

Paediatric Urology

Authors from John Hopkins University School of Medicine in Baltimore present their study into the identifying genetic and non-genetic factors contributing to the risk of bladder exstrophy-epispadias complex. They found it to occur most commonly as an isolated sporadic birth defect, and found no evidence of a single-gene effect or a common environmental factor.

Clinical and molecular characterization of the bladder exstrophy-epispadias complex: analysis of 232 families

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Accepted for publication 22 July 2004

OBJECTIVE

To identify genetic and nongenetic factors contributing to the risk of bladder exstrophy-epispadias complex (BEEC).

PATIENTS AND METHODS

In all, 285 families with BEEC were invited to participate in the study, and 232 of them were recruited. Epidemiological information was obtained from 151 of the consenting families, with a detailed clinical genetic examination of 94 probands. In all, 440 DNA samples were collected from 163 families for molecular analysis.

RESULTS

Most of the cases were sporadic and had no family history of BEEC. Among patients, 95% were Caucasian, and males were more common in both the epispadias group (M/F, 2.2, 29 patients) and the classic bladder-exstrophy group (M/F 1.8, 164), but in the cloacal exstrophy group the sex ratio was close to unity (1.1, 15). There was a statistically significant association with

advanced parental age ($P < 0.001$). Birth weight, gestational age and maternal reproductive history did not appear to be significantly different from those in the general population. Information on exposures to tobacco, alcohol and drugs was collected but none appeared to act as a risk factor. Karyotype analysis on 37 cases detected two chromosomal abnormalities, i.e. 46XY t(8;9)(p11.2; q13) and 47XYY. Molecular analysis of the HLXB9 gene, which causes Currarino syndrome, did not detect mutations in the blood or bladder DNA of 10 patients with bladder or cloacal exstrophy.

CONCLUSIONS

BEEC most commonly occurs as an isolated sporadic birth defect with a recurrence risk of $\ll 1\%$. There was no evidence of a single-gene effect or common environmental factor in this study population. In addition to race and advanced parental age, birth order may be a risk factor for BEEC. We suggest somatic mutations in a gene(s) within the pathway regulating bladder development may be the cause of BEEC.

KEYWORDS

bladder exstrophy, epispadias, cloacal, genetics, epidemiology

INTRODUCTION

The bladder exstrophy-epispadias complex (BEEC) represents a spectrum of urological abnormalities in which part or all of the distal urinary tract fails to close and is exposed on the outer abdominal wall. This rare congenital anomaly is thought to be a clinical spectrum ranging from isolated epispadias to classic bladder exstrophy (CBE), to its most severe form, cloacal exstrophy (CE) [1,2]. CE is also referred to as the 'OEIS' complex, an acronym for omphalocele, exstrophy of the bladder, imperforate anus and spinal defects [3]. Intermediate variants of BEEC have been described, including covered exstrophy, in which the bladder mucosa is covered with skin, but the underlying bladder and skeletal abnormalities are similar to CBE [4,5].

The reported incidence of BEEC varies, but the most commonly accepted values are 1 in 117 000 for male and 1 in 400 000 for female epispadias [1,2]; 1 in 30 000 live births for CBE [6]; and 1 in 200 000 to 1 in 400 000 for CE [2,7]. The incidence of CE among stillborn infants may be significantly higher than in live-born babies, ranging from 1 in 10 000 to 1 in 50 000 [8]. There is an overall greater proportion of males than females in BEEC, ranging from 2.3 : 1 to 6 : 1 in different reports [9,10]. According to the Birth Defects Monitoring Program of the Centers for Disease Control and Prevention (CDC), the birth prevalence of BEEC varies among the ethnic groups in North America. The highest rate of 8 per 100 000 was in Native American populations, while the lowest rate of 1 per 100 000 was in Asians [11].

