Low frequency of pathogenic allelic variants in the 46,XY differences of sex development (DSD)-related genes in small for gestational age children with hypospadias

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Abstract

Background: Hypospadias is a congenital disorder of male genital formation where the urinary opening is not on the head of the penis and genetic factors play an important role in the incidence of this early developmental defect in 46,XY individuals, in both isolated and syndromic forms. Children born small for gestational age (SGA) present a high frequency of hypospadias of undetermined etiology, ranging from 15 to 30%, but the detection of hypospadias' etiology remains low. **Patients and methods**: from a cohort of 46,XY DSD patients, we identified 25 SGA children with medium or proximal hypospadias; four of them with associated syndromic characteristics. DNA samples from subjects were studied by massively parallel targeted sequencing (MPTS) using a targeted panel. MLPA was used for molecular diagnosis in two children with clinical phenotype of Silver Russel syndrome. **Results**: Loss of DNA methylation (11p15 LOM) at ICR1 was identified in two out of four syndromic children. The other syndromic patient had 3M syndrome phenotype and two novel likely-pathogenic variants in compound heterozygous state were found in *CUL7* gene. The last syndromic subject had Mulibrey nanism and, a homozygous variant in *TRIM37* was identified in the patient and confirmed in heterozygous state in the mother. Among non-syndromic children seven rare heterozygous variants with uncertain significance in six DSD-related genes were identified: two children had *DHX37* variants associated with *GATA4* and *WWOX* variants, respectively; three children had heterozygous variants, in *WT1*, *IGF1R*, and *BMP8B* genes. **Conclusion:** Pathogenic or likely-pathogenic variants in DSD-related genes were not identified in non-syndromic SGA children with hypospadias, suggesting that multi-factorial causes, unknown genes or unidentified environmental factors (epigenetic defects), may be involved in the etiology of this condition.

Introduction

The child born small for gestational age (SGA) is defined as the one with birth weight and/or length 2 or more standard deviation (SD) below the population mean for gestational age. Approximately 10-15% of them do not recover growth in postnatal life. The underlying causes of pre and postnatal growth retardation are diverse including known genetic syndromes, such as Silver-Russell syndrome (SRS). However, hypospadias are presented even in cases that cannot be categorized in a particular syndrome (1,2). SRS is a clinical and (epi)genetic heterogeneous syndrome characterized primarily by pre and postnatal growth retardation. The most common molecular mechanisms are loss of methylation on chromosome 11p15 (11p15 LOM), occurring in 30-60% of patients. Some genotype-phenotype studies showed 11p15 LOM patients presenting more typical and severe presentations (3,4,5). Among the variable signs in patients with SRS, male genital abnormalities are presented in about 40% of the male patients with this syndrome (6). It is interesting to note that SGA patients also present a high frequency of genital atypia of undetermined etiology, ranging from 15 to 30% (7). Genetic factors play an important role in the incidence of hypospadias, but the detection of hypospadias' etiology remains low (8). Our aim is to investigate the genetic cause underlining hypospadias in syndromic and non-syndromic SGA children.

Materials and methods

Within a cohort of 272 subjects with 46,XY differences of sex development (DSD) followed in Clinical Hospital of University of São Paulo 59 have 46,XY DSD due to unknown cause. Among them, 30 are SGA children with medium or proximal hypospadias and were invited to participate in the study and 25 agreed to participate. Four of them had

associated syndromic characteristics. This study was approved by the local medical ethics committee and patients and/or guardians gave their informed written consent. All patients have 46,XY karyotype in at least 30 analyzed cells obtained from peripheral blood. DNA samples from subjects and their families were obtained from peripheral blood leukocytes and were studied by massively parallel targeted sequencing (MPTS) using a targeted panel.

Two versions of the custom panel of target genes were designed using the *Agilent SureDesign 2.0 tool* (Agilent Technologies, Santa Clara, CA, USA) (Table 1). Genes that participate in the process of sex development and growth were included. Exonic regions of the target genes were included, including an area up to 25 bp before and after each exon.

