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Research article

Can the external masculinization score predict the success of genetic testing in 46,XY DSD?

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Abstract: Genetic testing is judiciously applied to individuals with Disorders of Sex Development (DSD) and so it is necessary to identify those most likely to benefit from such testing. We hypothesized that the external masculinization score (EMS) is inversely associated with the likelihood of finding a pathogenic genetic variant. Patients with 46,XY DSD from a single institution evaluated from 1994–2014 were included. Results of advanced cytogenetic and gene sequencing tests were recorded. An EMS score (range 0–12) was assigned to each patient according to the team's initial external genitalia physical examination. During 1994–2011, 44 (40%) patients with 46,XY DSD were evaluated and underwent genetic testing beyond initial karyotype; 23% (10/44) had a genetic diagnosis made by gene sequencing or array. The median EMS score of those with an identified pathogenic variant was significantly different from those in whom no confirmed genetic cause was identified [median 3 (95% CI, 2–6) versus 6 (95% CI, 5–7), respectively ($p = 0.02$)], but limited to diagnoses of complete or partial androgen insensitivity (8/10) or 5α-reductase deficiency (2/10). In the modern cohort (2012–2014), the difference in median EMS in whom a genetic cause was or was not identified approached significance ($p = 0.05$, median 3 (95% CI, 0–7) versus 7 (95% CI, 6–9), respectively). When all patients from 1994–2014 are pooled, the EMS is significantly different amongst those with compared to those without a genetic cause (median EMS 3 vs. 6, $p < 0.02$). We conclude that an EMS of 3 or less may indicate a higher likelihood of identifying a genetic

cause of 46,XY DSD and justify genetic screening, especially when androgen insensitivity is suspected.

Keywords: disorders of sex development (DSD); external masculinization score (EMS); genetic testing; cytogenetics; 46,XY

1. Introduction

High-throughput sequencing methods have led the discovery of new genes and mutations in DSD and genetic analysis has firmly taken root in the clinical management of DSD [1]. Karyotype rather than phenotype is the focus in the new DSD classification scheme [2]. In practice however, genetic testing continues to be reserved and at the discretion of the medical geneticist.

The External Masculinization Score (EMS) (range 0–12) is a validated scoring system used to evaluate the degree of masculinization in an effort to standardize and succinctly communicate the phenotype of individuals with ambiguous genitalia. An EMS < 7 is considered ambiguous [3]. Since its introduction, the EMS has been widely integrated into the DSD assessment and literature. We hypothesized that the higher the discordance between phenotype and genotype, the greater the likelihood of identifying a genetic diagnosis. We retrospectively evaluated a cohort of individuals with 46,XY DSD and compared the EMS between those who did and did not have a confirmed genetic etiology for their genitourinary anomalies. We replicate our study with a recent cohort of 46,XY DSD patients evaluated from 2012–2014 using contemporary genetic techniques.

2. Methods and Materials

2.1. Historical cohort

This study cohort consisted of all patients referred to our institution during 1994–2011 who were evaluated by the multidisciplinary DSD team for abnormalities in sexual development. The most common reason for referral was ambiguous or atypical genitalia; delayed puberty was second most common. We excluded patients with sex chromosome DSD including 45,X/46,XY mosaicism and patients with large structural chromosomal abnormalities since in these cases, the genetic causes are detected on initial peripheral blood karyotype. Patients with incomplete medical records were also excluded.

A retrospective chart review was performed and the results of any genetic testing (ie: cytogenetic and gene sequencing tests beyond peripheral blood karyotype) was recorded. The general approach was determination of karyotype (including FISH for SRY) on all individuals who present to the clinic. Hormone studies done either in the minipuberty time period (if possible) or at puberty were also part of the initial visit. In those individuals who were prepubertal but older than 6 months of age, ACTH or HCG stimulation testing was performed to determine adrenal/pituitary/gonadal hormonal function. Array CGH was chosen as the initial test in those who had syndromic features or when a non-sex chromosomal abnormality was more likely. Based on the karyotype, hormone testing, physical examination, and imaging findings (i.e.: pelvic US to evaluate for mullerian structures), further targeted genetic testing was performed, but this was individualized based on the above. In

some instances, gonadal tissue was sent for touch prep FISH studies to evaluate for sex chromosomal mosaicism or chimerism. In selected cases before the year 2000, functional assessment of the androgen receptor was completed on a sample of tissue from the genitalia that was harvested at the time of surgery.