Animal models of BE have been developed and provide a better understanding of the developmental mechanisms of BEEC [12–14]. Normal embryological development of the bladder begins at 4–6 weeks of gestation, when the urogenital septum divides the cloaca into the anterior urogenital sinus and posterior anorectal canal. The cloacal membrane is invaded laterally by mesoderm to create the lower abdominal wall. Currently, the most accepted view is that premature rupture or abnormal development of the cloacal membrane is the cause of BEEC

[15,16]. The timing of the rupture may determine the severity within the BEEC spectrum; if the membrane ruptures before 4–6 weeks, CE ensues; if it ruptures after the urorectal septum has descended at 6 weeks, BE or epispadias occurs [1].

Although many descriptive epidemiology reports have been published [2,10,17], the causes of BEEC remain unknown. The reported risk factors include young maternal age [6], increased parity even after adjusting for age [18], and *in vitro* fertilization [19]. Possible genetic factors have been suggested, based on observations of rare familial cases, high but incomplete concordance in monozygotic twins, and a single report of increased recurrence risk of 1 in 70 for BEEC in the offspring of an affected parent [20–23]. These observations, along with the non-Mendelian inheritance of BEEC, suggest that spontaneous errors of development such as somatic mutation, or complex gene–environment interactions, may be responsible for BEEC.

We initiated a genetic study aiming to further clinically characterize BEEC and to identify the demographic, environmental and genetic factors that are associated with this complex and heterogeneous birth defect.

PATIENTS AND METHODS

The study was approved by the Institutional Review Boards of the Johns Hopkins Hospital, and was conducted in accordance with its guidelines. In all, 285 families with BEEC, most of them identified through our institutionally approved BEEC database of 815 patients, were invited to participate, and 232 were recruited. Of these, 155 were enrolled during clinical visits to the Paediatric Urology Clinic at the hospital. The rest of the families contacted us as a result of a study invitation placed on the web site of an Internet support group (<http://www.bladderexstrophy.com>), or were referred by an outside physician. Consent was obtained before parental interviews, clinical examinations, and/or sample collection.

An epidemiological questionnaire modelled after the National Birth Defect Prevention Study questionnaire developed by the CDC was completed by 151 families (<http://www.nbdpn.org/NBDPN>). Partial clinical and past medical information was available for the rest of the consenting families. We are

actively recruiting additional families for this study.

DNA AND RNA ISOLATION, PCR AND SEQUENCING ANALYSIS

Genomic DNA was isolated from whole blood, buccal swabs, mouth washes, or lymphoblastoid cell lines, using standard protocols (Puregene, Genra Systems, Inc., Minneapolis, USA). When available, discarded bladder tissue was collected during surgical procedures for DNA and RNA extraction. Total RNA from lymphoblastoid cell lines and bladder specimens was extracted using a standard Trizol protocol. RNA from blood was isolated with ZR whole-blood total RNA Kit (Zymo Research, Orange, CA). RNA preparations were purified (RNAeasy, Qiagen, Valencia, CA), quantified by spectrophotometry, and stored at –80 °C.

All exons of the HLXB9 gene (GenBank accession #AF107457) were amplified with at least 100 bp of flanking intronic sequences from bladder DNA obtained from five patients with CE, and genomic blood DNA of five individuals with CBE. The PCR protocols and primer sequences are available on request. PCR products were purified from gel bands or solution using a column purification kit (GFX™ PCR, Amersham Pharmacia Biotech Inc., Piscataway, NJ). Sequencing was carried out by the di-deoxy chain termination method using the Sequenase version 2.0 DNA sequencing kit (Amersham). The ABI Prism 3700 and 3730 automated fluorescent DNA analysers (PE Biosystems, Foster City, CA) were used for sequencing.

All results were assessed statistically with R version 1.8.1 [24]. The comparison of the male : female ratios to the expected 1 : 1 was assessed using a binomial test. All other tests of significance were based on the chi-square test.

