

# Genetics of Bladder-Exstrophy-Epispadias Complex (BEEC): Systematic Elucidation of Mendelian and Multifactorial Phenotypes

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**Abstract:** The Bladder-Exstrophy-Epispadias Complex (BEEC) represents the severe end of the urorectal malformation spectrum, and has a profound impact on continence, and on sexual and renal function. While previous reports of familial occurrence, increased recurrence among first-degree relatives, high concordance rates among monozygotic twins, and chromosomal aberrations were suggestive of causative genetic factors, the recent identification of copy number variations (CNVs), susceptibility regions and genes through the systematic application of array based analysis, candidate gene and genome-wide association studies (GWAS) provide strong evidence. These findings in human BEEC cohorts are underscored by the recent description of BEEC(-like) murine knock-out models. Here, we discuss the current knowledge of the potential molecular mechanisms, mediating abnormal urorectal development leading to the BEEC, demonstrating the importance of *ISL1*-pathway in human and mouse and propose *SLC20A1* and *CELSR3* as the first BEEC candidate genes, identified through systematic whole-exome sequencing (WES) in BEEC patients.

**Keywords:** Array, Bladder, Cloacal, Epispadias, Exome, Exstrophy, Pathway.

## 1. INTRODUCTION

The Bladder-Exstrophy-Epispadias Complex (BEEC) describes a spectrum of malformations, and has a profound impact on continence, and on sexual and renal function. The component findings of BEEC have variable severity, and include Epispadias (E) (Fig. 1; a,b), the mildest phenotype, and Classic bladder exstrophy (CBE) (Fig. 1; c,d), which is the intermediate and most common defect. Persistent cloaca (PC) or Urorectal Septal Malformation Sequence, characterized by a common cloacal cavity (including bladder and intestinal elements), usually imperforate anus, and without exstrophy [1, 2] and Cloacal Exstrophy (CE), which is often referred to as the OEIS complex (omphalocele, exstrophy, imperforate anus, and spinal defects) (Fig. 1; e) [3] may represent the more severe forms of BEEC; however, there are some who consider CE to have a different embryologic origin from CBE [4, 5].

In approximately one third of all BEEC cases, several urological malformations (e.g. ectopic kidney, renal agenesis, hydronephrosis) are present. Vesicoureteral reflux and obstruction of the ureteropelvic junction are observed frequently in both genders, and cryptorchidism is common in

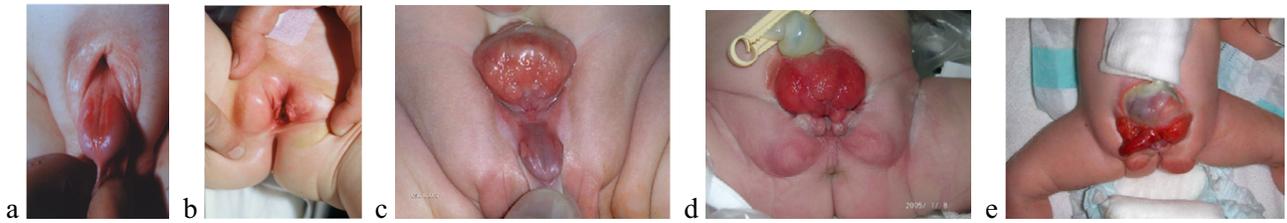
males [6]. Other malformations involving the gastrointestinal, skeletal, spinal and genitourinary systems (ambiguous genitalia) are reported frequently in CE [7-9]. Management of the BEEC is primarily surgical, and the main aims are the achievement of secure abdominal wall closure, urinary continence with preservation of renal function, and adequate cosmetic and functional genital reconstruction [10-12]. Following reconstructive surgery of the bladder during the neonatal period, continence rates of around 80% are expected during childhood. Additional surgery might be needed to optimize bladder storage and emptying function. In cases of final reconstruction failure, urinary diversion is necessary. In puberty, genital and reproductive functions are important issues [13, 14]. Psychosocial and psychosexual outcome and adequate health-related quality of life depend on long-term multidisciplinary care [15, 16].

Not a decade ago, the molecular elucidation of such rare and heterogeneous congenital malformation complexes as the BEEC seemed almost impossible. The aim of the present review is to illustrate, how recent systematic applications of array based and next generation sequencing techniques as well as modern functional pathway analyses have started to ultimately decipher the molecular basis even of such rare and heterogeneous congenital diseases as the BEEC.

