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Variation in the clinical and genetic evaluation of undervirilized boys with bifid scrotum and hypospadias

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Summary

Background—Bifid scrotum and hypospadias can be signs of undervirilization, yet boys presenting with these findings often do not undergo genetic evaluation. In some cases, identifying an underlying genetic diagnosis can help to optimize clinical care and improve guidance given to patients and families.

Objectives—The aim of this study was to characterize current practice for genetic evaluation of patients with bifid scrotum, and to identify approaches with a good diagnostic yield.

Methods—A retrospective study of the Boston Children's Hospital electronic medical records (1993–2015) was conducted using the search term “bifid scrotum” and clinical data were extracted. Data were abstracted into a REDCap database for analysis. Statistical analysis was performed using SPSS, SAS, and Excel software.

Results—The search identified 110 subjects evaluated in the Urology and/or Endocrinology clinics for bifid scrotum. Genetic testing (including karyotype, microarray, or targeted testing) was performed on 64% of the subjects with bifid scrotum; of those tested, 23% (15% of the total cohort of 110 subjects) received a confirmed genetic diagnosis. Karyotype analysis, when performed, led to a diagnosis in 17% of patients. Of the ten instances when androgen receptor gene sequencing was performed, a pathogenic mutation was identified 20% of the time.

Conclusion—This study demonstrated that the majority of individuals with moderate undervirilization resulting in bifid scrotum do not receive a genetic diagnosis. Over a third of the analyzed subjects did not have any genetic testing, even though karyotype analysis and androgen

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receptor (AR) sequencing were both relatively high yield for identifying a genetic etiology. Increased utilization of traditional genetic approaches could significantly improve the ability to find a genetic diagnosis.

Graphical abstract

Keywords

Bifid scrotum; Undervirilization; Disorders of sex development; Hypospadias

Introduction

Disorders of male genital development are congenital malformations of the penis and scrotum, and can vary in severity. Fortunately, advances in surgical technique have provided functional improvement for patients, if referred to an experienced surgical team [1,2]. In many cases, however, the underlying etiology of the developmental abnormality is unknown; this lack of a diagnosis may lead to significant distress for patients and their families [3,4]. Androgen receptor mutations provide a clear example of the impact of having a genetic diagnosis. Identification of an androgen receptor mutation allows for targeted clinical care, including the possible use of testosterone, fertility guidance, family testing, and assessment of risk to future children [5,6].

Most disorders of sex development (DSD) conditions can cause a range of phenotypes, from sex reversal to frankly ambiguous genitalia to mild undervirilization resulting in isolated hypospadias. Sex reversal and ambiguous genitalia typically trigger an evaluation for DSD, whereas isolated hypospadias does not, as most cases are thought to be non-hormonal in origin, making a DSD evaluation less likely to identify an underlying diagnosis. However, it is not entirely clear whether a DSD evaluation is warranted for the intermediate phenotype of hypospadias in combination with other signs of potential virilization such as cryptorchidism and/or bifid scrotum.

Although such undervirilization has been well managed surgically, progress in diagnosis has lagged. In many diseases, genetic testing to determine etiology has led to significant progress in understanding pathogenesis and even to targeted treatment, such as in congenital cardiac disease [9], intellectual disability [10], and hyperinsulinism/neonatal diabetes mellitus [11]. Recently, studies have focused on the genetics of DSD that result in frankly ambiguous genitalia [12–15], but relatively few studies have focused on the genetics of milder cases of undervirilization. However, there are recent reports suggesting that a significant number of these milder cases may also have underlying genetic etiologies, including *AR*, *NR5A1*, and *WT1* mutations [16].

Bifid scrotum was chosen as a proxy for undervirilization in this study to evaluate a group of subjects with a relatively narrow range of phenotypes. Bifid scrotum is a midline cleft in the scrotum and can be associated with incomplete fusion of the labioscrotal folds [7]. In the majority of cases, individuals with bifid scrotum also have hypospadias. One potential cause of this phenotype is insufficient testosterone secretion or action: fusion of the labioscrotal folds and urethral localization both take place during the first trimester under the influence of androgens [8]. As such, bifid scrotum with hypospadias may represent a DSD. Individuals with this phenotype are often seen in urology and/or endocrinology clinics and are not consistently classified as having a DSD.

