

ORIGINAL ARTICLE

Molecular diagnosis in hypospadias



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Received 15 July 2016; accepted 4 October 2016

Available online 14 October 2017

KEYWORDS

Hypospadias;
Penis;
Malformation;
Congenital;
Diagnosis;
Genetic;
Molecular

Abstract

Objective: The aim of this study is to perform a systematic review of the principal genetic and molecular diagnostic methods for hypospadias and their usefulness.

Methods: A Pubmed and EMBASE search was carried out using the following MESH terms: "molecular diagnosis", "genes", "hypospadias", "karyotype", "wgs", "fish", "chg", "sanger", "microarray", "mps", "wes", and "gwas".

Meta-analyses, systematic reviews, Cochrane reviews, clinical trials, narrative reviews and case series were included, between 2001 and 2016, in both Spanish and English. A total of 33 items were selected for review after reviewing titles, abstracts, and cross references.

Results: Hypospadias are the birth defect of the ventral aspect of the penis, accompanied by an ectopic location of the urethral meatus. 30% of all birth malformations in the newborns are urological malformations make up 30% of all birth malformations in newborns. Within the genetic and molecular test available for diagnosis, many are of varying usefulness. These include, among others, karyotyping, FISH, and Sanger sequencing.

Conclusions: Due to advances in technology, there are multiple molecular diagnosis methods that can widen the knowledge of the etiology of hypospadias. They also allow them to be used in the everyday practice for a complete study of patients.

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DOI of original article: <http://dx.doi.org/10.1016/j.uroco.2016.10.002>

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<http://dx.doi.org/10.1016/j.uroco.2017.09.006>

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PALABRAS CLAVE

Hipospadias;
Pene;
Malformación;
Congénita;
Diagnóstico;
Genético;
Molecular

Diagnóstico molecular en hipospadias**Resumen**

Objetivo: Realizar una revisión de la literatura sobre los principales métodos de diagnóstico de las hipospadias a nivel genético y molecular, y su utilidad en esta patología.

Métodos: Se realizó una búsqueda en las diferentes bases de datos como Pubmed y EMBASE con los siguientes términos MESH y sus combinaciones: «molecular diagnosis», «genes», «hipospadias», «karyotype», «wgs», «fish», «chg», «sanger», «microarray», «mps», «wes» y «gwas».

Se incluyeron artículos de metaanálisis, revisiones sistemáticas, revisiones de Cochrane, ensayos clínicos, revisiones narrativas y series de casos, entre 2001 y 2016, tanto en idioma español como en inglés. Se escogieron 33 artículos a partir de títulos, abstracts y referencias cruzadas que fueron incluidos dentro de esta revisión.

Resultados: Las hipospadias son el defecto en el desarrollo del aspecto ventral del pene acompañado de una ubicación ectópica del meato uretral. El 30% de las malformaciones congénitas de los recién nacidos corresponden a una malformación urológica. Dentro de las pruebas genéticas y moleculares que hay disponibles para su diagnóstico, existen múltiples de ellas de utilidad variable. Estas son el cariotipo, el FISH, la secuenciación de Sanger, entre otras.

Conclusiones: Gracias al avance de la tecnología, son múltiples los métodos de diagnóstico a nivel molecular que han permitido ampliar el conocimiento sobre las causas de hipospadias. Además, permiten en el ámbito de la práctica clínica diaria utilizarlos para realizar un estudio completo de los pacientes.

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Introduction

Hypospadias is a defect in the development of the ventral aspect of the penis, accompanied by an ectopic location of the urethral meatus.¹ It occurs in one in 250 live births, with a prevalence ranging from 4 to 43 cases per 10,000 births.^{2,3} Of the annual births, 2–3% present with some type of congenital malformation, among which urological malformations represent about 30%.⁴ In Colombia, the incidence of urological malformations has been shown to oscillate between 0.25 and 0.43%, hypospadias and cryptorchidism being the most frequent ones among them.⁴ According to data from the Colombian National Institute of Health, 48 cases of hypospadias were reported in 2012, corresponding to 1.99% of all congenital anomalies for the same year.⁵ Hypospadias has been linked to about 200 genetic syndromes.⁶

Taking into account the genes involved in the development of hypospadias, this article aims to review the literature on the main diagnostic methods for hypospadias at the genetic and molecular level and their usefulness.