### 1.1. Epidemiology

Among children of European descent, the overall birth prevalence for the entire BEEC spectrum has been estimated

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**Fig. (1).** a, b) boy and girl with epispadias; c, d) boy and girl with classic exstrophy of the bladder; e) boy with exstrophy of the cloaca (Ebert *et al.* 2009).

to be 1 in 10,000 [6]. While BEEC phenotypes have been observed as part of complex malformation syndromes, the majority of cases (approximately 98.5%) are classified as isolated [17-19]. Birth prevalences for the specific subtypes, including terminated pregnancies, have also been estimated. For E, an estimated prevalence of 1 in 117,000 in males and 1 in 484,000 in females has been reported [6]. On the other hand, Lloyd *et al.* [20] reviewed the American Kids' Inpatient Database (KID) and found a weighted (1997 – 2009) birth prevalence rate of 9.25 male E births per 100,000 in-hospital live births, with females uncaptured in KID. These authors also noted a 45% increase of E in the birth prevalence during this period.

For CBE, an estimated prevalence of 1 in 37,000 has been reported [21]. Here, Lloyd *et al.* [20] found a lower value of 1.68 CBE births per 100,000 in KID and a 33% decrease in the birth prevalence from 1997-2009. As for the observed increase of E, the authors suggested a shift in coding practices for the decrease in CBE, rather than a shift in epidemiology [20]. For CE, the estimated prevalence is 1 in 200,000 to 1 in 400,000 [8]; however, other studies suggest a higher prevalence of 1 in 80,000 to 1 in 159,000 [7, 22-26]. Prevalence ratios did not vary by maternal age [26]. The studies by Martinez-Frias *et al.* [8] and Caton *et al.* [25] support, that CE and CBE are different etiologic disorders, based upon their characteristic findings, prevalence and demographic characteristics. Findings of low birth weight, twinning, single umbilical artery, and infant mortality [8, 25], preterm births, and female sex [18] were associated with CE.

Due to the rarity of the phenotype, little is known about possible environmental risk factors. Reports by Wood *et al.* [27, 28] and Zwink *et al.* [29] suggest that assisted reproduction is a risk factor. Furthermore, maternal smoking and medical radiation during the first trimester were associated with a more severe phenotype, whereas periconceptual folic acid supplementation appeared to be protective [30]. Reefhuis *et al.* [31] found a positive association between use of clomid and CE (OR 5.4, 1.0-19.3).

## 1.2. Inheritance of the BEEC

Although familial occurrence is rare, 30 multiply affected families have been reported, and in some of these, the BEEC appears to follow a Mendelian mode of inheritance [32-35]. Nevertheless, the general consensus in the field is that - in the majority of patients - the genetic basis of the BEEC appears to be multifactorial [18]. For CBE, the reported recurrence risk among siblings ranges between 0.3-2.3%, in cases with non-consanguineous and non-affected parents [36, 37].

In their series, Shapiro *et al.* [36] reported the recurrence risk for the offspring of affected patients to be 1.4%. Hence, the recurrence risk for the offspring of affected patients and the risk of having a second affected child for parents who are non-consanguineous and non-affected shows an approximate 400-fold increase compared to the general population. Furthermore higher concordance rates among monozygotic compared to dizygotic twin pairs [34] emphasize a probable genetic etiology of the malformation. For CE, there are reports of recurrence in families [38]; increased occurrence among conjoined and monozygotic twins [39-45]; concordant conjoined twins [46]; and discordant dizygotic twins [47], all of which suggest a genetic basis for CE.

## 2. PATHOGENIC CONCEPT

BEEC has been recognized as one of the more intriguing phenotypes for mouse developmental researchers. The unique features of such a syndrome are its simultaneous onset of abnormalities of the bladder region and the upper (dorsal) part of external (ext.) genitalia.

### 2.1. Growth Factor Signals and Other Regulators Related with BEEC

Several essential growth factor signals for organogenesis are Hedgehog (Hh) and Wnt signals, which govern many developmental processes. Both signals are detected in the cloacal region, often in the cloacal membrane (CM) [48-50]. Such signals have been recently reported as being composed by the signal cascade, i.e., Hedgehog signal (Sonic Hedgehog (Shh))-Wnt signal-FGF (FGF8) by compound mutant analyses on embryonic ext. genitalia (genital tubercle; GT) and CM [51]. The Hedgehog signal (Shh as the ligand) and Wnt signal have been known to play roles in CM development [49, 52]. Alteration of these signals leads to abnormalities of or absence of CM formation, leading to severe caudal abnormalities [49]. Milder forms of CM abnormality such as partial (e.g., dorsal part) agenesis or abnormal orifice formation related to the CM may lead to BEEC phenotypes often with abnormal URS (urorectal septum) development [10, 52]. The timing of CM disruption was also suggested to influence these phenotypes [10, 52].

In addition to CM disruption, localized alterations in cell death either in the CM and/or in the PCM may have an effect on the phenotypes in mouse models of BEEC [10, 49]. However, the role of cell death, such as apoptosis, is still not well characterized, except for its possible role in the CM [48, 49].