Subgroups of "No Genetic Variant Identified" and "Genetic Variant Identified" were formed based on whether this genetic testing yielded a diagnosis. Diagnostic categories outlined by the DSD Consensus Statement were used [2,4]. An EMS was assigned to each patient according to clinical data collected during the team's initial external genitalia physical examination [3]. If gonads were not palpable on physical examination, they were scored "0" ("unknown") even if they were later discovered to be intra-abdominal. The EMS system as originally described does not account for penoscrotal transposition [3]; in practice however, we have noted that the urethral meatus in penoscrotal hypospadias in the presence of penoscrotal transposition could be interpreted as being in the perineal position rather than penoscrotal. Therefore a penoscrotal hypospadias with or without penoscrotal transposition would be scored be as a "1". To score the presence or absence of micropenis in 46,XY DSD, regardless of the assigned sex, we used the stretched length of the clitorophallus and compared this measurement to 2.5 standard deviations below age matched normal values [5].

2.2. Modern cohort

This cohort includes all 46,XY DSD patients evaluated during 2012–2014. Since 2012, the team has prospectively assigned an EMS during the initial 46,XY DSD patient assessment. The genetic assessment consists of SNP probe/CGH array as the initial test for patients with ambiguous genitalia and dysmorphic features, developmental delay, or other congenital anomalies. If complete androgen insensitivity (CAIS) is suspected, we prefer single gene testing as the initial test. In the absence of any of these aforementioned scenarios, a DSD panel is ordered. The DSD panel is done at UCLA and consists of whole exome sequencing that is masked to return results for only those genes known to be involved in DSD. As of 2012, the primary gene list covers:AKR1C4, AMH, AMHR2, AR, ARX, ATRX, CBX2, CYP11A1, CYP17A1, CYP21A2, DHH, DMRT1, DMRT2, FGFR2, FOXL2, HSD17B3, HSD3B2, LHCGR, MAMLD1, MAP3K1, NR0B1, NR5A1, POR, RSP01, SOX3, SOX9, SRD5A2, SRY, STAR, WNT4, WT1.

Statistical analysis was done using a 2-sided Mann Whitney U-test for ordinal variables and t-test for continuous variables; $p < 0.05$ was considered significant.

3. Results

During 1994–2011, 122 patients with 46,XY DSD were evaluated. Five were excluded because they had surgery elsewhere prior to the team's initial assessment and their EMS would have been altered and noncomparable. Two were excluded because they had missing data on physical exam to assign an EMS. Forty-four (40%) underwent genetic testing beyond karyotype during their assessment by the multi-disciplinary DSD team. The median EMS of those who underwent genetic testing beyond karyotype was 2.5 (range 0–9) and for those who did not have genetic testing beyond karyotype the median EMS was 6.0 (range 0–12) ($p = 0.18$).

The EMS for all these patients are shown in Table 1. For all patients included in the final analysis,

Table 1. Genetic testing and EMS of 46,XY DSD subgroups, modern cohort.

a) Genetic Variant Identified

Diagnosis	Gene Sequenced	SNP/CGH array	DSD Panel	Other	EMS
Ambiguous genitalia with MCA		✓			8
Ambiguous genitalia with MCA		✓		Methylation testing	2
Ambiguous genitalia with MCA	*ZEB2* for Mowat-Wilson				3
Androgen biosynthesis defect			✓		2
Complete gonadal dysgenesis			✓		3
CAIS	*AR*				0
PAIS	*AR*				0
PAIS	*AR*				7
Partial gonadal dysgenesis			✓		3

b) No genetic variant identified

Diagnosis	SNP/CGH array	DSD Panel	EMS
Ambiguous genitalia with MCA	✓		9
Ambiguous genitalia with MCA	✓		0.5
Micropenis	✓		9
Micropenis		✓	7
Micropenis		✓	9
Severe hypospadias	✓		6
Severe hypospadias	✓		6
Severe hypospadias	✓		6
Severe hypospadias		✓	9

genetic testing consisted of advanced cytogenetic methods (FISH or array comparative genomic hybridization (aCGH)), single gene sequencing, or gene panel testing (whole exome sequencing masked to assess only those genes known to lead to a DSD phenotype, also called the "DSD panel"). Given that the DSD panel has only recently entered clinical practice, only 3 patients, all in the "No Genetic Variant Identified" group, underwent this testing (Table 1). The median ages at initial DSD evaluation were 2.6 years compared to 0.22 years for those with or without a genetic diagnosis, respectively ($p = 0.06$). Of those who had genetic testing beyond karyotype, a genetic cause was identified in 10 (23%) patients with a median EMS 3 (95% CI, 2–6) and not identified in 34 (77%) with a median EMS 6 (95% CI, 5–7), $p = 0.02$ (Figure 1). Of those with a pathogenic genetic finding, 80% had androgen insensitivity and 20% had 5-AR deficiency (Table 1). In all but one case of androgen insensitivity (AIS), a genetic diagnosis was obtained with single gene sequencing. On the

