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Mutation screening of *TP63* related target genes*PERP* and *DSP* in Bladder ExstrophyEpispadias Complex.

Istiak Mahfuz[1,*], Stefan White[2], Wei Cheng[3]
[1]*Department of Zoology, Universityof Rajshahi, Rajshahi 6205, Bangladesh.*
[2]*White-Fox Science Consulting, New Zealand*
[3]*Chiron Healthcare Limited, Hong Kong SAR*
**Corresponding author*

Abstract
Background:*Bladder exstrophyepispadias complex (BEEC) is a rare and complex birth defect which involves the urinary, reproductive and intestinal tracts, as well as the musculoskeletal system. We have previously shown that TP63 is a candidate gene for BEEC, and thatexpression of the TP63 isoform ΔNP63 is reduced in BEEC patients. We have also identified ΔNP63 promoter in/del polymorphismsthat are associated with increased risk of BEEC. We hypothesisedthat the TP63>PERP>DSP pathway may be involved in BEEC.We analysedthe coding exons ofPERP and DSP to identify sequence variants that may contribute to BEEC pathogenesis.*
Methods:*Bioinformatic analysis was performed to design PCR primers to amplify PERP and DSP exons. For sequence variation detection, high resolution melting and Sanger sequencing were conducted.*
Results: *Screening of PERP and DSP coding exons in DNA samples from 22 BEEC patients did not identify any novel sequence variants. However, we did identify common variants in PERP exon 3 and DSP exons 1, 20, 23 and 24, validating our screening approach.*
Conclusions: *PERP and DSP play a vital role during development and embryogenesis. Although screening the coding sequence of these genes did not reveal any novel variants, but a role for these genesin BEEC pathogenesis cannot be excluded.*
Keywords: *Bladder Exstrophy, PERP, DSP, Development, Pathogenesis*

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I. Background

Bladder exstrophyepispadias complex (BEEC) is one of the most debilitating groups of congenital anomalies, and representsa major challenge in paediatric surgery and urology [1]. BEEC manifests as a cluster of ventral midline defects that exist in different phenotypes including exomphalos, epispadias, classic bladder exstrophy (CBE), and/or cloacal exstrophy (CE) [2]. Detailed information on different phenotypes of BEEC and environmental risk factors are limited or inconclusive [3].The prevalence of BEEC spectrum at birth is reported at 1/10,000, ranging from 1/30,000 for CBE to 1/200,000 for CE, with an overall greater proportion of affected males[4], with an overall greater proportion of males affected. Without treatment, affected individuals continue to suffer from lifelong physiological and psychological problems including incontinence, genital disfigurement, sexual dysfunction, renal impairment, depression and low self-esteem [1].

Although, the pathogenesis of BEEC is still unclear, but in comparison with general population the high incidence of BEEC among siblings, offspring and identical twins suggests involvement of one or more genetic components.Further evidence of genetic involvement comes from twin studies, with monozygotic twins significantly more prone (62%) to BEEC than dizygotic twins (11%) [3,5-7]. Based on our previously published murine *p63*$^{-/-}$ knock-out model of BEEC (the only genetic model of the condition), *TP63* has been considered as a BEEC candidate gene [8]. *TP63*exist in two major isoforms; the pro-apoptotic *TAP63* and anti-apoptotic *ΔNP63*. We have previously reported that *ΔNP63* is predominantly expressed in normal murine and human ventral bladder urothelium[8-9].Expression analysis of BEEC tissues shows down-regulation of *ΔNP63,* and up-regulation of *TAP63*[9-10]. However, sequence analysis of *TP63* exonsdid not reveal any variants that may contribute to human BEEC[10]. Recently we found that *ΔNP63* promoter in/del polymorphismsare associated with a significantlyincreased risk of human BEEC [9].However, we failed to identify any significantgenetic variation in *TAP63* promoter region that could contribute to the risk of BEEC pathogenesis [11].