RESULTS

Based on direct examination and/or available clinical records, we determined the primary diagnosis in 232 cases; epispadias was diagnosed in 33 (14%), CBE in 180 probands (78%), and CE in 19 (8%). Consistent with the proposed hypothesis that BEEC represents a clinical spectrum encompassing epispadias, CBE and CE, there were intermediate phenotypes in several patients. One patient

Diagnosis (n)	Males	Females	M/F ratio	TABLE 1 The sex distribution
Epispadias (29)	20	9	2.2	
CBE (164)	105	59	1.8	
CE (15)	8	7	1.1	
Total (208)	133	75	1.8	

three CE patients with unilateral kidney agenesis).

Seven patients with BEEC had anomalies outside the BEEC spectrum. These included four with cardiac defects, i.e. a ventriculo-septal defect (two), ventriculo-septal defect and patent foramen ovale, and bicuspid aortic valve. Mild digital anomalies (syndactyly and clinodactyly) were present in two cases, and one male with CBE had moderate maxillary hypoplasia.

Information about the sex of the affected person was available for 208 probands (Table 1). Overall, the proportion of males (133) was higher than females (75), giving a M/F ratio of 1.8 for the entire cohort. When analysed by diagnostic category, the male predominance was most evident for the epispadias group (Table 1), but this was only marginally significant ($P = 0.06$) because there were too few in the group. The proportion of males was higher in the CBE group, which was statistically significant ($P < 0.001$), and lowest in the CE group. Thus there appeared to be a trend towards increasing male prevalence with decreasing severity of BEEC.

Information on maternal age was available for 201 families; the mean (range) age was 29.5 (16–42) years. The mean maternal age was highest in the CE group (31.3 years) and was 29.6 and 29.3 for the epispadias and CBE groups, respectively. These differences among the three groups were not statistically significant (Fig. 1A). The overall distribution of births across specific maternal age groups showed a trend for older mothers among those in the BEEC group that was statistically different from the general population data available from CDC reports ($P < 0.001$; Table 2). There were very few mothers (2%) younger than 20 years in our study group, contrary to the suggested hypothesis that young maternal age may be a risk factor for BEEC.

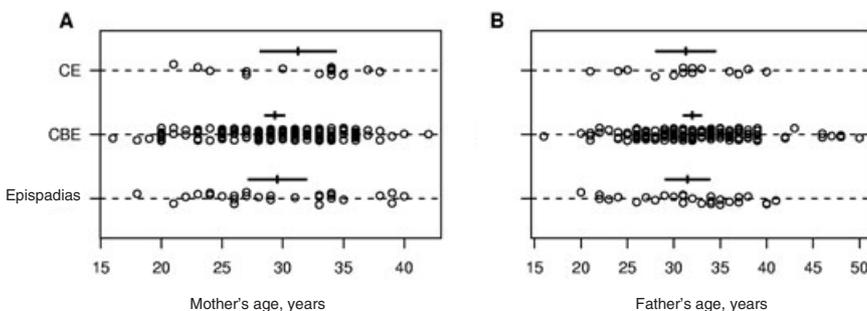
We also analysed birth prevalence for selected birth defects in specific maternal age groups, available from the CDC annual National Vital Statistic Reports and the US Census Bureau (<http://www.cdc.gov/nchs>; and <http://www.census.gov/prod/www/statistical-abstract-us.html>). We used the mean of the published data for three years (1995, 1998 and 2001) to compare with the study population, as most of the probands were aged <10 years. There was no association of

TABLE 2 The distribution of BEEC and other selected birth defects in specific age groups

Group	Age groups, years						Total
	<20	20–24	25–29	30–34	35–39	>39	
Age distribution, %							
Parental, for GP and BEEC							
General population: mothers	12	25	27	23	11	2	11 857 075*
BEEC	2	14	34	35	13	2	201
General population: fathers	5	20	27	26	15	6	11 857 075
BEEC	0.50	9	24	33	20	7	195
Maternal, for selected birth defects							
Spina bifida	14	29	26	22	8	1	2 642
RA/S	12	25	29	23	11	N/A	1 047
O/G	28	34	19	13	6	N/A	3 321
CL/P	13	27	26	22	10	2	9 505
DA	17	29	24	19	9	2	9 566

Values are based on the National Vital Statistics Reports and the US Census Bureau reports for 1995, 1998 and 2001. The data are presented as a mean percentage and the total indicates the total number of cases. *The distribution of the birth defects is adjusted for the total number of births for which information was available (11 695 309). Present data are in italics. SB, spina bifida; RA/S, rectal atresia/stenosis; O/G, omphalocele/gastroschisis; CL/P, cleft lip/palate; DA, digital anomalies (polydactyly, syndactyly, adactyly).