Hedgehog and Wnt signals have been also known to affect the nearby Peri cloacal mesenchyme (PCM) through

epithelial-mesenchymal interaction. By such interaction, the PCM is influenced by the CM to develop its characteristic mesenchymal gene expression, such as *Bmp* (bone morphogenetic protein) 4 [49]. In addition to these changes in gene expression, regulation of mesenchymal differentiation has been suggested. Thus, alteration of hedgehog signals by several genetic combinations has been shown to display BEEC like phenotypes. In fact, several mouse hedgehog-signal mutants display BEEC-related abnormalities. Developmental genes such as *Alx4* (paired type homeobox gene) have also been shown to genetically interact with hedgehog signals [53]. *Alx4* has been shown to interact with the hedgehog pathway, particularly in limb formation and digit development by the ZPA (zone of polarizing activity). *Alx4* mutant mice have been shown to display several defects affecting the coordinated developmental process in the formation of the ventral body wall and external genitalia, like bladder abnormalities with epispadias-like phenotypes together with upper GT defects.

Similar phenotypes are described in *Gli3* mutant mice, which is a negative transcriptional regulator of hedgehog signaling [53]. Various genetic mutants of *Alx4* and/or *Gli3* have been shown to display defective body wall closure. In such mutants, abnormal bladder and ext. genitalia phenotypes with reduced *Alx4* or together with reduced *Gli3* are partly rescued in some genetic backgrounds by “reducing” hedgehog (*Shh*) gene dosage [53]. Thus, the orchestration of the hedgehog signaling pathway with its modifier-genes needs to be further investigated.

In contrast to BEEC in mouse models, there have been several genes reported to be responsible for the development of body wall abnormalities including body wall closure. Hedgehog signaling has also been shown to be essential for body wall closure with abnormalities related to omphalocele [54]. However, the down-stream target genes remain to be elucidated.

In addition to growth factor signals, there have been several genes found to be responsible for body wall abnormalities including body wall hernia or gastroschisis [55]. Currently, only a few genes have been identified as being responsible for BEEC like phenotypes. One such example is the *Ap2* gene, which has been suggested as one of the essential genes for body wall closure. *Ap2alpha*-deficient mice have been reported as showing phenotypes related to body wall closure and upper GT formation [56]. In addition to *Ap2*, *TP63* has been also suggested as showing similar phenotypes. *TP63* is an essential regulator of skin development and epithelial formation. In addition, the *TP63* KO mouse displayed defective body wall and upper GT phenotypes [57]. However, it remains still unclear whether the reported mutants display highly penetrant BEEC-like phenotypes.

Most recently, Chari *et al.* [58] demonstrated a potential cross regulation of the *Shh* and *TP63* pathways in keratinocytes that maintains epidermal homeostasis by controlling both, proliferation and differentiation.

Although both genes have been relatively well characterized with respect to their interacting genes during epithelial development, little is known regarding their interacting genes and, as outlined in chapter 5, their contribution to BEEC-like phenotypes.

## 2.2. Developmental Cell Lineages Related with BEEC

Orchestrated and complex *in vivo* phenotypes can be also analyzed in mouse models using cell lineage analysis. Hedgehog and *Wnt* signals have been known to affect the nearby mesenchyme, the Peri-cloacal mesenchyme (PCM) through epithelial-mesenchymal interaction. An important breakthrough for mouse BEEC models was the use of tissue lineage analysis using hedgehog (HH) signal-responding cell analysis [59]. The lineage for hedgehog-responding cells was monitored by *Gli1* gene-marked cell imaging. Mesenchyme close to the developing cloaca (PCM) was shown to contribute to the bladder region and upper part of the external genitalia [59]. This study opened the door for lineage analysis in the mouse, which was essential to understand syndromic abnormalities such as BEEC.

Another line of work should also be noted for its progress. Several cell lineages including the cloacal region have been suggested to contribute to bladder (in addition to other pelvic organs) and ext. genitalia. One lineage includes the *Isl1* type developmental gene-positive cell lineage. The *Isl1* gene, also discovered in a genome-wide association study and meta-analysis as a top CBE candidate gene (see chapter 5), has been suggested to regulate limb development as it was determined to be upstream of *Hand* - *Shh* in hindlimb formation. Recently *Isl1* was shown to be expressed in hindlimb and GT (genital tubercle) [60]. In fact, alteration of the *Bmp 4* (Bone morphogenesis protein) gene in this cell lineage resulted in abnormalities in ext. genitalia and bladder.