Materials and methods

A Pubmed and EMBASE search was carried out using the following MeSH terms: “molecular diagnosis,” “genes,” “hypospadias,” “karyotyping,” “WGS,” “FISH,” “CGH,” “Sanger,” “microarray,” “MPS,” “WES,” and “GWAS.”

We included meta-analyses, systematic reviews, Cochrane reviews, clinical trials, narrative reviews, and case series from 2001 to 2016, in both Spanish and English.

We selected 33 articles based on titles, abstracts and cross references, which were included in the present review.

Results and discussion**Embryology and associated genes**

Urethral development begins with the formation of the urogenital sinus during the sixth week of gestation, followed by the growth of the genital tubercle (GT), which occurs in a proximal–distal and dorsal–ventral manner, along with the formation of the urethral plate and epithelial tubulogenesis. GT, together with the urethral plate epithelium (UPE), has a polarizing activity, which guides mesenchymal and epithelial interactions.⁷

Sonic Hedgehog Protein expressed in the UPE is required for the initiation and growth of GT.³ Proteins, such as homeobox (HOX) and Sonic Hedgehog, guide and regulate the whole process. Fibroblast growth factors (FGFs) and Wnt5 promote development and growth, while bone morphogenetic proteins (BMPs) play a role in apoptosis.^{3,7}

The testicle develops the ability to produce androgens, which promote the organogenesis of the male reproductive tract and intervene in the fusion of the urethral folds.

The masculinization of the external genitalia is triggered mainly by dihydrotestosterone, where the genital tubercle differentiates into glans, the urethral folds into the body of the penis, and genital eminences into the scrotum. Simultaneously, Sertoli cells secrete anti-Müllerian hormone, which induces the regression of the Müllerian duct. These cells are

essential for the functioning of Leydig cells and seminiferous tubules.⁸

The HOXA13 gene is an important regulator in the embryological process and its mutations; it is responsible for syndromes such as the hand-foot-genital syndrome, which includes hypospadias as a manifestation. This gene is expressed in the mesenchyme of the GT and UPE. Studies in mice have been able to confirm how the loss of HOXA13 function affects the formation and proliferation of the mesenchyme surrounding these structures, with subsequent consequences on the distal fusion of the meatus. Likewise, it has been proven to be essential for programmed cell death, and necessary for the fusion, growth and closure of the penile urethra.⁹

Fibroblast growth factor 8 (FGF8) or androgen-induced growth factor is expressed during the initial growth of the GT and UPE, increasing the expression of FGF10, BMP and HOXD. Alterations of these genes disrupt the maturation of the urethral epithelium, predisposing to hypospadias.⁷

The androgen receptor contains a transcription initiation region called CpG islands. These CpG islands are important since they are DNA methylation sites, which play a key role in the control of gene expression and activity, genomic imprinting and X-chromosome inactivation. Hypermethylation of CpG islands in the promoter region of the steroid receptor gene has been associated with the transcriptional inactivation of genes, and is functionally seen as an inactivation mutation, which may result in altered virilization.¹⁰

The gene encoding SRD5A, essential for the production of 5 α -reductase enzyme, is important because this enzyme is expressed during male genital development around the ventral portion of the remodeling urethra and it converts testosterone to its more potent form, dihydrotestosterone, which induces the formation of the external genitalia.³

Genes responsible for the balance between androgens and estrogens appear to be important. Estrogen receptors ESR1 and ESR2 are expressed in the developing genital tubercle, and different SNPs in the encoding genes have been associated with hypospadias.⁷ In turn, activating transcription factor 3 (ATF3) is an estrogen-responsive gene that shows strong up-regulation in patients with hypospadias.