It was hypothesized that few children receive a genetic diagnosis, yet still receive comprehensive surgical and medical management. This hypothesis was based upon clinical experience that only a small fraction of individuals with bifid scrotum, regardless of whether they had additional clinical features, received a diagnosis to explain the underlying mechanism of disease. As syndromic features would potentially point toward possible genetic etiologies, it was also hypothesized that those patients with additional clinical findings were more likely to have additional evaluation and workup. To test these hypotheses, a retrospective chart review of children with bifid scrotum with and without hypospadias was performed to document the characteristics and genetic evaluation of these males at a tertiary care referral center. The review also sought to identify the differences in diagnostic evaluation and outcome between those with isolated genitourinary abnormalities and those with syndromic features.

Methods

Cohort

A text-based search system (i2b2) [17] was used to identify subjects seen in the Division of Endocrinology and/or Department of Urology at Boston Children's Hospital between 1993 and 2015 using the search term "bifid scrotum". Additional subjects were prospectively identified through review of Endocrine and/or Urology schedules between December 2014 and May 2015. All subjects in the cohort were actively followed by Endocrinology, Urology, and/or Genetics through at least 2004, with the vast majority being seen more recently. Subject characteristics including gender, race, ethnicity, age at first evaluation, gestational age at birth, severity of hypospadias, genetic evaluation, serum testosterone measurements, and presence of non-genitourinary abnormalities were extracted from review of medical records. Hypospadias was dichotomized as proximal/middle (perineal, scrotal, penoscrotal

and penile) and distal (coronal and glanular) to reflect the severity of the hypospadias. Categories of non-genitourinary abnormalities (syndromic features) included cardiac, renal, neurologic, neuro-developmental, gastrointestinal, musculoskeletal, ophthalmologic, endocrine, otolaryngologic, or growth abnormalities.

Statistical analysis

Study data were collected and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted at Boston Children's Hospital [18]. The Boston Children's Hospital Institutional Review Board approved this research. Microsoft Excel, SPSS, and SAS 9.4 software were used for statistical analysis. Groups were compared using Fisher's exact test and two tailed *t*-tests.

Genetic evaluation

Genetic evaluation was designed to include any of the following: karyotype, chromosomal microarray, or targeted genetic evaluation, including sequencing and deletion testing. None of the patients had whole-exome or whole-genome sequencing. The vast majority of the targeted genetic testing has taken place since 2005 (only four targeted tests sent prior) but karyotypic analysis has been performed throughout the study time period.

Results

Description of cohort

A search of the Boston Children's Hospital patient database and of the Urology and Endocrine clinic schedules identified 110 subjects with bifid scrotum. Nearly half (46%) of the subjects were seen by both Endocrine and Urology providers, while 11% were seen only in the Endocrine clinic and 43% were seen only in the Urology clinic. Table 1 shows the characteristics of the bifid scrotum cohort as a whole and also of the subset with isolated genitourinary abnormalities (62%) and the subset with additional non-genitourinary abnormalities (38%). Subjects with non-genitourinary abnormalities in addition to their bifid scrotum are referred to through the rest of this publication as syndromic subjects. Presence or absence of micropenis was not reliably reported in the cohort, and therefore not included in the analysis. Non-genitourinary abnormalities were seen in 42 subjects, some with multiple abnormalities: cardiac (13), neuro-developmental (nine), growth (nine), neurologic (eight), ano-rectal malformations (eight), musculoskeletal (six), renal (four), otolaryngologic (four), endocrinologic (one), and ophthalmologic (one).

A little less than one-third of the subjects (31%) had orchidopexy for unilateral or bilateral undescended testes. This was similar between the groups, with 29% of the isolated genitourinary group having orchidopexy compared with 33% of those with non-genitourinary abnormalities.