other hand, of those who did not have any pathogenic genetic finding, the diagnoses varied- ambiguous genitalia with multiple congenital anomalies [MCA] (4), hypogonadotropic hypogonadism (6), isolated hypospadias (9), micropenis (3), and mixed gonadal dysgenesis [MGD] (3) (Table 1).

During 2012–2014, 40 patients with 46,XY DSD were eligible and 18 (45%) underwent advanced genetic testing which included SNP arrays or the DSD panel in addition to single gene sequencing. Of these, 9 (50%) had a genetic cause of their 46,XY DSD identified and their median EMS was 3 (95% CI, 0–7) compared to a median EMS of 7 (95% CI, 6–9) for those who did not have a genetic cause identified ($p = 0.05$). If all patients from both cohorts are pooled together, the EMS is significantly different amongst those with compared to those without a genetic cause (median EMS 3 vs. 6, $p < 0.02$).

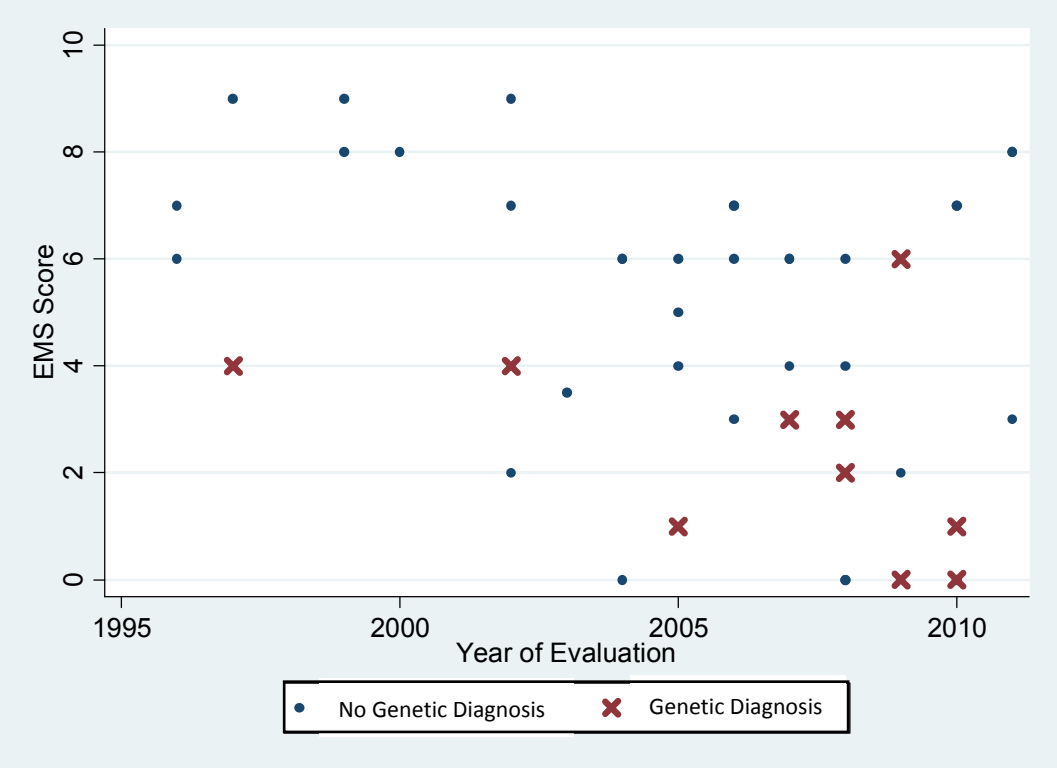

Figure 1. The EMS and results of genetic testing of the 46,XY DSD cohort.

4. Discussion

The molecular etiology of 46,XY DSD has historically been identified in only ~ 20% of cases [6] but with advances in genetic technology the rate of genetically diagnosing 46,XY DSD will likely increase. It is possible that in the future, those with an identifiable genetic cause will be categorically separated from those with no genetic cause identified and the classification of DSD will continue to evolve [7]. From 2012–2014, we saw a 50% success rate of genetic testing compared to 23% previously during 1994–2011.