The panel-based sequencing was performed in the Illumina MiSEQ platform (Illumina, Inc., San Diego, CA, USA). Paired-end reads were aligned to the hg19 assembly of the human genome with BWA-MEM. Variants were called and annotated with Platypus and ANNOVAR. Sanger sequencing was used to segregate the variants in the family. Multiplex Ligation-dependent Probe Amplification specific for Silver-Russel and Beckwith-Wideman syndromes (SALSA MLPA ME030 BWS/RSS) (MRC-Holland, Amsterdam, Netherlands) was used for molecular diagnosis in both children with SRS phenotype. The identified variants were classified according to American College of Medical Genetics (ACMG) criteria (9).

The targeted panel sequencing data were screened for rare variants (minor allele frequency < 0.1% in the public databases: Genome Aggregation Database (gnomAD) (10,11), 1000 Genomes (12), and in the Brazilian population database (ABraOM) (13), located in exonic and consensus splice site regions. Subsequently, the filtration pipeline prioritized potentially pathogenic candidate variants (loss of function variants and variants classified as pathogenic by at least three *in silico* programs).

The sequencing reads carrying candidate variants were visually confirmed using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA). Candidate variants were segregated in the available family members by Sanger method.

Results

Syndromic SGA children: The 11p15 LOM was identified in two clinical SRS children by MLPA, confirming the clinical diagnosis of SRS. The other syndromic patient has 3M syndrome phenotype, and presents two likely pathogenic variants (p.Trp1622* and p.Gln897Hisfs*23) in compound heterozygosis state in *CUL7* gene. Sanger sequencing confirmed the heterozygous p.Gln813fs variant in his mother. The child is a SGA boy, born from non-consanguineous parents, with disproportionate short stature, proximal hypospadias, unusual facial features (hypoplastic midface; short, broad neck). The last syndromic subject is a SGA child, born from consanguineous parents, with triangular face, hypoplastic midface, frontal bossing, short stature, proximal hypospadias, feeding difficulties, and early respiratory distress. The homozygous p.Tyr341Ilefs*16 variant in *TRIM37* was identified in the patient. Sanger sequencing confirmed the heterozygous p.Tyr341Ilefs*16 variant in his mother (Table 2).

Non-syndromic SGA children: Seven rare heterozygous variants with uncertain significance in six DSD-related genes were identified in five patients: *DHX37* variants (p. Val717Ile and p.Ala737Thr) were found associated with *GATA4* p.Pro407Arg and with *WWOX* p.Ttyr85Asp, respectively, in two children; three children each have heterozygous variants, the *WT1* p.Cys350Arg, the *IGF1R* p.Arg1337Cys, and the *BMP8B* p.Arg116Cys variant (Table 3). Sanger sequencing confirmed the occurrence of the heterozygous variant *BMP8B* p.Arg116Cys in the patient's mother.

Discussion

SGA patients present a high frequency of hypospadias without a known etiology (8). Some studies believe that this low diagnosis could be explained because this condition has yet unknown genes and/or environmental factors involved (14, 15, 16). The present study investigated a possible molecular cause of hypospadias in syndromic and non-syndromic children.

In the syndromic SGA patients with hypospadias, the molecular diagnosis confirmed the clinical phenotype. Defects in *CUL7* gene are a known cause of 3M syndrome type 1, confirming the clinical features of the patient. The protein encoded by *CUL7* interacts with *TP53*, *CUL9*, and *FBXW8* proteins. *TRIM37* encodes a protein of the tripartite motif (TRIM) family, whose members are involved in diverse cellular functions such as developmental patterning and oncogenesis; mutations in *TRIM37* are associated with Mulibrey nanism, a serious autosomal recessive disorder. No defect in other gene associated with 46,XY DSD diagnosis was identified in the syndromic children.

SRS patients are characterized by a large spectrum of features and signs, varying from complex to milder phenotypes. Several clinical scoring systems have been proposed in the last few years. The First International Consensus Statement on diagnosis and management of SRS indicated the use of the Netchine-Harbison clinical scoring system (NH-CSS) for SRS clinical diagnosis. The most common molecular mechanisms are loss of methylation on chromosome 11p15 (11p15 LOM), occurring in 30-60% of patients, and maternal uniparental disomy for chromosome 7 (upd(7)mat), occurring in 5-10% of patients (3, 4, 5). Among the variable signs in patients with SRS, male genital abnormalities are presented in about 40% of the male patients with this syndrome (6). It is interesting to note that SGA patients also present a high frequency of genital atypia, ranging from 15 to 30%, of undetermined etiology (7). We

hypothesized that genital atypia in SRS is not directly related to the epigenetic cause of the syndrome but it could share the same genetic mechanism of hypospadias in SGA children.

