (IGV) (Broad institute, California, USA) (Thorvaldsdottir et al., 2013).

In one patient we did not detect a pathogenic variant and we therefore manually searched aligned genome sequencing reads for stretches of homozygosity around the ZEB1 locus indicative of a heterozygous deletion. Importantly, we used the soft clip function of the IGV software to visualise the likely breakpoints. Primers were designed at each end (forward 5´-CTTTCTGCTGAGGCCATTTC-3´ and reverse 5´-TGGGTGACTAGAGCCAGACC-3´ primer) and the PCR product was Sanger sequenced to confirm the breakpoints. Control data on copy number variations in the ZEB1 genomic region were mined from the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home; accessed December 2018).

A schematic diagram of the pipeline used to process genomic DNA samples in our study is shown in Supplementary Figure S1.

2.4. Paternity testing

Paternity testing was performed in families C31 and C32 to prove de novo origin of the detected mutations using an established set of 16 forensic markers (Ensenberger et al., 2010).

2.5. Functional assessment of pre-mRNA splicing

To assess the effect of a selected variant on pre-mRNA splicing, cDNA of an affected individual II:2 from family C36 was amplified and analysed by direct sequencing, spanning a region of ZEB1 exons 4-7 using forward 5´-CTGAGGCACCTGAAGAGGAC-3´ and reverse 5´-TTGCAGTTTGGCATTCA-3´ primer.

2.6. Review of mutations associated with PPCD3

We performed a comprehensive and manually curated review of all publicly available literature reporting ZEB1 PPCD-associated variants. All variants listed in this article are
annotated in accordance with transcript NM_030751.5 and GRCh37/hg19 human genome assembly.

3. RESULTS
The clinical, demographic and sequencing data of 19 affected individuals from 12 families with PPCD3 are summarized in Table 1.
Slit-lamp examination showed characteristic bilateral corneal signs in all affected subjects. Only 9 eyes of 5 individuals (26.3%) (mean age 27±11.9; range 8-40) had a BCVA of 1.0, while moderate or severe visual impairment (i.e., BCVA <0.33) was present in 10 eyes of 7 individuals (36.8%) (mean age 39±16.5, range 20-59). Two individuals had nystagmus, including subject II:2 from family C34 who was noted to have cloudy corneas from 6 weeks of age. Four individuals (21%) had a keratoplasty in at least one eye. None of the participants in this study had been diagnosed with glaucoma. Two individuals out of 19 reported that they had undergone surgery for hernia and one for hydrocele. None of the probands had signs of cognitive deficiency consistent with agenesis or hypoplasia of corpus callosum (Jang et al., 2014; Chaudhry et al., 2017).
In total, we identified ten novel heterozygous variants in ZEB1 that were evaluated as pathogenic: c.454del, c.482-2A>G, c.646G>T, c.1045G>T, c.1498_1501del, c.1669C>T, c.1918C>T, c.2577del, c.2650C>T (Figure 1, 2) and a large ~0.34 Mb deletion (Figure 3). Two known ZEB1 mutations were also found; c.689_690del and c.1576dup (Figure 1). In concordance with previous observations (Krafchak et al., 2005; Evans et al., 2015; Liskova et al., 2016) all affected individuals had a heterozygous loss-of-function allele. The c.1576dup mutation was observed in an individual who also had a unique c.469C>G; p.(His157Asp) variant, predicted to be benign by four out of the five software tools we used (Supplementary Table 1). Unfortunately, familial samples were not able available to determine the phase of these variants in the affected proband. In samples analysed by massively parallel
sequencing no potentially disease-associated variants were found in other genes known to
be associated with corneal endothelial dystrophies.

Of note, the proband from family C31 with sporadic disease underwent screening by Sanger
sequencing, followed by exome sequencing and genome sequencing. Visualising the aligned
genome sequencing reads in IGV using a soft clip function enabled us to identify likely
deletion breakpoints (Supplementary Figure S2). Sanger sequencing across the region
confirmed the presence of the deletion encompassing \textit{ZEB1} exons 1-7 and part of exon 8
(chr10:hg19:g.31,476,838_31,812,958del) (Figure 3).