As suggested previously, *Shh* is expressed in the CM and *Bmp4* is expressed in the nearby mesenchyme (PCM). The *Isl1* gene can mark a rather broad area of PCM, GT and caudal embryonic regions together with hindlimb [60]. How the alteration of *Bmp* signals in this *Isl1* cell lineage can lead to defective caudal mesenchyme remains to be investigated. Generally, epithelial (endoderm)-derived hedgehog signals “relayed” toward mesenchymal genes such as *Bmp4* have been suggested [61]. Regarding *Bmp* signals, *Msx* genes have been long suggested as interacting with *Bmp* signals and its mutants show body wall abnormalities [62].

Regarding cell lineage contributions, the possible connection between bladder organogenesis and umbilical cord for body wall formation remains to be elucidated.

The umbilical cord (UC) is located adjacent to the cloacal membrane/cloaca, and an unstable or defective cloacal membrane/cloaca could be one of the causes of such syndromes. Proper umbilical cord development is essential for development of the umbilical region, connecting the maternal environment and the embryo. Some developmental genes are suggested to function within such processes.

In the lower body region, cells derived from the anterior (upper) part of body wall, the aPCM region, have been reported to contribute to the upper part of genitalia [53]. Such cell lineages can also explain simultaneous onset of BEEC phenotypes. Dye induced cell marking experiments revealed the presence of cell migration from the umbilical cord region (aPCM) or infra-umbilical mesenchyme region towards upper part of ext. genitalia. Genetically marked experiments or genetic modulation of such cell populations would be neces-

sary to verify such significance. Part of these candidate genes were reported in the case of *Alx4* but some other genes showed similar expression patterns, including *Tbx4*, *Tbx3*, *Pitx1*, and *Mab2112* genes [53]. Redundancy among such genes may exist for their developmental functions.

### 2.3. Merits and Perspectives on Mouse Models for BEEC

Complex *in vivo* phenotypes can be also analyzed in mutant mouse models. Given that such phenotypes often “phenocopy” those of human patients, there are often no questions regarding the usefulness of analyzing mutant mouse models. However, essential genes and growth factor signaling pathways simultaneously governing multiple tissues are often expressed in the very early embryo (for example gastrula). Thus, introduction of a mutation in these important regulatory genes is often expected to lead to the early embryonic lethality prior to organogenesis. In general, conditional gene inactivation systems would be effective and necessary to analyze such processes. However, cell lineages are only currently being identified for both genital and bladder regions. Therefore, conditional mutant approaches based on cell lineages such as *Bmp* signals in *Isl1* cells, or other effector genes in hedgehog responding cells, will be necessary in the future.

Another factor to consider is the potential species-specific differences related to BEEC phenotypes between mice and humans. General remarks on BEEC phenotypes between human and mouse are species dependent morphogenic events. It should be noted that reproductive organ formation, particularly for GT (genital tubercle; embryonic ext. genitalia) formation, displays species variations. Although there are no questions regarding the importance of mouse model analysis, one should also pay attention to potential differences between mouse and human phenotypes. Such differences are partly derived from the anatomical/developmental differences of the two species. The upper (dorsal) part of the ext. genitalia is suggested as formed by species-specific developmental processes, such as the formation of penile bones, mesenchymal and prepuccial differentiation. Hence, the extent of phenotypic similarity one can identify with respect to GT formation including its abnormalities, such as epispadias, requires attention. Fundamental developmental pathways for upper GT formation could be conserved between human and mice. Some pelvic organs, pelvic urethral curvature and pelvic muscles also show species dependent changes.

Regarding such “fundamental” developmental processes, BEEC phenotypes should be regarded not as just simple simultaneous bladder and genital region abnormalities but they may offer good models to understand organogenesis. Organs should develop in coordination. Thus, it is expected that multiple phenotypes may arise during such processes. The broader spectrum of BEEC and research on these embryologic mechanisms may include the caudal end of phenotypes including the perineum plus anterior portion of body wall, and midline abnormalities including bladder regions. Continuous efforts to analyze mouse mutant series in addition to cell lineage analysis will gradually provide insights into understanding these intriguing phenotypes.

### 3. COPY NUMBER VARIATIONS (CNVs)

Since the BEEC represents a rare and severe birth defect, with life-long impairments including reduced reproduction rate, one can assume a significant fraction of *de novo* events among these patients. The experience in medical genetics suggests that the mutational events will comprise genomic alterations of different size ranging from small changes affecting single nucleotides to large alterations resulting in losses or gains of several thousand to millions of base pairs.