Studies of methylation role in specific disorders, as well as its influence on phenotypes would be helpful to increase our understanding about external genitalia development, contributing to expand the molecular etiology of hypospadias.

In non-syndromic SGA children, seven rare heterozygous variants with uncertain significance in six DSD-related genes were identified in these patients. The causes of hypospadias could be attributed to defects in genetic factors or considered syndrome-associated hypospadias (14, 17, 18). However, the association of these variants of uncertain significance with the phenotype could not be established in the absence of functional studies.

Conclusion

In conclusion no proven genetic defects clarify the etiology of hypospadias in syndromic and non-syndromic SGA children, suggesting that multi-factorial causes, unknown genes or unidentified epigenetic defects, may be involved in the etiology of this condition.

References

1. LEE, P. A., et al. International Small for Gestational Age Advisory Board consensus development conference statement: management of short children born small for gestational age, april. 2001. Pediatrics 111.6 (2003): 1253-1261.
2. CLAYTON, P. E. et al. Management of the Child Born Small for Gestational Age through to Adulthood: A Consensus Statement of the International Societies of Pediatric Endocrinology and the Growth Hormone Research Society. The Journal Of Clinical Endocrinology & Metabolism, [s.l.], v. 92, n. 3, p.804-810, mar. 2007. The Endocrine Society. http://dx.doi.org/10.1210/jc.2006-2017.
3. BARTHOLDI, D et al. Epigenetic mutations of the imprinted IGF2-H19 domain in Silver-Russell syndrome (SRS): results from a large cohort of patients with SRS and SRS-like phenotypes. Journal Of Medical Genetics, [s.l.], v. 46, n. 3, p.192-197, 18 mar. 2009. BMJ. http://dx.doi.org/10.1136/jmg.2008.061820.

4. BRUCE, Sara et al. Clinically Distinct Epigenetic Subgroups in Silver-Russell Syndrome: The Degree ofH19Hypomethylation Associates with Phenotype Severity and Genital and Skeletal Anomalies. The Journal Of Clinical Endocrinology & Metabolism, [s.l.], v. 94, n. 2, p.579-587, fev. 2009. The Endocrine Society. http://dx.doi.org/10.1210/jc.2008-1805.
5. EGGERMANN, T. et al. Broad Clinical Spectrum in Silver-Russell Syndrome and Consequences for Genetic Testing in Growth Retardation. Pediatrics, [s.l.], v. 123, n. 5, p.929-931, 13 abr. 2009. American Academy of Pediatrics (AAP). http://dx.doi.org/10.1542/peds.2008-3228.
6. Wakeling EL, Brioude F, Lokulo-Sodipe O, O'Connell SM, Salem J, Bliek J, Canton AP, Chrzanowska KH, Davies JH, Dias RP, Dubern B. Diagnosis and management of Silver–Russell syndrome: first international consensus statement. Nature Reviews Endocrinology. 2017 Feb;13(2):105.
7. MOREL, Y et al. Aetiological diagnosis of male sex ambiguity: a collaborative study. European Journal Pediatric, [s. L.], v. 161, n. 1, p.49-59, jan. 2002.
8. KALFA, Nicolas; PHILIBERT, Pascal; SULTAN, Charles. Is hypospadias a genetic, endocrine or environmental disease, or still an unexplained malformation? International Journal Of Andrology, [s.l.], v. 32, n. 3, p.187-197, jun. 2009. Wiley. http://dx.doi.org/10.1111/j.1365-2605.2008.00899.x.
9. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, Committee ALQA. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405-424
10. Brownstein CA, Beggs AH, Homer N, Merriman B, Yu TW, Flannery KC, Dechene ET, Towne MC, Savage SK, Price EN, Holm IA, Luquette LJ, Lyon E, Majzoub J, Neupert P, McCallie D, Jr., Szolovits P, Willard HF, Mendelsohn NJ, Temme R, Finkel RS, Yum SW, Medne L, Sunyaev SR, Adzhubey I, Cassa CA, de Bakker PI, Duzkale H, Dworzy Ski P, Fairbrother W, Francioli L, Funke BH, Giovanni MA, Handsaker RE, Lage K, Lebo MS, Lek M, Leshchiner I, Macarthur DG, McLaughlin HM, Murray MF, Pers TH, Polak PP, Raychaudhuri S, Rehm HL, Soemedi R, Stitziel NO, Vestecka S, Supper J, Gugenmus C, Klocke B, Hahn A, Schubach M, Menzel M, Biskup S, Freisinger P, Deng M, Braun M, Perner S, Smith RJ, Andorf JL, Huang J, Ryckman K, Sheffield VC, Stone EM, Bair T, Black-Ziegelbein EA, Braun TA, Darbro B, Deluca AP, Kolbe DL, Scheetz TE, Shearer AE, Sompallae R, Wang K, Bassuk AG, Edens E, Mathews K, Moore SA, Shchelochkov OA, Trapane P, Bossler A, Campbell CA, Heusel JW, Kwitek A, Maga T, Panzer K, Wassink T, Van Daele D, Azaiez H, Booth K, Meyer N, Segal MM, Williams MS, Tromp G, White P, Corsmeier D, Fitzgerald-Butt S, Herman G, Lamb-Thrush D, McBride KL, Newsom D, Pierson CR, Rakowsky AT, Maver A, Lovre IL, Palanda IA, Peterlin B, Torkamani A, Wedell A, Huss M, Alexeyenko A, Lindvall JM, Magnusson M, Nilsson D, Stranneheim H, Taylan F, Gilissen C, Hoischen A, van Bon B, Yntema H, Nelen M, Zhang W, Sager J, Zhang L, Blair K, Kural D, Cariaso M, Lennon GG, Javed A, Agrawal S, Ng PC, Sandhu KS, Krishna S, Veeramachaneni V, Isakov O, Halperin E, Friedman E, Shomron N, Glusman G, Roach JC, Caballero J, Cox HC, Mauldin D, Ament SA, Rowen L, Richards DR, Lucas FA, Gonzalez-Garay ML, Caskey CT, Bai Y, Huang Y, Fang F, Zhang Y, Wang Z, Barrera J, Garcia-Lobo JM, Gonzalez-