A Genome Wide Expression Profiling (GWEP) study has been carried out in human exstrophic and normal bladder tissues [12]. This studyidentified a number of BEEC pathogenesis-related genes,mainly associated with desmosome (a protein family required for cell-cell adhesion)structure/function and/or cytoskeletal assembly.This study also noted that the *P53* effector related to *PMP22* (*PERP*) and Desmoplakin (*DSP*) may be

consideredas promising BEEC candidate genes. Among the desmosomal proteins, *DSP* is ubiquitously expressed[13-14], and has an important role in early embryogenesis [15]. Like *p63*, *Perp*and *Dsp*are expressed in stratified epithelia during embryogenesis [16-17].*Perp*$^{-/-}$ null mice die of dehydration shortly after birth through blistering in the stratified epithelium [16],similar to *p63*$^{-/-}$ null mice[18]. On the other hand,*DSP*mutations can lead to epidermolysis bullosa[19], a blistering skin condition that has previously been reported in BEEC patients [20]. The role of *Perp*and *Dsp*in *p63* regulated development is to maintain epithelial integrity. We have previously speculated that disruption of the *P63>PERP>DSP* pathway may result in a loss of epithelial integrity during bladder development, and eventually lead to BEEC [21]. This study screenedthe coding sequence of *PERP* and *DSP*in a cohort of BEEC patients.

II. Materials And Methods

DNA Samples

Buccal DNA samples of BEEC patient were collected from India, Bangladesh, China, Australia, Spain, Canada and USA. All samples were obtained with informed consent from the parents or guardians and ethics approval from the respective institutions(Australian samples, Royal Hospital for Children, Melbourne, Number# HREC28140A). DNA isolation from buccal swabs was carried out using the BuccalAMP™DNA Storage and Extraction kit obtained from Epicentre® as per the manufacturer's protocol. The DNA quantity and quality of each sample was determined using a Nanodrop® ND-1000 spectrophotometer.

Primer Design

Exon specific primers were designed using Light Scanner Primer Design Software (Version 1.0.r.84) and Primer3 web tool (available at http://bioinfo.ut.ee/primer3/). An*insilico* PCR (available at http://genome.ucsc.edu/cgi-bin/hgPcr?command=start) was performed to confirm the specificity for each designed primer pair. Primers are listed in Table S1. For *PERP*,3primer pairs were designed for each of the3exons, and for *DSP* a total of 30 primer pairs were designed for 24 exons(Figure 1). All primer pairs were optimized using a Labnet multi-gene PCR Machine (Model: Multigene Gradient, Catalogue number TC 9600-G-230v).

High Resolution Melting (HRM)

The majority of the *PERP* and *DSP* exons were screened using HRM analysis.HRM Curve prediction with designed primers were performed using uMeltsoftware [22]. HRM was performed on DNA samples as previously described [23] using a LightScanner HR96 machine with a melting range of 70–98 °C. Curve analysis was performed using the LightScanner HRM analysis software (Version2.0.0.1331). Normalized and different curves were generated by plotting time ('x' axis) and fluorescence ('y' axis), andused to identify potential sequence variants. A baseline was established from the most common curveson a difference plot,allowingaberrant curves to be determined and analysed for sequence variation. Gel Electrophoresis was conducted to check the amplification quality. The samples were then purified using ExoSAP-IT reagent following manufacturer's protocol (USB Corporation) to remove unused primers and enzyme, followed by Sanger sequencing.

Sanger Sequencing

PCR reactions were performed with HotStart-IT Taq Master Mix (2X) in some exons of *PERP* and *DSP*, as a high GC content renderedthe sequences unsuitable for HRM analysis. Reaction conditionsfollowed theHotStart-IT Taq Master Mix (2X) Protocol by USB corporation. The quality and quantity of amplified products were checked using gel electrophoresis.

Amplified samples were purified using ExoSAP-IT reagent following manufacturer's protocol (USB Corporation). Potential sequence variants were confirmed through Sanger sequencing on an Applied Biosystems 3130xl Genetic Analyzer at the Gandel Charitable Trust Sequencing Centre of Monash Health Translation Precinct, Melbourne, Australia. Sequence chromatograph trace files were viewed using the Sequence Scanner from Applied Biosystems. SNP identification was done visually, and sequences were aligned to the human genome (hg19) using NCBI-BLAST [24] and UCSC-BLAT[25].