FIG. 1. Plot of maternal (A) and paternal age (B) at birth by diagnostic categories; the open circles show each parental age and the solid line above each category the mean and 95% CI.



initially diagnosed with epispadias was later found to have an intermediate variant phenotype where skin covered the BE. In addition to BE, two patients had a single defect within the CE spectrum (spina bifida and omphalocele) and another had gastroschisis.

In addition to the surgical evaluation and review of previous medical documentation,

detailed clinical genetics examinations were performed on 94 patients with BEEC. None of them had developmental delay or neurological symptoms unrelated to the BEEC phenotype (i.e. seizures, ataxia, facial nerve anomalies, etc.). There were no reports of learning disability, although there was no formal assessment. Five probands had congenital renal defects (dysplastic kidney, duplicated kidney, and

older mothers with spina bifida, rectal atresia/stenosis, and omphalocele/gastroschisis, three birth defects that could be a part of the CE phenotype. Indeed, based on this analysis, omphalocele/gastroschisis appeared to be associated with younger mothers; 28% of the cases were born to mothers aged <20 years, a segment of the population accounting for only 12% of all mothers. A population-based study of abdominal wall defects in Australia also reported that omphalocele was significantly more common in mothers aged <20 years [18]. The distribution of cleft lip and/or palate and digital anomalies (birth defects that are outside of the BEEC spectrum) was no different from that in the general population (Table 2).

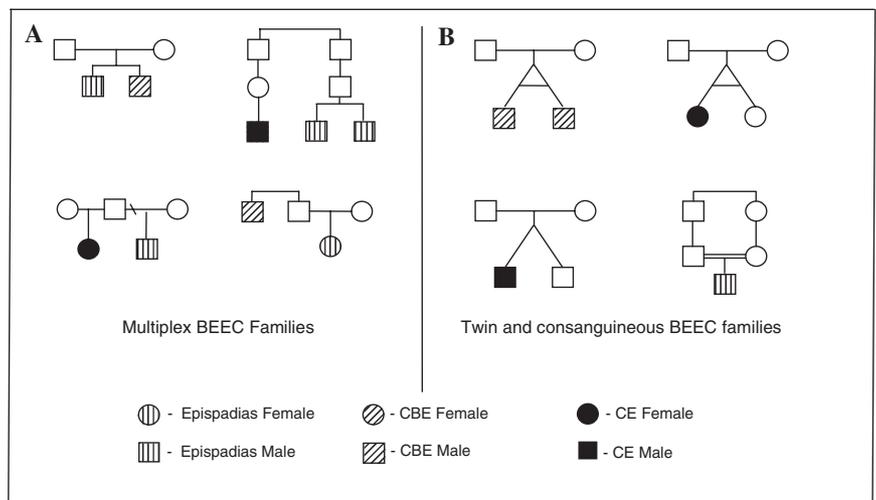
The mean (range) paternal age was 32 (16–50) years; there were no differences in the mean paternal age among the epispadias, CBE and CE groups (31.5, 32 and 31.3 years, respectively; Fig. 1B). Again, the paternal age in these BEEC families was greater than in the general population ($P < 0.001$; Table 2).

Most probands (95%) were Caucasian; as in the general population, the mean birth weight was 3319 g (98 children) and the mean gestational age at delivery was 39.2 weeks (105). Only four of the 151 families (2.7%) reported any relative affected by BEEC (Fig. 2A). Sibling data were available for 200 of the participating families, and they had a total of 259 unaffected children, in addition to the BEEC probands. Four probands had a total of seven biological children, all of whom were unaffected. There were three twin pairs in these families, two of whom were monozygotic. Concordance for BEEC was present in only one of the twin monozygotic pairs. Consanguinity was reported by one family, where parents were first cousins (Fig. 2B).