In accordance with this hypothesis, several earlier studies using conventional karyotyping report larger structural chromosomal aberrations in BEEC [35]. In 2004, Thauvin-Robinet and colleagues reported an infant with CE carrying a relatively large 9q34.1-qter deletion [63]. With the introduction of array based techniques over the past decade additional microaberrations have been found in BEEC patients including patients with severe CE phenotypes as well as the milder CBE phenotype. In 2005, Kosaki *et al.* reported the first smaller deletion in a CE patient comprising chromosomal region 3q12.2-q13.2 spanning 13Mb [64]. While this aberration was still visible on conventional karyotyping, in 2010 El-Hattab *et al.* reported another, much smaller deletion (2.4Mb) in a CE patient comprising chromosomal region 1p36.33 [65]. This aberration was not visible on conventional karyotyping and the deleted region harbored approximately 70 RefSeq genes. Among these genes, the authors discussed *NOC2L*, *DVLI1*, the human homolog of the *Drosophila* dishevelled gene (*dsh*), encoding a cytoplasmic phosphoprotein that functions as a mediator of the WNT signaling pathway [66, 67], and *MMP23B* as possible candidate genes. Despite these reports, no single chromosome abnormality has been shown to be associated with CE.

Independently, in 2010, Draaken *et al.* [68] and Lundin *et al.* [69] identified two unrelated CBE patients carrying a 22q11.2 microduplication using array-based analysis. Subsequently, Draaken *et al.* [70], using multiplex ligation-dependent probe amplification and microarray-based analyses identified four additional patients with 22q11.21 duplications in 244 unrelated BEEC patients, including 217 CBE patients. The identified duplications were of variable size. Pooling data of all three studies (eight duplications in 313 CBE patients) yielded a combined odds ratio of 31.86 (95% confidence interval, 4.24-1407.97). Array-based sequence capture and high-throughput targeted re-sequencing established, that all breakpoints resided within the low-copy repeats 22A to 22D. Comparison of the eight duplications revealed a 414 kb phenocritical region, harboring 12 validated RefSeq genes. Characterization of these 12 candidate genes through whole-mount *in situ* hybridization (WISH) of mouse embryos at embryonic day 9.5 and 10.5 suggested *CRKL*, *THAP7*, and *LZTR1* as CBE candidate genes. A ninth CBE patient carrying a 22q11.21 duplication was reported by Pierquin and Uwineza [71].

In 2011, Vlangos *et al.* reported their copy number variation (CNV) study in a cohort of 13 CE patients using two different platforms, a 250K SNP array and a 244K Oligonucleotide array [72]. The oligonucleotide array showed increased sensitivity over the SNP array; however, the majority of CNVs that were detected were observed multiple times in the Database of Genome Variants (<http://dgv.tcag.ca/>)

dgv/app/home). Other CNVs did not contain any known genes or were determined to be inherited from an unaffected parent. The authors concluded from this study that CE is unlikely to be caused by a single recurrent genomic CNV. In the same study, 14 candidate genes were analyzed based on their known or predicted role in caudal development in humans or mice [72]. Ten novel variants were found in seven genes, six of which were within introns and did not uncover a cryptic splice site. The remaining variants included a missense variant in the *IHH* gene and an 18bp duplication in the *FGF8* gene, both of which were carried by an unaffected parent. Two variants were located in the 5' untranslated region of the *WNT3* gene, and the only available parent for analysis did not carry the variant. Based on these data, the authors concluded that none of the variants that were identified provided a likely genetic explanation for the CE phenotype in these patients. However, it could not be ruled out that some of the rare variants could be associated with the CE phenotype and have reduced penetrance in the parents.

Further array-based molecular karyotyping by Draaken *et al.* [73] in an additional 110 individuals with BEEC identified one additional *de novo* 0.9 Mb 19p13.12 microduplication, involving 20 validated RefSeq genes. WISH of the corresponding mouse orthologous genes on embryos at gestational days 9.5 and 10.5, with particular emphasis on the ventrolateral trunk, showed that only transcripts for orthologs of human *CASP14*, *AKAP8*, *SYDE1*, *BRD4*, *CYP4F22*, and *WIZ* were found to be specifically expressed in the hindgut and/or caudal region. Of these, *WIZ* was prioritized over the other genes, showing expression in the developing genital region. However, subsequent Sanger sequencing of *WIZ* in the complete BEEC cohort did not reveal any pathogenic alterations affecting the coding region of *WIZ*.

In 2014, Söderhäll *et al.* [74] reported on a case with CBE and an unbalanced X chromosome rearrangement that was further characterized with array-based CGH analysis. The X chromosome rearrangement consisted of a gain of chromosomal material in region Xq26.3->qter and loss in region Xp22.12->pter. This aberration was also carried by the patients' mother and sister, neither with CBE. All three have disproportionate short stature, as expected due to the deletion of one of the copies of the *SHOX* gene. X-inactivation studies revealed a complete skewed inactivation pattern in carriers. Crossover events in the maternal germline furthermore resulted in different genetic material on the rearranged X chromosome between the index patient and her sister suggesting an X-linked genetic risk factor for CBE. Most recently, Jorgez *et al.* [75] reported on a 10-year-old boy with CBE carrying a 66 kb deletion, which deleted only one copy of *OTX1*. The boy was identified in a cohort of 30 BEEC patients using NimbleGen 36720 aCGH. None of the remaining 29 BEEC subjects or the 85 controls without GU defects presented with CNVs in *OTX1*.