III. Results

PERP and *DSP*exons were screened for possible sequence variants in 22BEEC buccal swab DNA samples (Table 1). No novel sequence variants were identified in both gene that could contribute to BEEC pathogenesis. However, two common SNPs (*rs648396* and *rs648802*) in *PERP* exon 3 were identified in 13 patients. Sequencing of *DSP* exons 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21 and 22 did not identify any sequence variants. *DSP* exon 1 showed a common insertion SNP (*rs17133512*) in 9 patients). We also found common SNPs in exon 20 (*rs2064217* in 4 patients), exon 23 (*rs28763969* in 2 patients) and exon 24 (*rs2744380* in 5 patients and *rs11558731* in 8 patients). Figure 2 and 3 show HRM difference curves and a sequence trace of the common synonymous variant (*rs2806234*) in *DSP* exon 20.

Table 1. Common SNPs identified during the screening of *PERP* and *DSP* exons.

Exons	Screening Technique	Variants
PERP Exon 3	Sanger	*rs648396, rs648802*
DSP Exon 1	Sanger	*rs17133512*
DSP Exon 20	HRM	*rs2064217*
DSP Exon 23	Sanger	*rs28763969*
DSP Exon 24	Sanger	*rs2744380, rs11558731*

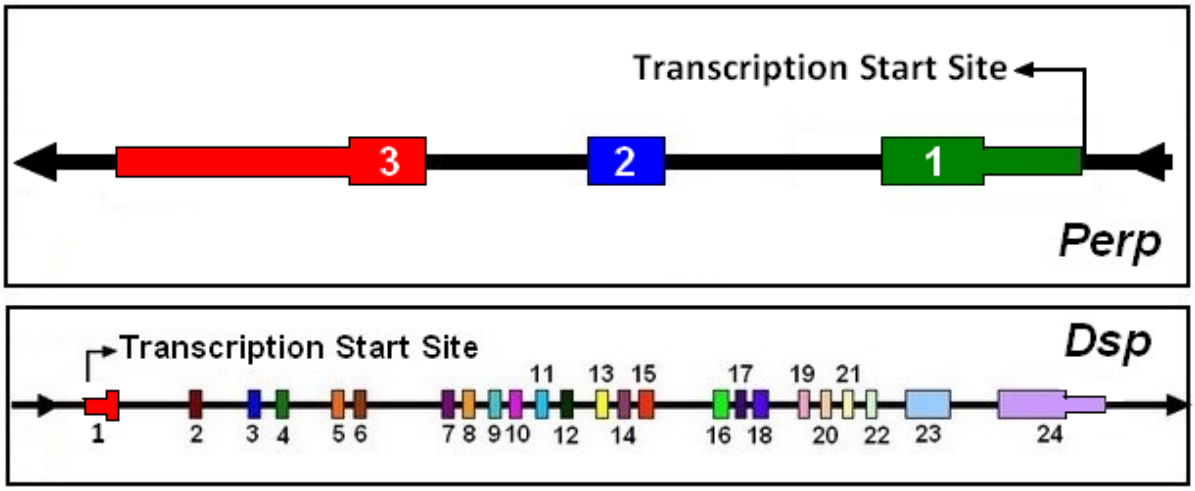

Figure 1. Schematic diagram of the *PERP* and *DSP* genes, showing the different exons.