Twenty-six of 151 probands (17%) had first-, second- or third-degree relatives with congenital anomalies unrelated to BEEC. Of interest, midline defects and oral clefts were present in 15 families, with spina bifida (five), cleft lip (four), hypospadias (two), and one each of imperforate anus, sacral dimple, umbilical hernia and extra vertebrae.

In all, 49% of the BEEC probands were first-born; in contrast, a previous study of 173 case-parent trios with cleft lip and palate reported that only 25% of those affected were born from a first pregnancy [25]. To

FIG. 2. Panel A shows the pedigrees of four multiplex BEEC families (clockwise from top left; brothers, second cousins/brother pairs, niece-uncle pair, and half-siblings). Panel B shows two monozygotic twin pairs, one of which is concordant for CBE, a consanguineous family, and a discordant fraternal twin pair.



determine whether being firstborn confers an increased risk for BEEC, we obtained birth-order information from the annual National Vital Statistic Reports and averaged the data for 1995, 1998 and 2001 (Table 3). The observed differences when comparing parity between BEEC with the general population was marginally significant ($P = 0.08$), again probably because the sample was too small.

There were no significant effects in the analysis of exposures to tobacco, alcohol and drugs during the pregnancy. We obtained exposure information from 151 BEEC families. Smoking (any amount) was reported by 22 mothers (15%) which was not significantly different from the 13% incidence reported by CDC for all mothers giving birth in 1995, 1998 and 2001. Exposure to alcohol (any amount) was reported by 41 women (27%), mostly limited to a few drinks before confirmation of the pregnancy. None of the present case mothers reported excessive drinking or a history of alcoholism. According to the birth certificate information published by CDC, $\approx 1\%$ of pregnant women giving birth in 1995, 1998 or 2001 reported alcohol use (any amount). However, this self-reported information may under-report actual rates of exposure, and a more detailed study by the CDC found an alcohol exposure rate of 12.8% among women who delivered in 1999 [26].

A previous report suggested a possible association between *in vitro* fertilization and BEEC [19]. To address this issue we analysed

TABLE 3 Birth order of the BEEC probands compared to the general population

Birth order, %	General population	BEEC
Pregnancy		
First	40	49
Second	32	31
Third	16	15
Fourth	6	3
Fifth	2	1

the information on reproductive history available for 206 patients; this included the total number of pregnancies, miscarriage history, assisted reproductive techniques and hormonal medications. Two babies with BEEC (epispadias and CBE) were born after *in vitro* fertilization procedures and three other (two with CBE and one with CE) were conceived through artificial insemination with donor sperm. In six families the pregnancies occurred after stimulating ovulation with drugs. The overall proportion of mothers who reported having had one or more miscarriages was 18%. The highest incidence of miscarriages was in the epispadias group (34%) and was statistically different from both the general population and the CBE and CE subgroups ($P = 0.02$). The miscarriage incidence in the CE group was 27%, but because the group was small this difference from the general population was not statistically significant (Table 4).

BEEC subgroups, n (%)	Number of miscarriages		TABLE 4 <i>The history of miscarriages</i>
	None	≥1%	
Epispadias	19 (66)	10 (34)	
CBE	139 (86)	23 (14)	
CE	11	4	
Total	169 (82)	37 (18)	

Our long-term objective is to identify possible genetic causes of BEEC. In preparation for future linkage and association studies, we collected 440 DNA samples from 163 participating BEEC families. Karyotype analysis on 39 cases detected two chromosomal abnormalities, 46XY,t(8;9)(p11.2; q13) and 47XY.