Since the WT1 and SF1 genes are essential for the embryonic development of the renal and urogenital systems, mutations in these genes cause more severe alterations. SF1 mutations were found in severe penoscrotal hypospadias cases with cryptorchidism, while WT1 mutations have been described in cases of penoscrotal hypospadias and micropenis.³

Etiology

Hypospadias can be syndromic or non-syndromic. 10–30% of the cases are attributed to known genetic syndromes.^{11,12} The vast majority have idiopathic, multifactorial origin, with genetic, hormonal and environmental alterations.^{6,12} Exposure to environmental chemicals causes epigenetic dysregulation in the fetus, altering acetylation or normal methylation of genes.⁸ Up to 30% of hypospadias have identified molecular causes.³

Alterations can be found in genes responsible for phallic development, gonadal synthesis of steroids, or the response

to these hormones and their receptors.¹³ Genetic theory is reinforced by the fact that hypospadias is seen in family groups in about 10% of cases, and recurrence in male children of affected parents in 15% of cases.^{2,8,13} On the other hand, genetic alterations can be inherited in 57–77%, with equal transmission through maternal or paternal lines.¹¹

Molecular studies

Karyotyping: It should be used as an initial diagnostic approach in any patient presenting with syndromic or non-syndromic hypospadias. This recommendation is due to the fact that chromosomal alterations can be evidenced in 3% of male patients with genital anomalies associated with isolated cryptorchidism, in 7% with hypospadias, and in 13% presenting with a combination of both.¹⁴ It is essential in the study of disorders of sex development (DSD) associated with hypospadias, such as Klinefelter syndrome, which has an XXY karyotype, hypospadias with micropenis with XXY karyotype,¹⁵ or 46,XX testicular disorder of sex development (a rare genetic syndrome associated with microorchidism, hypospadias, and gynecomastia).¹⁶ Karyotyping also helps in the diagnosis of mixed gonadal dysgenesis or 45,X/46,XY mosaicism, which occurs with severe hypospadias associated with other genital malformations.¹⁷ In these cases, early diagnosis is essential because the latter condition is related to an increased risk of malignancy by 20%.¹⁸

Fluorescence in situ hybridization (FISH): It detects nucleotide sequences in cells or tissues. There are few case reports in the literature that use this technique as a diagnostic method. The most relevant findings found to cause hypospadias are: (3;4) translocation,¹⁹ dicentric Y chromosome,²⁰ or partial interstitial deletion of the long arm of chromosome 1.²¹ All these etiologies were found in case reports of patients with surgically difficult-to-treat hypospadias, therefore it may be a diagnostic option for patients who needed multiple surgical corrections in search of these syndromes. Its use is not recommended in isolated hypospadias cases.

Comparative genomic hybridization (CGH): It allows the detection of amplifications and deletions in the smallest chromosomal regions. However, it does not detect balanced mutations.²² This technique allows the detection of trisomies and large chromosomal abnormalities (such as karyotype), but it also detects small, submicroscopic imbalances such as deletions, duplications or triplications.²³ It has been shown that the use of CGH has increased the ability to detect chromosomal abnormalities by up to 18%.²³ The most important use of CGH, especially the array CGH, is the detection of chromosomal abnormalities, especially genomic imbalances present in 6% of congenital defects. Consequently, performing these studies in neonates may increase the possibility of early detection of chromosomal abnormalities consistent with a genetic/genomic disorder, especially when there are no clinical features that could help to identify the cause.²³ The above, especially in cases of syndromic hypospadias.

DNA sequencing (Sanger method) or the chain termination method: It is based on the use of DNA polymerase to synthesize DNA strands that have a specific termination, generating fragments of all possible sizes, which can be

distinguished by the type of labeling used or by the incorporation of a specific terminator.²⁴ As a starting point, a primer complementary to the DNA of interest initiates the synthesis of the complementary strand by adding deoxynucleotide triphosphate (dNTP), which extends until the addition of a modified nucleotide called terminator or ddNTP (dideoxynucleotide triphosphate), which is fluorescently labeled and does not contain an OH group at the 3'-OH end to further extend the strand.²⁵

Direct sequencing of exons that encode genes involved in hypospadias has been used in multiple studies to identify different mutations. Kalfa et al. identified four genomic variants of the ATF3 gene, present in 10% of the patients in their study and in none of the control groups. The described variants were: a heterozygous missense mutation in exon 3 (L23 M), and three genomic variants without transduction (C53070T, C53632A, Ins53943A) close to exon 6.²⁶ Wang et al. found mutations in the SRD5A2, WT1 and AR genes in 27% of the patients, which were associated with hypospadias and also with micropenis.²⁷ Other polymorphisms have been reported in the MALMD1, ATF3, SF1 genes.^{28,29} This technique has identified the greatest number of variants responsible for hypospadias, which is why its use in syndromic or non-syndromic pathology is recommended after performing karyotyping.