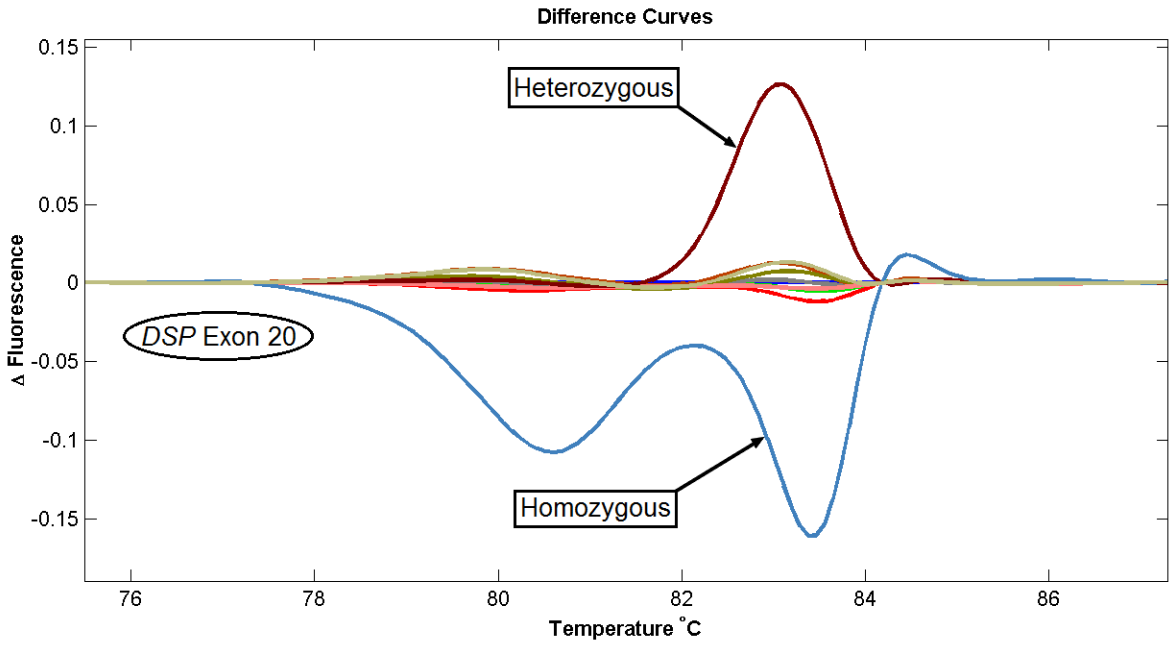

Figure 2. HRM analysis, showing a common synonymous heterozygous and homozygous variant (rs2064217) found in *DSP* exon 20.

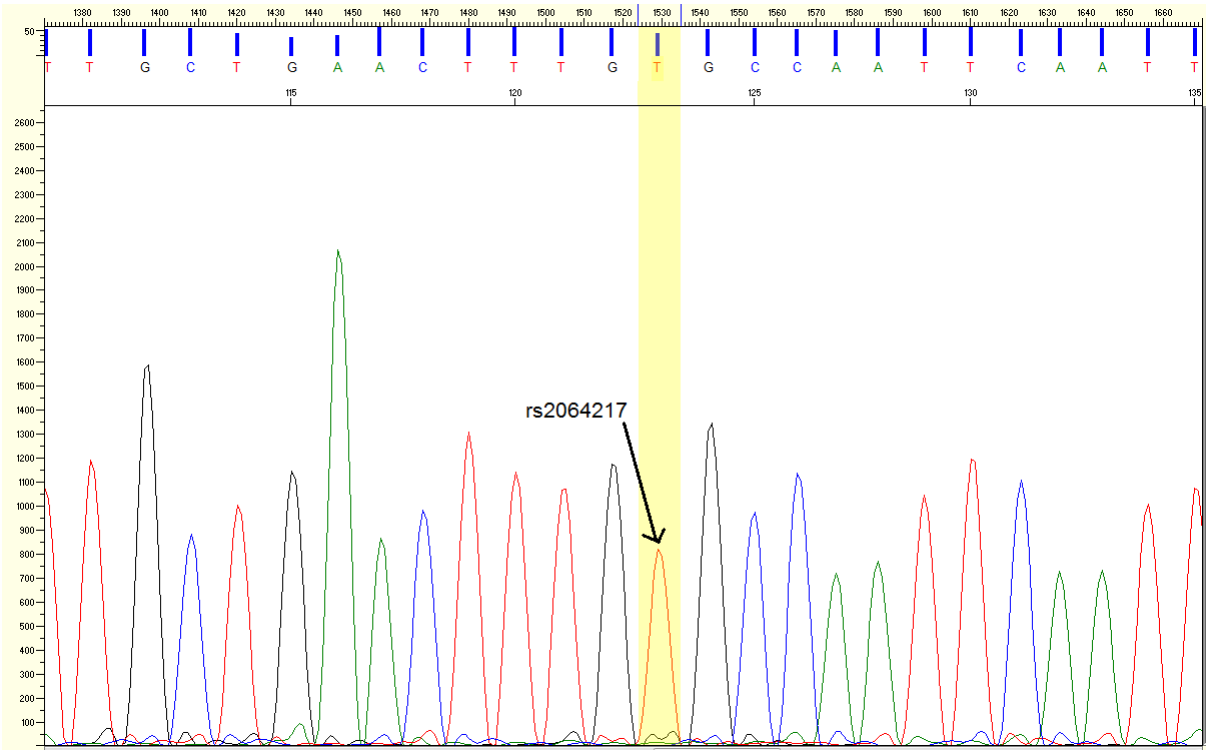

Figure 3: Sequence trace of *DSP* exon 20, with the common synonymous homozygous variant (rs2064217) indicated by an arrow.