We considered the *HLXB9* gene as a possible candidate, as mutations of this gene have been associated with Currarino syndrome [27]. This autosomal dominant congenital anomaly occurs by abnormal dorsal-ventral patterning and includes sacral defects, anorectal anomalies, and presacral teratoma or meningocele, conditions that resemble the anomalies seen in BEEC [28]. Considering that somatic mosaicism in the bladder would not be detectable in genomic DNA extracted from peripheral lymphocytes, we tested DNA obtained from bladder specimens of five patients with CE. No blood samples from these patients were available and blood genomic DNA from five patients with CBE was also analysed. All sequences were reviewed by two independent investigators and no obvious disease-causing mutations were detected. That there were several single-nucleotide polymorphisms within *HLXB9* excluded the possibility of large deletions in the gene. These polymorphisms were present in samples from normal controls and are unlikely to confer increased susceptibility to BEEC. The present data suggest that mutations of *HLXB9* are not a common cause of BEEC, although changes in more distant regulatory regions or within introns of this gene cannot be excluded.

DISCUSSION

There are limited data on the epidemiology, risk factors and genetics of BEEC, perhaps because of its low birth prevalence. The data collected from the present cohort of patients adds to the increasing publications on this subject. There were several patients with intermediate phenotypes between the

recognized clinical entities of epispadias, CBE and CE in the study population. These clinical observations support the hypothesis that BEEC is a clinical spectrum with variable severity, and perhaps common risk factors and pathological mechanisms [2].

Unilateral renal agenesis was found in 28% of the CE cases reported in a previous study [8], and we detected a solitary kidney in three of 14 patients with CE, which suggests that CE occurs earlier than CBE and within the developmental period of kidney formation at or before the fifth week of gestation. Based on this information, genes involved in kidney development should be considered as possible candidates for CE. Apart from anomalies known to be part of the BEEC spectrum, very few of the present patients had anomalies outside the urogenital system and none of them had developmental delay. This confirms that BEEC occurs as an isolated sporadic developmental defect that is not associated with the involvement of other organ systems [10].

As expected from previous publications [9,10], males were more commonly affected and the M/F ratio of the entire cohort was 1.8. However, there was no male excess in the CE group. While this may be a random observation because the group was relatively small, it is possible that female gender confers some level of protection against BEEC that could be overcome by a stronger or earlier genetic insult in patients with CE.

There was a trend to advanced parental age in the BEEC group; the distribution of probands in specific parental age groups was statistically different from that of the general population ($P < 0.001$), corroborating the observation of increased parental age in BEEC (Table 2). This is in contrast to the commonly cited statement that a maternal age of <20 years is a risk factor for BEEC, which is based on a registration of congenital anomalies among 6.3 million newborns [6]. There was a discrepancy between the observed and expected cases of epispadias (17

vs 13) and CBE (16 vs 11) among mothers aged <20 years, according to these authors, but it was only just statistically significant. One possible explanation for the apparently older parents is that we sampled only a subgroup of the BEEC population, and cannot exclude the possibility of selection bias, as most of the study participants were recruited from a tertiary medical centre or were self-referred through a web-based support group. Further prospective studies with appropriate control families are needed to resolve the discrepancy of these observations.

The presence of four multiplex families suggests some genetic component for the cause of BEEC. Although the pedigree structure of these families may be consistent with an autosomal recessive inheritance, the family data from the cohort, and from previous reports, is not compatible with Mendelian transmission. Because there were so few multiplex families with BEEC, a formal segregation analysis is unlikely to identify a specific mode of inheritance. The high proportion of sporadic BEEC families could reflect either genetic or environmental factors controlling the risk. In accordance with the proposed hypothesis that CBE and CE are two different expressions of a primary developmental field defect [2], defects in any one of several genes involved in a caudal developmental pathway are expected to produce the BEEC phenotype. Thus, there is a high likelihood of causal heterogeneity and many factors determining the risk of BEEC.

The incidence of anomalies outside the urogenital system among relatives of the BEEC probands was 17%. While this incidence appears to be high, especially for oral clefts and for anomalies involving the midline, this result is difficult to interpret in the absence of an appropriate control group, but it indicates the need to obtain a detailed medical and genealogical history in all BEEC families.