Microarray: It is a recent technique that allows the simultaneous measurement of the expression of hundreds of genes using messenger RNA (mRNA) analysis. It is carried out by hybridization between a specific probe and the target molecule. It was mentioned earlier that the use of array CGH may be useful in the early diagnosis of hypospadias, especially in the context of genetic disorders associated with it.

Next-generation sequencing or parallel mass sequencing: Through DNA fragmentation and subsequent ligation, adapter sequences are added to the ends of the fragments. These fragments are amplified clonally and are grouped as entities to be sequenced. This test allows the detection of all types of genomic variation in a single study. By identifying differences in the DNA sequence, genetic variants can be detected when comparing the DNA of a study individual with a reference DNA.³⁰ Recent advances in hypospadias have identified new associated genes and possible causal variants; additionally, studies in animals have brought to light new knowledge on the molecular genetics of normal and abnormal development of the penis.¹¹

Whole exome sequencing (WES): This technique has allowed the identification of deletions in cases of gonadal dysgenesis and disorders of sex development that include hypospadias as one of their manifestations. This low-cost study has been reported to help the early identification of the variant causing the alteration. An example is deletion due to a shift of the reading frame, located in exon 6 of NR5A1, reported by Eggers et al.; this gene has been identified as a key gene in determining gonadal sex.³¹ This diagnostic method can be performed in cases of syndromic hypospadias, in which no cause has been established in previous studies.

Whole genome sequencing (WGS): There are no articles reported in the literature that describe its use for the diagnosis of hypospadias. Van der Zanden et al., one

of the main researchers on the subject, conclude in their articles that due to its high costs, this technique is only used to identify monogenic causes. As time goes by and costs decrease, it will be possible to use it in large cohorts with isolated hypospadias in order to identify the genes involved.³

Genome-wide association study (GWAS): This diagnostic method is useful to identify the loci that can be etiological. Geller et al. performed a GWAS in 1006 cases of surgically confirmed hypospadias and in 5486 controls, with the subsequent identification of 18 loci associated with hypospadias and 4 suggestive associations, especially the loci connected to genes involved in embryological development. The most significant loci found in the study were close to members of the homeobox family, especially HOXA, IRX5, IRX6, and ZFX3. HOXA4 has been extensively documented as the cause of isolated hypospadias, and HOXA13 as the cause of hand-foot-genital syndrome.³² Van der Zanden et al., using GWAS, reported 436 patients with hypospadias and 494 controls, and determined that variants in the diacylglycerol kinase kappa gene are strongly associated with hypospadias, mainly to anterior and medium hypospadias. Therefore, they conclude that the stratification by hypospadias phenotype based on the location of the urethral opening in future genetic studies may reduce genetic heterogeneity and improve the results.³³

Conclusions

There are different genes involved and identified as causing syndromic and non-syndromic hypospadias. We have performed a literature review on these genes, as well as on different methods of genetic and molecular diagnosis, and the causes described with these methods in the literature.

To complement the study of patients with this pathology, it is advisable to start with karyotyping as an initial diagnostic approach, essential in patients with disorders of sex development. The sequencing of exons can be performed in cases of syndromic or non-syndromic hypospadias following a negative karyotyping result, since it has the ability to detect different causative genes that were previously reported. Whole exome sequencing is useful in patients with disorders of sex development for early identification of the causative variant. It is also possible to perform comparative genomic hybridization in the case of neonates suspected of having a genetic/genomic disorder and when clinical features do not help to identify the disorder. In embryos, where a genetic disorder is suspected, the microarray may be helpful for an early diagnosis of hypospadias and the syndrome itself in order to establish prenatal treatment. In the case of patients who have already undergone multiple surgical procedures with difficult-to-treat hypospadias, FISH can be helpful to identify the associated disorder. Genome-wide association study, whole genome sequencing and next-generation sequencing are more advanced and costly techniques that are used to find new associated genes, which will improve our knowledge and management of this pathology in the future.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interests

Authors have no conflict of interests to declare.

Acknowledgments

We thank the Institute of Genetics at the Pontificia Universidad Javeriana for its support and collaboration in the preparation of this document.

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