Lamuno D, Llorca J, Rodriguez MC, Varela I, Reese MG, De La Vega FM, Kiruluta E, Cargill M, Hart RK, Sorenson JM, Lyon GJ, Stevenson DA, Bray BE, Moore BM, Eilbeck K, Yandell M, Zhao H, Hou L, Chen X, Yan X, Chen M, Li C, Yang C, Gunel M, Li P, Kong Y, Alexander AC, Albertyn ZI, Boycott KM, Bulman DE, Gordon PM, Innes AM, Knoppers BM, Majewski J, Marshall CR, Parboosingh JS, Sawyer SL, Samuels ME, Schwartzentruber J, Kohane IS, Margulies DM. An international effort towards developing standards for best practices in analysis, interpretation and reporting of clinical genome sequencing results in the CLARITY Challenge. Genome Biol.15(3):R53.

11. http://gnomad.broadinstitute.org/.
12. Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR, Consortium GP. A global reference for human genetic variation. *Nature*. 2015;526(7571):68-74.
13. Naslavsky MS, Yamamoto GL, de Almeida TF, Ezquina SAM, Sunaga DY, Pho N, Bozoklian D, Sandberg TOM, Brito LA, Lazar M, Bernardo DV, Amaro E, Duarte YAO, Lebrão ML, Passos-Bueno MR, Zatz M. Exomic variants of an elderly cohort of Brazilians in the ABraOM database. *Hum Mutat*. 2017;38(7):751-763.
14. Baskin LS, Ebbers MB. Hypospadias: anatomy, etiology, and technique. Journal of pediatric surgery. 2006 Mar 1;41(3):463-72.
15. Bouty A, Ayers KL, Pask A, Heloury Y, Sinclair AH. The genetic and environmental factors underlying hypospadias. Sexual Development. 2015;9(5):239-59.
16. Joodi M, Amerizadeh F, Hassanian SM, Erfani M, Ghayour-Mobarhan M, Ferns GA, Khazaei M, Avan A. The genetic factors contributing to hypospadias and their clinical utility in its diagnosis. Journal of cellular physiology. 2019 May;234(5):5519-23.
17. Van der Horst HJ, De Wall LL. Hypospadias, all there is to know. European journal of pediatrics. 2017 Apr 1;176(4):435-41.
18. MANSON JM, CARR MC. Molecular epidemiology of hypospadias: review of genetic and environmental risk factors. Birth Defects Research Part A: Clinical and Molecular Teratology. 2003 Oct;67(10):825-36.