Presence and severity of hypospadias

The present study examined how frequently subjects with bifid scrotum also had hypospadias. For individuals with only genitourinary (GU) findings, 93% had more severe proximal/middle hypospadias, whereas a smaller majority (76%) of the subjects with

syndromic findings had proximal/middle hypospadias (Table 1). Two individuals (3%) with isolated bifid scrotum had mild or no hypospadias, while eight syndromic subjects (19%) had mild or no hypospadias. In total, the majority (86%) of subjects with bifid scrotum had severe hypospadias.

Genetic evaluation and diagnosis

To determine how often genetic testing led to a diagnosis, all genetics tests performed for individuals in the cohort were reviewed (Table 2). It was found that 15% of all subjects had a confirmed genetic diagnosis. Of the 64% of subjects who had any type of genetic testing – including karyotype, microarray, or targeted genetic testing – 23% received a genetic diagnosis. These diagnoses included sex chromosome abnormalities diagnosed by karyotype ($n=9$) or microarray ($n=1$), non-sex chromosome abnormalities ($n=3$), androgen receptor mutations ($n=2$), and Smith-Lemli-Opitz syndrome ($n=1$). Seven of the 16 cases with genetic diagnoses (44%) required orchidopexy to treat unilateral or bilateral undescended testes. These seven cases were identified from genetic testing performed on 28 out of the 34 subjects who had orchidopexy. Of the 76 individuals who did not have a record of orchidopexy, 41 had genetic testing and nine received a genetic diagnosis. Of the 69 subjects who had karyotype testing sent, 17% were found to be abnormal. Of the isolated GU subjects, a geneticist saw 22% at least once, while a geneticist saw 60% of the syndromic subjects. Of those who saw a geneticist, 90% had genetic testing as part of their evaluation compared with 50% of those who were not seen in the Genetics clinic.

Karyotype

Karyotypes were the most frequent genetic studies performed and were seen at comparable rates in the syndromic and isolated genitourinary groups (67% and 60%, respectively). Subjects seen only in the Urology clinic had karyotypes performed 26% of the time, compared with 83% of the time for those seen only in the Endocrine clinic and 92% of the time for subjects seen both in Urology and Endocrinology clinics. Subjects seen by a geneticist had karyotypes performed 88% of the time compared with 49% of those who did not see a geneticist.

Chromosomal microarray

Seventeen subjects had chromosomal microarray testing, and one was found to have a pathogenic copy number variant. The majority of this testing was performed in the syndromic group (33%; 14 out of 42) rather than in the isolated GU group (4%; 3 out of 68; $P<0.0001$). The diagnostic yield of microarray for the entire cohort was 6%, compared with 7% for the sub-group with additional syndromic findings. None of the non-syndromic subjects received a diagnosis based on microarray results.

Targeted testing

Targeted gene-specific or deletion testing was conducted on a number of genes/regions, including *AR*, *SOX9*, *SRY*, *DHCR7*, *NLGN4*, *FGD1*, *MID1*, *STK9*, *NLGN3*, *NR5A1*, *SRD5A2*, *FMR1*, and 22q11 deletion. About a quarter of the cohort (24%) had single gene or targeted deletion testing, much of which (39%) was androgen receptor sequencing.

Androgen receptor (*AR*) sequencing was performed on 10 subjects, with 20% ($n=2$) receiving an androgen insensitivity diagnosis.

Prematurity

Based on prior reports of an association between prematurity and undervirilization [19], gestational age at birth was examined within the cohort. Subjects with missing data on gestational age were omitted from this analysis. Of the present cohort of 110 subjects, 83 had data on gestational age, and 51% of those 83 subjects ($n=42$) were born prior to 37 weeks. Of the subjects with isolated GU findings and a known gestational age, 44% (30 of 68 subjects with available data) were born before 37 weeks, while 60% (21 of 35 subjects) with additional syndromic abnormalities were born before 37 weeks ($P=0.18$). The average gestational age of those with available data across the cohort was 35.9 weeks. The average gestational age for the isolated GU cases was 36.3 weeks compared with 35.2 weeks for syndromic cases ($P=0.23$). In terms of etiology, four premature subjects received genetic diagnoses, with two isolated GU and two syndromic cases.