In samples that underwent exome sequencing, the sequencing depth of the \textit{ZEB1} gene
(NM_030751.5) exceeded 20x in at least 97.7% of the coding region across all samples
analysed (n=5). In samples subjected to genome sequencing, the sequencing depth of the
entire \textit{ZEB1} region (NG_017048.1) exceeded 20x in at least 97.9% of the region across all
samples analysed (n=3).

In family C36, analysis of the \textit{ZEB1} transcript in the blood of an individual who had a splice
site variant, c.482-2A>G, demonstrated that the mutation alters pre-mRNA splicing, resulting
in exon 5 skipping and insertion of a premature termination codon, described as
r.482_684del, p.Gly161Glufs*9 (Figure 2).

None of the novel pathogenic mutations located in \textit{ZEB1} coding region or the unique variant
p.(His157Asp) were found in gnomAD, Database of Genomic Variants or in data specific to
the Czech population. Segregation analysis within the families also supported their
pathogenicity (Figure 1-3). As parents in families C31 and C32 were clinically unaffected and
did not carry \textit{ZEB1} mutations detected in their children, we suspected \textit{de novo} origin of the
mutations, which was subsequently confirmed by paternity testing (Figure 1,3).

A schematic representation of all PPCD3-associated \textit{ZEB1} variants that have been reported
to date, including this study, is provided in Figure 4. In total, 49 mutations have been
identified in 54 families of various ethnic backgrounds. All are predicted to be null alleles and
hence support the hypothesis that PPCD3 arises due to \textit{ZEB1} haploinsufficiency. Further
details on the identified mutations are shown in Supplementary Table 2.
4. DISCUSSION

In this study we report ten novel and two recurrent mutations in ZEB1, as well as the first genetic study of PPCD3 patients from Slovakia and Switzerland. Importantly, we show that massively parallel sequencing is a useful tool, particularly genome sequencing, to genetically diagnose the condition. We also demonstrate for the first time that it is possible to experimentally determine the effect of ZEB1 variants on pre-mRNA splicing using whole-blood derived RNA. In addition, we put our work in context by providing a comprehensive review of all disease-associated ZEB1 variants reported to date.

All ZEB1 reported pathogenic variants either introduce premature termination codons or comprise large deletions predicted to completely disrupt the gene transcription (Krafchak et al., 2005; Aldave et al., 2007; Liskova et al., 2007; Vincent et al., 2009; Liskova et al., 2010; Nguyen et al., 2010; Bakhtiare et al., 2013; Liskova et al., 2013; Lechner et al., 2013; Jang et al., 2014; Evans et al., 2015; Liskova et al., 2016). Although it has been anticipated that aberrant ZEB1 transcripts are likely eliminated by the mRNA nonsense mediated decay surveillance pathway, cellular studies have shown that ZEB1 disease-causing variants may, in addition to the reduced protein production, also result in impaired nuclear localization of the encoded protein (Chung et al., 2014).

Of the 49 ZEB1 pathogenic variants that have been described, five (10%) were large deletions (Liskova et al., 2016; Chaudhry et al., 2017). Their size varies considerably (0.071-3.3 Mb), and importantly they can evade detection by exome sequencing, qPCR or SNP arrays, depending on primer or SNP locations. Genome sequencing overcomes these limitations. In proband from family 31 it has allowed us to detect the fifth unique deletion in association with PPCD3 and to precisely visualize and map its breakpoints. On this basis we decided to use genome sequencing as the first line approach in two other probands.

Massively parallel sequencing also enabled us to screen multiple genes associated with corneal endothelial disease, which could be useful in determining the genetic cause in cases of bilateral corneal edema of uncertain aetiology (Bakhtiari et al., 2013; Evans et al., 2015).
We also conducted transcript analysis using blood-derived RNA to show for the first time that a novel splicing mutation c.482-2A>G causes aberrant pre-mRNA splicing.