Medical geneticists have long played an active role with the DSD team but genetic testing has and continues to be selectively applied due to many factors. The decision to perform genetic testing can be driven by clinical suspicion of the diagnosis, the likelihood of finding a genetic abnormality,

an inconclusive clinical evaluation, the impact of testing on management, and the desire of the family to more accurately define a recurrence risk. In order to establish and improve the yield of genetic testing, we evaluated our case series of 46,XY DSD and questioned whether a higher phenotype discordance from the 46,XY karyotype was associated with a pathogenic genetic finding.

The EMS is a standardized way of recording and conveying the degree of virilization on physical examination [3]. Deeb et al. evaluated whether the EMS could be used to distinguish between individuals with PAIS with or without a mutation in *AR* and did not find any correlation between genotype and phenotype in this group of patients [7]. Indeed, the phenotype of 46,XY DSD can widely vary even among individuals who have the same pathogenic variant. The EMS has also been applied to help guide clinical management of gonadectomy in individuals with 45,X/46,XY mosaicism; the degree of virilization is inversely correlated with tumor risk with a 52% incidence of tumor or preneoplastic gonadal markers in individuals with 45,X/46,XY who have an EMS < 7 compared to a 13% incidence in individuals with 45,XY/46,XY who have an EMS > 7 [8]. In our study, we applied the EMS to our cohort of individuals with 46,XY DSD to determine if a lower EMS (higher genotype-phenotype discordance) was associated with any identifiable genetic cause for 46,XY DSD. We found that the group who had an identifiable genetic cause during their DSD evaluation had a lower EMS compared to those without an identifiable genetic cause and this trend appears consistent between historical and modern cohorts despite changes in genetic technology over time.

It remains unclear which individuals with 46,XY DSD should undergo what type of initial genetic test. It is reasonable to assume that in some cases, genetic testing was done to confirm rather than to explore for an etiology for the 46,XY DSD. Only 50% of our modern cohort had a genetic variant identified and the testing carried out in this group was more homogenous, consisting of usually SNP arrays or the DSD panel. This rate reflects the complexity of 46,XY DSD encountered in clinical practice and the broad range of 46,XY DSD patients referred to a DSD team (Table 1 and Table 2). Furthermore, approaches to genetic testing in this patient population likely differ between

Table 2. Genetic testing and EMS of 46,XY DSD subgroups, historical cohort.

a) Genetic Variant Identified

Diagnosis	Gene Sequenced		aCGH	Other	EMS
	AR	5-AR			
CAIS	✓				0
CAIS	✓				1
CAIS	✓				1
CAIS	✓				1
CAIS	✓				2
5-AR Deficiency	✓	✓	✓		3
5-AR Deficiency	✓				6
PAIS	✓				4
PAIS	✓	✓			4
PAIS				AR binding	4

b) No genetic variant identified

Diagnosis	Gene Sequenced			aCGH	Other	EMS
	AR	5-AR	Other			
Ambiguous genitalia with MCA					FISH subtelomeres	3
Ambiguous genitalia with MCA				✓		6
Androgen Biosynthesis Defect					Touchprep gonad biopsy	5
Androgen Biosynthesis Defect			CDKN1C; DAX-1			4
CAIS	✓					0
Hypogonadotropic hypogonadism				✓	FISH Fragile X	6
Hypogonadotropic hypogonadism					FISH Kallman X region	6
Hypogonadotropic hypogonadism					FISH Kallman X region	9
Hypogonadotropic hypogonadism			DSD gene panel			9
Leydig Cell Hypoplasia	✓		Kallmann, LH receptor			2
MGD			DHA, NR5A1		FISH gonad touchprep	6
Micropenis				✓	FISH subtelomeres	3
Micropenis					Methylation for Prader-Willi	8
Micropenis		✓				8
PAIS	✓				FISH subtelomeres	0
PAIS	✓					2
PAIS		✓				6
PAIS	✓		DSD gene panel		AR binding	7
Severe Hypospadias	✓		Y chromo-some			7
Severe Hypospadias			Y chromo-some			7
Severe Hypospadias					SKY analysis	7
Severe Hypospadias			WT1			8
Severe Hypospadias	✓					6
Severe Hypospadias	✓					6
Severe Hypospadias			DSD gene panel			4
Severe Hypospadias		✓				7
Severe Hypospadias		✓				7
Severe Hypospadias, 2 UDT					FISH gonad touchprep	6
Severe Hypospadias, 2 UDT	✓	✓	SRY, HLXB9			4
Severe Hypospadias, 2 UDT	✓	✓				2
Vanishing Testes				Fragile X	FISH subtelomeres	0
Vanishing Testes					FISH gonad touchprep	9