#### 4. IDENTIFICATION OF SINGLE GENE MUTATIONS USING WHOLE-EXOME SEQUENCING (WES)

The previous findings from CNV analyses provide a proof of concept that rare *de novo* mutations contribute to the

formation of the BEEC in humans. To identify smaller *de novo* dominant mutations/small CNVs, the use of next-generation sequencing, specifically whole-exome sequencing (WES) seems plausible. Yet, to the best of our knowledge, no study has been published to date on WES in the elucidation of the BEEC. Here, we present our first approach to the discovery of BEEC candidate genes using WES. Besides the aim to identify smaller *de novo* dominant mutations, we also sought to identify inherited autosomal-recessive mutations.

WES was performed in eight patients with CE, representing the most severe form of the BEEC spectrum. The CE phenotype was chosen, since to our knowledge, no report of adult patients with CE and biological children has been published [35]. This is probably due to the severe malformation of the external and internal reproductive system as well as the co-occurring defects (imperforate anus, spinal defects, renal anomalies). The lack of reproduction in CE patients is suggestive of an underlying dominant *de novo* event or rare autosomal-recessive disease causing genes. Prior to exome sequencing, all patients underwent CNV analysis to exclude the presence of larger causative CNVs [68, 73]. All eight case-parent trios were analyzed by WES as outlined elsewhere [76]. All variants detected with a frequency >1% in our in-house data were not further followed. We first searched for heterozygous *de novo* variants and identified five, yet unknown, non-synonymous amino acid substitutions in three patients (Table 1). These variants were confirmed by Sanger sequencing and were all absent in the respective parents. Several publicly available prediction programs consistently characterized three of these variants in *PRPF38A*, *PRPF8* and *SLC20A1* as disease causing (Table 1). While *Slc20a1* is expressed in the mouse urinary tract system (UTS) at embryonic Theiler Stage 22 implicating a developmental role in mouse UTS, expression was regionally restricted. Further, extensive data mining in human, mouse and zebrafish databases provided no evidence for the involvement of *PRPF38A* or *PRPF8* in BEEC etiology. Moreover, the p.Arg1681Trp variant identified in *PRPF8*, encoding the pre-mRNA-processing-splicing factor 8, is likely to be a neutral variant, since mutations in this gene have been associated with autosomal dominant retinitis pigmentosa type 13, a feature not observed in our CE patient. Hence, here only the p.Gly237Arg substitution in *SLC20A1* remains the most promising pathogenic variant found.

We then focused on rare recessive, homozygous or compound heterozygous variants in the patients. Whereas none of the patients were found to be homozygous for any of the variants, we detected compound heterozygosity in four genes in different patients (Table 1). Sanger sequencing confirmed all these variants and confirmed maternal and paternal transmittance for each of the two variants. For all four genes, at least one variant was predicted to be benign. In three patients, compound heterozygosity was found in the genes encoding wolframin-1, senataxin and erythrocytic spectrin beta-I, respectively. Mutations in these genes are found to be associated either with different forms of autosomal dominant Wolfram syndrome, autosomal recessive spinocerebellar ataxia-1/autosomal dominant juvenile amyotrophic lateral sclerosis type 4, or manifesting clinically as types of hemolytic anemia, features not observed in these isolated CE patients. Hence, here only the variants in the cadherin receptor

Table 1. Heterozygous *de novo* and compound heterozygote variants detected by WES in a total of eight CE patients.