There have only been two recurrent ZEB1 mutations reported. In this study we have documented the de novo origin of c.689_690del; p.(His230Arg*7), previously found in an American family (Bakhtiari et al., 2013). We have also identified c.1576dup; p.(Val526Glyfs*3) in a patient of South Asian ethnicity, previously reported in three families from North America (Krafchak et al., 2005; Bakhtiari et al., 2013; Lechner et al, 2013) and one British family (Evans et al., 2015). Interestingly, this patient (B13-II:1) also harboured an additional unique missense variant in ZEB1 p.(His157Asp). Unfortunately, we were not able to determine the phase of this variant with respect to c.1576dup to assess its contribution to the phenotype. However, in silico analysis suggests that it is likely benign and, if not, we hypothesise that it is located on the same allele as the loss-of-function variant given that bi-allelic loss-of-function ZEB1 alleles are embryonic lethal (Takagi et al., 1998; Liu et al., 2008).

The de novo mutation rate amongst PPCD3 probands is unknown, partly because individuals with PPCD3 can be asymptomatic, and some subjects even show incomplete penetrance for the disease, and thus a reported family history can be unreliable (Krafchak et al., 2005; Liskova et al., 2007; Liskova et al., 2010; Bakhtiari et al., 2013; Evans et al., 2015). Herein, we identified de novo mutations in two of 12 families studied, resulting in an incidence rate of 16.6% in this study. However, this figure may be an underestimate because although there was no family history reported in families B10-B12 and SW2, the parents of the probands were unavailable for examination or testing.

ZEB1 null alleles are found at an extremely low frequency in large-scale public databases such as gnomAD and the Database of Genomic Variants. As they lack individual-level data without details of ophthalmic examination, the presence of such variants in these datasets does not exclude their potential pathogenic role (Cooper et al., 2011; Coe et al., 2014).

In summary, given that ZEB1 deletions of various sizes as well as non-coding pathogenic variants in GRHL2 and OVOL2 are present in a significant number of PPCD cases, we
consider genome sequencing to be a time and cost-effective approach to identify the molecular genetic cause of PPCD.
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Figure 1: Disease-causing ZEB1 point mutations and small heterozygous deletions identified in ten families. Affected individuals are shown as black symbols. Individuals who tested positive for a particular mutation in each family are labelled with +/- and those who were negative with -/- . The proband from family C32 harbour a known mutation c.689_690del which arose de novo ; three affected individuals from family C34 carried a novel mutation c.646G>T; in family SK1 three individuals had novel mutation c.1045G>T; in family B9 two affected individuals had a novel mutation c.2577del and in families B10, B11 and B12 the probands were found to each harbour a different novel mutation c.1918C>T, c.1669C>T and c.2650C>T, respectively. The proband from family B13 carried a recurrent mutation c.1576dup. Two Swiss families SW1 and SW2 carried novel c.1498_1501del and c.454del, respectively.

Figure 2: Assessment of the c.482-2A>G ZEB1 variant on pre-mRNA splicing in family C36. Pedigree (A), a novel heterozygous mutation located in a canonical splice site (B). PCR of blood derived cDNA (C) from a control individual (line 1 marked as C) and the patient sample (Line 2, marked as P) showing two different products. The smaller fragment was found to represent skipping of exon 5 (D) due to aberrant pre-mRNA splicing (r.482_684del) (NM_030751.5, NG_017048.1). Direct sequencing of the PCR amplified products confirming expected splicing patterns in the control sample (E) and one wild type and one aberrant transcript resulting in exon 5 skipping in the patient sample (F).

Figure 3: A schematic representation of the de novo partial heterozygous ZEB1 deletion identified in family C31. The breakpoints of the deletion were identified, and the corresponding sequence chromatogram is shown.

Figure 4: Schematic diagram of the ZEB1 gene showing 49 heterozygous pathogenic mutations identified to date in patients with posterior polymorphous corneal dystrophy type 3. Larger deletions are shown as a bar with coordinates of the deleted region (GRCh37/hg19). Mutations identified in the current study are highlighted in red, novel variants are underlined. Description of coding and splicing mutations are based on reference sequence NM_030751.5, NG_017048.1 and numbering starts at the translation initiation site.