

# Description of a novel variant in the *MAMLD1* gene in isolated hypospadias

## *Descripción de una nueva variante del gen MAMLD1 en hipospadias aislado*

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### Abstract

**Introduction** Hypospadias is a multifactorial malformation. Among previously described genes, the *MAMLD1* has recently been identified in association with the development of hypospadias. So far, there are no studies in Latin America addressing this gene. The aim of this paper is to describe the presence of variants in exon 3 of the *MAMLD1* in our population.

**Materials and Methods** Using the Bio-Repository's database of the Human Genetics Institute, we identified 51 patients between 2008 and 2012 with varying degrees of isolated hypospadias. Exon 3 was sequenced looking for polymorphisms. These were assessed with *in silico* prediction algorithms to describe the effect of these changes on the function and structure of the protein.

**Results** Four patients with 3 variants were identified. The p.S364P variant has not been previously described and has a deleterious effect. The other two, rs41313406 and rs61740566, have been previously described and are not considered variations with harmful effect on the protein function. The genotype-phenotype correlation varies in severity between the variables described.

**Conclusions** For the first time a Latin American population is molecularly analyzed. The p.S364P variation has never been described before. Variations with different degrees of hypospadias are described.

### Keywords

- ▶ genitourinary anomalies
- ▶ hypospadias
- ▶ MAMLD1
- ▶ genetic variant

### Resumen

**Introducción** El hipospadias es una malformación multifactorial. Dentro de los genes asociados descritos, el *MAMLD1* se ha identificado recientemente como asociado al desarrollo de esta enfermedad. Hasta el momento no hay estudios en Latinoamérica sobre este gen. El objetivo del presente trabajo es describir la presencia de variantes en el exón 3 del *MAMLD1* en nuestra población.

**Material and Métodos** Basados en los registros del Biorrepositorio del Instituto de Genética Humana, se identificaron 51 pacientes entre el año 2008 y el 2012 con diferentes

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**Palabras-Clave**

- ▶ anomalías genitourinarias
- ▶ hipospadias
- ▶ *MAMLD1*
- ▶ variante

grados de hipospadias aislado. A estos se les realizó la secuenciación del exón 3. Los polimorfismos identificados fueron evaluados con algoritmos de predicción *in silico* para evaluar el efecto de estos cambios sobre la función y estructura de la proteína.

**Resultados** Se encontraron 4 pacientes con 3 variantes. La variante p.S364P no ha sido descrita previamente y tiene un efecto deletéreo. Las otras 2, rs41313406 y rs61740566, han sido descritas previamente y no se consideran variaciones con potencial de efecto deletéreo sobre la función de la proteína. La correlación fenotipo-genotipo varía en severidad entre las variables descritas.

**Conclusiones** Por primera vez se analiza molecularmente una población latinoamericana, encontrando la variante p.S364P no descrita previamente, y 2 variantes ya descritas previamente.

**Introduction**

Hypospadias are considered a multifactorial disease given the interaction between molecular events and the environment. Molecular mechanisms are complex. To date, there are around 26 genes associated with the development of hypospadias. Recently, the *MAMLD1* gene (Mastermind like domain containing protein 1") association with hypospadias was identified.<sup>1</sup> Originally known as *CXorf6* (Chromosome X open reading frame 6) or *F18*, the *MAMLD1* is a gene first described by Laporte et al. in 1997 in patients with ambiguous genitalia during the study of the X-linked myotubular miotrofia.<sup>2,3</sup> A decreased expression of the *MAMLD1* generates a reduction in testosterone levels in early periods of organogenesis around the 8–12 weeks of gestation. A decrease of 25–30% in the gene expression reduces levels of testosterone to a 50–60% of normal values, but never becomes undetectable.<sup>5</sup> This explains the association with the development of the hypospadias. It has been described the expression of the gene in other tissues like skeletal muscle, brain, placenta and heart.<sup>1</sup> Interaction of *MAMLD1* and co-activation with other genes (*STAR CYP11a1*, *CYP17a1*, *HSD3b1*, *INSL1*, *SF1*, *HES3*, *STAT3*), in knock-out mice with a normal development of genitals, has shown a more complex molecular function of this gene.<sup>2</sup> Genotype-phenotype correlation is not always the same because patients with the same mutations present different degrees of clinical severity.<sup>4</sup> On the other hand, patients with mutations in this gene have normal levels of testosterone after birth and hCG stimulation test is not altered due to the deletion of the *MAMLD1*.<sup>6</sup>

Given the complexity of the functions and interactions of *MAMLD1* and processes yet to characterize as mentioned above, it is necessary to continue further studies on this gene. The purpose of this work is to make a description of variants in exon 3 of the gene *MAMLD1* in Colombian patients affected with hypospadias.