Discussion

The experience at Boston Children's Hospital over the past two decades offers insights into both the characteristics and work-up of male subjects with bifid scrotum, which is a potential indicator of undervirilization. These subjects were seen by providers with different areas of clinical focus and underwent variable diagnostic evaluations. The variability may reflect the way in which these patients are classified, at times grouped with patients with isolated genitourinary issues such as hypospadias, while at other times considered to have disorders of sex development (DSD). The review of medical records indicated that the extent of genetic work-up differs depending on whether subjects were seen by both an endocrinologist and urologist when compared to seeing only a urologist. This may reflect differing diagnostic strategies amongst specialties, or it may be due to confounding by clinical presentation, with more significant cases being sent for multidisciplinary evaluation and also meriting a more comprehensive evaluation.

In the present cohort, almost two-thirds of subjects had genetic testing and approximately one-quarter of those subjects received a genetic diagnosis. The majority of the diagnoses were made with chromosomal analysis. Androgen receptor sequencing also yielded diagnoses, with two confirmed cases out of 10 subjects who received such testing. These results indicate that karyotype analysis and *AR* sequencing appear to be high-yield tests in individuals with the bifid scrotum phenotype. Relatively few subjects had *AR* sequencing as part of their clinical workup, and, although this would need to be validated, it seems likely that some of the 100 subjects who did not have *AR* sequencing would have had a pathogenic variant identified if it had been performed. Similarly, karyotypic analysis of the 41 untested subjects could potentially have led to additional diagnoses. Both chromosomal abnormalities and androgen insensitivity diagnoses would have the potential to change evaluation and treatment. Individuals with sex chromosome abnormalities, such as Turner syndrome variants, benefit from cardiac, renal and audiologic evaluations [20] and may also be at risk for gonadoblastoma. Diagnosis of partial or complete androgen insensitivity is important,

given the risk of testicular tumors, effect on fertility, potential need for hormonal replacement, implications for puberty, and risk for family recurrence [6].

Additional research that is focused on subjects with bifid scrotum and/or hypospadias is needed to determine the true yield of genetic testing in this population. Some genital abnormalities may be caused by environmental or epigenetic factors, or random developmental events, but an additional unrecognized fraction may carry diagnoses that could be identified using whole-exome or whole-genome sequencing. Recent exome sequencing studies of DSD associated with more severe undervirilization demonstrate a reasonable diagnostic yield [12–14], which is comparable to other rare disorders when modern genomic approaches are used for diagnosis [21]. This may indicate an opportunity to use similar approaches to advance knowledge of the genetics of hypospadias and manifestations of undervirilization. There will likely be overlap with DSD genes, potentially with milder mutations in genes known to be associated with gonadal or genital development such as *NR5A1* [22]. Indeed, the present authors recently performed whole-exome sequencing in 10 subjects with bifid scrotum and hypospadias, and identified *NR5A1* mutations in two of these subjects [23]. There may also be distinct genes/loci identified, as suggested by a recent genome wide association study of hypospadias that identified 22 loci, many of which do not lie near genes known to be related to DSD [24].

The present findings also support some previously reported observations. It has been noted in the past that there is an association between prematurity and hypospadias/undervirilization, although the pathophysiology is not well understood [19,25,26]. This association may potentially be related to hCG, placental function, and/or LH receptor signaling in the first trimester. For those with documented gestational ages in the present cohort, the average was 35.9 weeks, which was approximately 4 weeks earlier than a standard full-term pregnancy.