Patient ID	Complete Phenotype of the CE Patients	Chromosomal Location	Gene	Gene Function (Associated Disease)	cDNA Position, Substitution and Consequence	Known SNP (dbSNP142), Minor Allele Frequency (MAF)	Predicted Consequence <sup>1</sup>	Transmission
<b>Heterozygous <i>de novo</i> variants</b>								
19	bladder exstrophy, anal atresia, inguinal hernia, abnormal penis, enlargement of the pubic symphysis	1p32.2	<i>PRPF38A</i>	pre-mRNA-processing factor 38A	c.514G>A, p.Glu72Lys	-	D,D,D,D	-
341	omphalocele, tethered cord, Arnold-Chiari malformation, ventricular septal defect, permanent foramen ovale, os sacrum dysplasia, bipartite scrotum, inguinal hernia, epilepsy	14q21.2	<i>MIA2</i>	melanoma inhibitory activity protein 2	c.896A>C, p.Glu299Ala	-	B,B,D,-	-
		17p13.3	<i>PRPF8</i>	pre-mRNA-processing-splicing factor 8 (AD <sup>2</sup> retinitis pigmentosa type 13)	c.5041C>T, p.Arg1681Trp	-	D,D,D,-	-
		19q13.33	<i>NUCB1</i>	nucleobindin 1	c.281A>G, p.His94Arg	-	D,B,D,B	-
407	omphalocele, anal atresia, tethered cord, lipoma, atrial septal defect, duplex kidney (right), sacrum dysgenesis	2q13	<i>SLC20A1</i>	sodium-dependent phosphate transporter 1	c.709G>A, p.Gly237Arg	-	D,D,D,-	-
<b>Compound heterozygous variants</b>								
19	see above	14q23.3	<i>SPTB</i>	erythrocytic spectrin beta-1 (AD <sup>2</sup> spherocytosis type 2; AR <sup>3</sup> forms of elliptocytosis 3 and anemia, neonatal hemolytic, fatal and near-fatal)	c.2480G>A, p.Arg827Gln c.3282G>A, p.Glu1095Lys	rs150013838 (MAF: 0.051) rs201653621 (MAF: 0.046)	B,B,B,- D,B,D,-	paternal maternal
131	cloacal exstrophy, lipomeningocele (lumbosacral) with tethered cord, uterus duplex, os sacrum dysplasia, hip dysplasia, malformed feet	4p16.1	<i>WFS1</i>	wolframin-1 (AD <sup>2</sup> forms of Wolfram syndrome type 1, Wolfram-like syndrome and deafness)	c.41A>G, p.Gln14Arg c.2452C>T, p.Arg818Cys	rs142651446 (MAF: 0.00184) rs35932623 (MAF: 0.0042)	B,B,B,B D,D,D,D <sup>4</sup>	maternal paternal
406	bladder exstrophy, omphalocele, anal atresia without fistula, tethered cord, bilateral hydronephrosis, me-gaureter, uterus duplex, vaginal septum	3p21.31	<i>CELSR3</i>	cadherin, EGF-like, laminin G-like, seven pass G-type receptor 3	c.5470G>A, p.Val1824Met c.6950T>C, p.Met2317Tyr	rs141484230 (MAF: 0.00079) rs200387155 (MAF ?)	B,D,B,D D,D,B,D	maternal paternal
407	see above	9q34.13	<i>SETX</i>	probable helicase senataxin (AR <sup>3</sup> spinocerebellar ataxia-1; AD <sup>2</sup> amyotrophic lateral sclerosis 4, juvenile)	c.472T>G, p.Leu158Val c.3287A>G, p.His1096Arg	rs145438764 (MAF: 0.0016)-	B,B,D,D B,B,B,B	maternal paternal

<sup>1</sup>prediction from publically available programs in the order Mutation Taster, MutPred, PolyPhen-2 and SIFT: B, benign or polymorphism; D, disease causing or probably damaging;<sup>2</sup>AD, autosomal dominant; <sup>3</sup>AR, autosomal recessive; <sup>4</sup>benign according to the NCBI ClinVar database.

gene *CELSR3* seem to remain as candidates for disease association. This gene is involved in planar cell polarity signaling, organizing multicilia in individual cells (single-cell polarity) [77].

Early somatic mutations in mesenchymal bladder precursors, as well as epigenetic events in relevant tissues might also contribute to the disease. Moreover, our previous twin study provided evidence for environmental effects on disease formation [34]. Altogether, one may expect a model of gene-gene and/or gene-environment (or -epigenetic) interaction, underlying BEEC etiology.

Further investigation of both, *SLC20A1* and *CELSR3* is warranted, including screening of larger CE patient cohorts in order to identify additional mutation carrying patients as well as functional murine and zebrafish studies.