Table 1. Genes included in both custom panels.

Target genes											
ACBD7	ARX	BMP15	CYP11A1	DND1	FST	HNF1B	KATNBL1	NANOS3	POR	SOX10	SUPT3H
ACVR1B	ATRX	**BMP8B***	CYP17A1	ESR1	GADD45G	HPGDS	LEF1	NCOA1	PRKACG	SOX13	TCF21
ACVRL1	BBS1	BMPR1B	CYP19A1	FAM189A2	GATA4	HSD11B1	LGR5	NCOA2	PTGDS	SOX7	TRIM32
AKR1C2	BBS10	CAMK1D	CYP26B1	FBLN2	GATA6	HSD17B3	LHCGR	NCOA3	PUM2	SOX9	**TRIM37**
AKR1C3	BBS12	CBLN1	DHCR7	FGD1	GDF9	HSD3B2	LHX1	NR0B1	RSPO1	SRA1	UBE3A
AKR1C4	BBS2	CHD7	DHH	FGF9	GDNF	**IGF1R**	MAMLD1	NR3C1	RSPO2	SRD5A2	USP34
AMH	BBS4	CITED2	DHX37	FGFR2	GPR56	IHH	MAP3K1	NR5A1	SIX1	SRY	WT1
AMHR2	BBS5	CITED4	DMRT1	FOXL2	GPR83	INHBB	MKKS	PAPPA	SIX4	STAG3	WWOX
AR	BBS7	CTNNB1	DMRT2	FRAT1	GTF2F1	IRX3	MSX1	PDGFA	SMAD4	STAR	ZFPM2
ARL6	BBS9	**CUL7**	DNAJC15	FSHR	HHAT	KATNAL1	NANOS2	PDGFRB	SMARCE1	STIM1	

*In bold, genes included only in the second version of the panel with significant variants.

Table 2. Variants found in syndromic children with hypospadias

Syndromic subject	Phenotype	Diagnosis	Gene	Variant	Protein
1	Growth failure, feeding difficulties, body asymmetry, protunding forehead, hypospadias	Silver-Russell syndrome	*H19* DMR	11p15 LOM	-
2	Growth failure, feeding difficulties, body asymmetry, protunding forehead, hypospadias	Silver-Russell syndrome	*H19* DMR	11p15 LOM	-
3	Proximal hypospadias, ocular hypertelorism, short nasal base, short neck, low cartilage ear pavement, small hands, single palmar fold, clinodactyly; difficulty growing and weight gain from birth	3M syndrome	*CUL7*	c.4866G>A and c.2691delA	p.Trp1622* and p.Gln897Hisfs*23
4	Proximal hypospadias, triangular face, frontal bossa, middle face hypoplasia, enlarged filter,artiuclar foruxitude, hypospadias, and short stature	Mulibrey nanism	*TRIM37*	c.1020delC	p.Tyr341Ilefs*16

Table 3. Uncertain significance rare heterozygous variants in SGA children with proximal hypospadias

Subject	Phenotype	Gene	Variants	Protein
1	Proximal hypospadias	*WT1*	c.1048T>C	p.Cys350Arg
2	Proximal hypospadias, horizontal nystagmus, discrete epicanthus, broad nose base, umbilical and inguinal hernia, triangular face, weight gain difficulty	*IGF1R*	c.4009C>T	p.Arg1337Cys
3	Proximal hypospadias	*BMP8B*	c.346C>T	p.Arg116Cys
4	Proximal hypospadias, micropenis and bilateral cryptorchidism	*DHX37*	c.2149G>A	p. Val717Ile
		GATA4	c.1220C>G	p.Pro407Arg
5	Proximal hypospadias and bilateral cryptorchidism	*DHX37*	c.2209G>A	p.Ala737Thr
		WWOX	c.253T>G	p.Tyr85Asp