It is also important to recognize some of the limitations of this study. As a retrospective chart review, the gathered information was limited by the information found in the electronic medical records (EMR). It was attempted to minimize this limitation by focusing on characteristics that were reliably reported, and this led to the focus on bifid scrotum as a commonly reported sign of undervirilization. Hypospadias presence and severity were also reported in the majority of cases, but penis size and testicular location were not as frequently reported. Different practitioners may have also used different definitions of “bifid scrotum”. A standard definition for bifid scrotum is the presence of a midline cleft in the scrotum, but there may have been variability as to when providers decided to use this term, and this may in turn have introduced some variability into the phenotypes of subjects in this study. Although outside records were reviewed when available, it is possible that some outside records were not uploaded into the EMR and that the analysis may not reflect the full extent of all testing. Another challenge was evaluating genetic testing over a period of time when the availability of testing evolved. Changes over time may lead to an underestimate of current rates of targeted genetic testing and chromosomal microarray, which are forms of testing used with increasing frequency over time. That being said, all subjects were actively being seen through to at least 2004, a time at which targeted sequencing of some genes, including the androgen receptor, were clinically available at the present institution. It is

noted that the use of a karyotype, an approach that has been in routine use for decades, should be less affected by the time frame encompassed by this study.

Conclusion

This analysis demonstrated that genetic testing yields a diagnosis for many individuals with bifid scrotum. Identification of sex chromosome mosaicism and partial androgen insensitivity has significant implications on anticipatory guidance for patients and families, as well as on clinical evaluation and management. These findings serve as a reminder that a finding of hypospadias in conjunction with cryptorchidism (whether unilateral or bilateral) or hypospadias in the context of additional non-GU anomalies warrants genetic testing. These findings further suggest that patients with isolated bifid scrotum or with hypospadias and bifid scrotum in the absence of cryptorchidism or anomalies outside the GU system may also benefit from genetic evaluation, although further study is needed to determine the yield of genetic testing in such patients. Recent advances in technology for genetic evaluation, including whole-exome and whole-genome sequencing, could further improve the diagnostic yield and could thereby change the care of these patients in the future.

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Table 1

Bifid scrotum cohort characteristics.

	Age at 1 st visit (years)	Gestational age (weeks) [*]	% born prior to 37 weeks [*]	Hypospadias			
				Proximal or middle	<i>n</i>	Distal or none	<i>n</i>
Total bifid cohort							
	0.43	35.85	50.6%	86.4%	95	9.1%	10
Isolated GU subjects	0.35 ^a	36.34 ^b	42.9% ^c	92.6% ^d	63	2.9%	2
Syndromic subjects	0.56	35.17	60.0%	76.2%	32	19.0%	8
							4.8%
							2

^a $P=0.514$

^b $P=0.23$

^c $P=0.18$

^d $P=0.006$, Fisher's exact

^{*} Missing gestational age data on 27 subjects (20 syndromic, 7 isolated genitourinary (GU))

^{**} Statistical comparisons between isolated genitourinary (GU) subjects and syndromic subjects

Genetic evaluation of entire bifid scrotum cohort, subdivided into genitourinary only and syndromic sub-groups.

Table 2

		Total cohort	n	Genitourinary only	n	Syndromic	n	two sided P-value *
Any genetic evaluation		63.6%	70	60.3%	41	69.0%	29	0.42
	Karyotype	62.7%	69	60.3%	41	66.7%	28	0.68
	Chromosomal microarray	15.5%	17	4.4%	3	33.3%	14	<0.0001
	Gene-specific or targeted deletion testing							
		23.6%	26	19.1%	13	28.6%	12	0.35
	AR sequencing	9.1%	10	11.8%	8	4.8%	2	0.31
Confirmed genetic etiology of group/ sub-group		14.5%	16	10.3%	7	21.4%	9	0.16
Confirmed genetic etiology of those with any genetic evaluation		22.9%	16	17.1%	7	31.0%	9	0.25
	AR diagnosis of those tested	20.0%	2	25.0%	2	0	0	1

* Comparisons between isolated genitourinary and syndromic subjects using Fisher's exact test