IV. Discussion

PERP is a promising candidate gene for BEEC pathogenesis

PERP has a critical role in epithelial stratification,as well as in cell-cell adhesion during development [16,26]. A previous report showed that *Perp* is a direct downstream target of the BEEC candidate gene *Tp63*[26]. In this study, we did not find any potential sequence variants in the coding region of *PERP*, but it is possible that intron 1 of this gene may have some valuable information on the disease phenotype. Ihrie and colleagues showed that *TAp63* and *ΔNp63* both transactivate the *Perp* reporter construct to a similar level, and this function is mediated largely through the p53/p63 consensus element in intron 1. Intron 1 is the major p53-responsive site in *Perp*[16], anda complete analysis of the intronic sequences may reveal non-coding variants that regulate *Perp* expression.

As *PERP* is a *TP63* target gene, it is possible that itsfunction is impaired in BEEC patients. As described by Beaudry and colleagues, *PERP* induction is compromised in some patients with Ankyloblepharon Ectodermal Dysplasia and Cleft Lip/Palate (AEC) [27-28], which is also an associated anomaly of BEEC [29-30]. Interestingly, AEC is caused by mutations in the *TP63*, specifically in the Sterile Alpha Motif (SAM) domain. A role for *PERP* in BEEC is further supportedby the findings of Qi and colleagues, where they found that *PERP* is overexpressed in the bladder tissue of BEEC cases compared to unaffected bladder[12].

Possible involvement of other desmosomal proteins in BEEC

Other desmosomal proteins like Desmoglein and Desmocolin (found in extracellular core of desmosome), and also the Plakoglobin and Plakophilins (found in outer dense plaque of desmosome) may beinvolvedin BEEC pathogenesis. These desmosomal components facilitate contact and adherence to neighbouring cells [31]. GWEP study by Qi and colleagues showed that Desmin (*DES*) and Desmulin (*DMN*) are the two most under-expressed genes in BEEC [12]. The muscle-specific intermediate filament (IF) protein is encoded by these two genes. Not surprisingly, the IFs interact with the C-terminal domain of *DSP* and form a connection with desmosomal plaques, which lead to epithelial stratification via cell-cell adhesion [13,15]. Therefore, disruption of interactions between *DSP* and IFs during embryogenesis might contribute to BEEC progression.

Alternative signalling pathwaysin BEEC pathogenesis

According to Qi et al. [12], additional genes such as *SYNPO2* and *WNT5A* might also be involved in BEECpathogenesis. A recent report showed that a 32kb intergenic region between *WNT3* and *WNT9B* harbour regulatory elements for CBE [32]. The *WNT3* promoter region also contains binding motifs forseveral transcription factors [33], some of which are differentially expressed in human new-born bladder exstrophy tissue and have a

vital role in embryonic urorectalseptation process [12,34]. Furthermore, the promoter region has been shown to contain regulatory elements that regulate WNT signalling via p63 [35]. The study of Liu and colleagues also suggests a functional relation between *Tp63* and *Wnt5a*,where both of them seem to be regulated by a common upstream pathway [36]. It is therefore tempting to speculate that both the WNT pathway and TP63 pathway[21]may be interlinked in BEEC pathogenesis.Moreresearch is needed to prove such interactions. Study of promoters and enhancers of *PERP* and *DSP*,although not part of this study, may help shed further light on the disease pathogenesis.

V. Conclusion

Our study did not identify any sequence variation in *PERP* and *DSP* coding regionsthat may contribute to BEEC pathogenesis.However, to ascertain the complete picture of *PERP* and *DSP* involvement, more research and a large sample cohort is needed to identify disease causing mutations for such rare highly heterogeneous disorder which in turn may focus light on the interaction and regulation of these genes.

COMPETING INTERESTS

The author(s) declare that they have no competing interests.

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