## 5. ANALYSIS OF CANDIDATE GENES AND GENOME WIDE ASSOCIATION STUDIES (GWAS) IN THE CONTEXT OF MULTIFACTORIAL PHENOTYPES

As outlined above, the general consensus in the field is that, in the majority of BEEC patients, the genetic basis appears to be multifactorial [18]. To account for this multifactorial genetic basis, candidate gene association studies and more recently GWAS provided the basis to investigate the contribution of candidate genes or regions to the formation of multifactorial phenotypes. For the BEEC, only a few candidate gene association studies have been performed to date [78-80] with the most important being the one conducted by Wilkins *et al.* [80]. Based on their previous observations, an inactivation of the N-terminal truncated isoform of *p63*, *deltaNp63*, in mouse embryos, caused the formation of a CBE phenotype in a subset of litters [57]. Wilkins *et al.* investigated the contribution of *TP63* to the formation of human BEEC [80]. The authors hypothesized that *TP63* is involved in human BEEC pathogenesis. Re-sequencing of the *deltaNp63* promoter in 163 BEEC patients and 285 ethnicity-matched controls showed seven single nucleotide polymorphisms and four insertion/deletion (indel) polymorphisms. These indel polymorphisms were associated with an increased risk of BEEC. Significantly the sites of indel polymorphisms differed between Caucasian and non-Caucasian populations. A 12-base-pair deletion was associated with an increased risk only in Caucasian patients whereas a 4-base-pair insertion was only associated with BEEC in non-Caucasian patients. This association however, was inconsistent over the different exstrophy populations (Asian, Australian, Canadian) tested. In an attempt to replicate this finding, Qi *et al.* [79] performed a candidate gene association study of *TP63* in 154 Caucasian BEEC patients and their unaffected parents. Using 109 selected tagging SNPs localized within *TP63* with a minor allele frequency >0.01, they found nominally significant associations between BEEC and six SNPs and four haplotype blocks including or near these significant SNPs; however, none of these results remained significant after correction for multiple testing. Moreover, Darling *et al.* [81] did not observe any *TP63* promoter mutation in 112 BEEC patients. On the other hand, we detected that PERP, a tetraspan protein and a direct *TP63* downstream

target, was 22.2-fold overexpressed in human bladder sets [82]. PERP specifically localizes to the desmosomes, where it extends through the plasma membrane to link neighboring cells and whole-mount in situ hybridization studies have demonstrated prominent PERP expression in bladder [83].

Our GWAS (see below) however, detected no evidence for an involvement of *TP63*, *PERP* or any other desmosomal gene at a genome-wide level [84]. Hence, the desmosomal and cytoskeletal dysregulation observed in postnatal tissue [82] may not reflect the early embryonic situation. Otherwise, the dysregulation of various *TP63* isoforms in newborn exstrophy bladder tissue might be due to defects in factors regulating alternative *TP63* expression and splicing. Hence, supporting evidence for a contribution of *TP63* and desmosomal genes in the etiology of BEEC needs further investigation.

The first GWAS, comprising 218 cases and 865 controls and 78 trios in total, all of European descent identified suggestive evidence for association with CBE across the authors' discovery and follow-up samples. Interestingly, *WNT3* and *Wnt9b* have been both associated with urogenital anomalies in humans and mice respectively [85, 86]. The most associated SNP within this region resides 4 kb next to the *WNT3* promoter (<http://promoter.cdb.riken.jp/>), a region highly conserved among amniotes. Of note, variants in the *WNT3* 5'UTR were also identified in the CE cohort reported by Vlangos *et al.* [72]. Nakamura *et al.* identified several potential transcription factor-binding motifs to exist within the *WNT3* promoter region [87], several of which were previously found to be differentially expressed in human newborn bladder exstrophy tissue and known to be important for promotion of the embryonic urorectal septation process [82, 88]. Finally, this region has been shown to contain regulatory elements which regulate Wnt signaling via *p63* [89]. Thus, it is tempting to speculate that there are regulatory domains within this intergenic region that are able to modulate Wnt signaling via a conserved *WNT3-WNT9B-p63* regulatory module in the context of urorectal and urogenital development.

In our second GWAS we analyzed 110 CBE patients and 1,177 controls of European origin. Here, an association was found with a region of approximately 220kb on chromosome 5q11.1. This region harbors the human *ISL1* (*ISL LIM homeobox 1*) gene. Multiple markers in this region showed evidence for association with CBE, including 84 markers with genome-wide significance [84]. We then performed a meta-analysis using data from our first GWAS (see above). This meta-analysis also implicated the 5q11.1 locus in CBE risk. A total of 138 markers at this locus reached genome-wide significance in the meta-analysis, and the most significant marker (rs9291768) achieved a *P* value of  $2.13 \times 10^{-12}$ . Murine expression analyses detected *Isl1* expression in the genital region within the critical time frame for human CBE development. Genital regions with *Isl1* expression included the peri-cloacal mesenchyme and the urorectal septum.

Hence, our second GWAS identified the first genome-wide significant locus for CBE at chromosomal region 5q11.1, and provided strong evidence for the hypothesis that *ISL1* is the responsible candidate gene in this region.

## 6. CONCLUSION

The systematic and comprehensive application of modern array based and next generation sequencing techniques in large BEEC cohorts has started to identify putative disease causing genes and regions in the human genome for both Mendelian and multifactorial BEEC phenotypes. These studies will ultimately provide new diagnostic possibilities, and allow precise estimation of recurrence risk in affected families. Parallel functional analysis of the respective embryonic pathways provides a more and more profound understanding of the molecular biological mechanisms underlying the normal and disturbed embryology of the human urogenital system. Furthermore, understanding the respective embryonic pathways will subsequently help to explain related urorectal and genitourinary malformations, such as anorectal malformations, vesicoureteral reflux, ectopic pelvic kidney, renal agenesis, and cryptorchidism.

## CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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