Severe congenital anomalies occur in 3% of all live births, but the underlying causes of 65–75% of these birth defects are still unknown. Among the possible mechanisms are polygenic (gene-gene) and multifactorial (gene-environment) interactions, an effect of a teratogenic agent, or spontaneous errors of development caused by de novo germ cell or somatic cell genetic event [29]. Multifactorial and polygenic malformations, e.g. cleft lip and palate, spina bifida and congenital heart

defects, generally have an increased recurrence risk of $\approx 3\%$ [30]. In contrast, the recurrence risk for the BEEC families in this study was $<1\%$. One possible mechanism for BEEC is a somatic mutation event. The incomplete monozygotic twin concordance, and the low recurrence risk for a second affected child, may be consistent with this hypothesis. An early insult that affects not only the bladder but the mesenchymal precursors of the kidney and the cells populating the gonads, may account for the solitary kidney in CE patients and the reported 400-fold greater chance for an affected individual to have an affected offspring [31]. A recent report of a patient with CE and chromosomal tissue mosaicism [32] also suggests that BEEC can occur as a result of a somatic event in the mesenchyme at the stage of the three germ-layer embryo.

Ascertainment of possible teratogenic exposures was limited by sample size and lack of an available control group, but we found no significantly greater risk for either tobacco or alcohol consumption. More than 50 drugs, chemicals and physical agents have proven teratogenic potential [29]. None of them, except alcohol, was documented among the participating BEEC families. Considering the dose-response curve of alcohol and the well-defined phenotype of fetal alcohol syndrome, it is very unlikely that the reported alcohol consumption in the present group is related to BEEC. Although control data were available through CDC reports for many variables, direct comparisons were not possible because of the disparate sample size and SEM compared to our smaller series. In addition, data ascertained from a retrospectively administered questionnaire are potentially inaccurate. Recall bias may also have affected data collected from mothers in the epidemiological questionnaire.

Overall, it is estimated that $\approx 15\%$ of all clinically recognized pregnancies end in early spontaneous abortion. The proportion of women who have a miscarriage increases with age, from 12% for women aged <20 years, to 26% for women aged >40 years [33]. There was a significantly greater rate of miscarriage in the epispadias group that cannot be attributed to greater age. In the absence of pathology data to determine the cause of the miscarriage, a possible explanation for this may be that the aborted embryo may have had a more significant midline defect incompatible with life.

No obvious causal mutations were identified in the *HLXB9* gene. Direct sequencing analysis of many potential candidate genes for sporadic conditions such as BEEC is an inefficient and costly approach. In the absence of multiplex families suitable for linkage studies, the analysis of similar complex birth defects poses unique challenges and requires more elaborate statistical methods than the LOD score method used for monogenic traits. Association studies using statistical methods based on the transmission disequilibrium test for linkage disequilibrium have been widely used for dissecting complex traits, e.g. birth defects, when sufficiently large study samples are available [34–36], and should be considered for future genetic dissection of BEEC.

ACKNOWLEDGEMENTS

We thank all patients and their family members for participating and generously donating their time and biological specimens for this study. We are grateful to Barbara Ward, President of The Association for The Bladder Exstrophy Community web-support group for hosting an invitation for research participation (<http://www.bladderexstrophy.com/>). Many thanks to Heiko Reutter, Children's Hospital Medical Center, Bonn, Germany and Valerie Murphy, University College, Cork, Ireland for their stimulating discussions and plans for future collaborations. This work was supported by the Johns Hopkins University School of Medicine General Clinical Research Centers grant #M01-RR00052, from the National Center for Research Resources/NIH and by grant K23 DE00462 (S.A.B.).

CONFLICT OF INTEREST

None declared. Source of funding: Johns Hopkins – GCRC.

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Abbreviations: (BE)EC, (bladder exstrophy)-epispadias complex; CBE, classic bladder exstrophy; CE, cloacal exstrophy; CDC, Centers for Disease Control and Prevention.