centers. For example, Baetens et al. evaluated 32 children with 46,XY DSD using aCGH, targeted copy number analysis of *SRY*, *SOX9*, *NROB1*, *WNT3*, and *NR5A1*, targeted sequencing of *AR*, *NR5A1*, *WT1*, and as indicated, *SRY*, *HSD18B3*, *and SRD5A2genes*. Applying this type of screening method, they did not find any difference in diagnostic yield between patients with more (EMS > 7) or less (EMS < 7) severe phenotypes but their study base was restricted to 46,XY children younger than 2 years with atypical genitalia who were assigned the male sex [9].

The type of genetic testing performed in individuals with a DSD diagnosis has evolved to include advanced cytogenetics and gene panel testing [1]. Single gene testing, however, remains relevant in this field, especially when androgen insensitivity is suspected. One patient with AIS evaluated in 1997 had this diagnosis confirmed with AR binding studies, although this mode of evaluation has since been replaced with gene sequencing or gene panel testing. Given the lag time for new testing to translate into clinical practice, only three patients in the historical cohort had a gene panel performed but no genetic cause for their features was identified.

Our study is limited by the fact that genetic testing was individualized for each patient and based on different immeasurable or non-standardized factors which affect genetic testing. Genetic testing depends on the individual's hormonal evaluation, expert opinion, insurance authorization, and the availability of genetic tests. Clinical genetics evaluations in our DSD program were managed between 3 faculties and the rationale for testing was not measured and likely varied. For example, if AIS was suspected such as in an apparent female with delayed menarche, elevated testosterone levels, and 46,XY chromosome as well as young girls with 46,XY karyotype and Sertoli cell function. Then genetic testing in this case would have been to confirm rather than to explore for a genetic cause. PAIS was suspected in 46,XY individuals with serum testosterone, LH, and FSH within normal range for a pubertal male with decreased response to testosterone and exclusion of other 46,XY DSD disorders. 5-alpha-reductase deficiency was suggested by an elevated testosterone: dihydrotestosterone ratio greater than 20:1 in individuals raised male or in prepubertal females who have Sertoli cell function, no evidence for testosterone biosynthesis defect, and normal androgen receptor. Our current approach is that if there is no apparent high-yield single gene testing based on clinical history, exam, family history, hormone testing, and imaging, then a panel test is recommended (i.e.: 13 gene Flugent panel for hypogonadotropic hypogonadism or DSD panel. Often times though additional genetic testing while recommended cannot be performed because of lack of insurance authorization.

Another potential confounder that we were unable to adjust for was the differential likelihood of genetic testing yield associated with each 46,XY diagnosis. For example, 80% of women with CAIS and 30% of individuals with PAIS will have an identifiable pathogenic variant in AR [10] compared to the polygenetic and multifactorial etiologies for isolated severe hypospadias or hypogonadotropic hypogonadism. It is possible that cases without a pathogenic variant identified were classified as such simply due to lack of an appropriate test. Future studies of genotype-phenotype correlation could improve upon these points by clarifying the rationale for testing and ensuring that groups to be compared share the same potential outcome for genetic testing (i.e.: similar hormonal profiles).

Despite these limitations, our observations are based on a team's 30 years' experience and suggest that a lower EMS may be associated with genetic testing yield. This study informs on how we can improve the genetic testing pathway for 46,XY DSD patients. As sequencing technologies continue to improve and disseminate, our study suggests the EMS may be useful in guiding who and which genetic testing will be useful (i.e.: is it necessary to test for 35 genetic variants when AR or

5AR sequencing would arrive at the same finding?). This is particularly helpful in today's climate of insurance denials for genetic testing and laboratory genetic testing utilization analyses. Until it is standard practice to screen all individuals with 46,XY DSD for a genetic cause, the yield of testing may be guided by the EMS whereby identifying a genetic diagnosis is most likely with a score of 3 or less.

5. Conclusion

Genetic screening may be justified in cases of 46,XY DSD with an EMS of 3 or less given a higher likelihood of identifying a genetic cause.

Conflict of Interest

All authors declare no conflict of interest in this paper.

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