**Material and Methods****Population characteristics**

During the period from January 2008 to October 2012, patients with isolated hypospadias were recruited. All types of clinical severity were included using Duckett's classification. Patients were selected from the DNA Bank of urological

congenital malformations of the Institute of Human Genetics at the Pontificia Universidad Javeriana. Excluded cases were all syndromic hypospadias registered in the DNA Bank. All recruited cases were captured when surgery was scheduled to correct the congenital defect. All cases had a signed consent by the parents or legal representatives before being included in the study. Once patients were admitted to the study, a questionnaire was filled. The pool of demographic information included data from the patient and his parents, description of the malformation and diagnostic methods, prenatal history and detailed technical information about the surgical procedure. At the time of venipuncture during anesthetic induction, the blood sample was taken from a peripheral vein (3–5 mL) in tubes with anticoagulants. Subsequent extraction of DNA was made. Storage of samples was at –20°C. DNA extraction was performed using the method of salting-out with the Promega Wizard® kit. Extracted DNA was evaluated with spectrophotometry to determine the concentration and absorbance between 260 and 280 nanometers (GeneQuant Amersham Biosciences®).

**Sample and data processing**

Once DNA was extracted, PCR for exon 3 of the *MAMLD1* gene was done. This exon was divided into three fragments through the design of primers with the program Primer3®. Amplification according to standardized protocols in the laboratory of Molecular Biology and Genetics at the Institute of Human Genetics at the Pontificia Universidad Javeriana was made using a thermal cycler from BIO-RAD® iCycler. Agarose gels were used stained with 1% ethidium bromide and visualized using UV light to reveal the size of the fragments.

Subsequently purification was performed using Pure-Link® PCR Purification kit and Affimetrix® ExoSAP-IT for PCR product cleanup. Purified samples were then verified using electrophoresis in agarose 1%. Sequencing by Sanger method was performed with the ABI PRISM® 3730XL Analyzer (96 capillary type). Finally, analysis of the sequences was performed using Sequencer® 5.0 software. Prediction analysis in-silico was conducted using Polyphen®, SIFT®, SNP&GO®, Mutation-taster® and Mustab®.

Results were analyzed according to the classification of the degree of the hypospadias. A population analysis was

performed calculating the allele frequencies by direct counting method and Harlequin® 3.1 to assess the Hardy Weinberg equilibrium.

## Results

During the study period 67 cases were collected. This corresponds to 34% of the total number of individuals included in the Bank. Of these, 51 samples for analysis were processed. Average age at the time of surgery was 5 years ( $\pm 4$  years). The 50.9% of the cases came from Cundinamarca and the remaining from different departments of the country. According to the classification of Duckett, the most frequently operated degree was scrotal (35.29%), followed by penile hypospadias (25.49%).

Sequencing analysis identified 3 changes in exon 3 of the *MAMLD1* gene in 4 out of 51 analyzed individuals (**►Table 1** and **►Fig. 1**). Two changes were identified in patient number 21. The first identified change was p.S364P. This variant has not been previously described. There is only information of two cases of this variant in the latino population in the Exome Aggregation Consortium database. A frequency of 0.0002148 in latinos and a  $MAF = 2.291e - 05$  has been described. To assess the potential impacts of this variant in the protein, a damaging impact in 3 out of 5 algorithms was identified (**►Table 1**). The second change is the same as the one identified in patient number 130. In patient number 14 the variant p.P334S (rs41313406) was identified. According to prediction bioinformatic algorithms, this change has a potential impact on the function of the protein (**►Table 1**). This same variant was identified in patient number 64. The third change, p.V432A

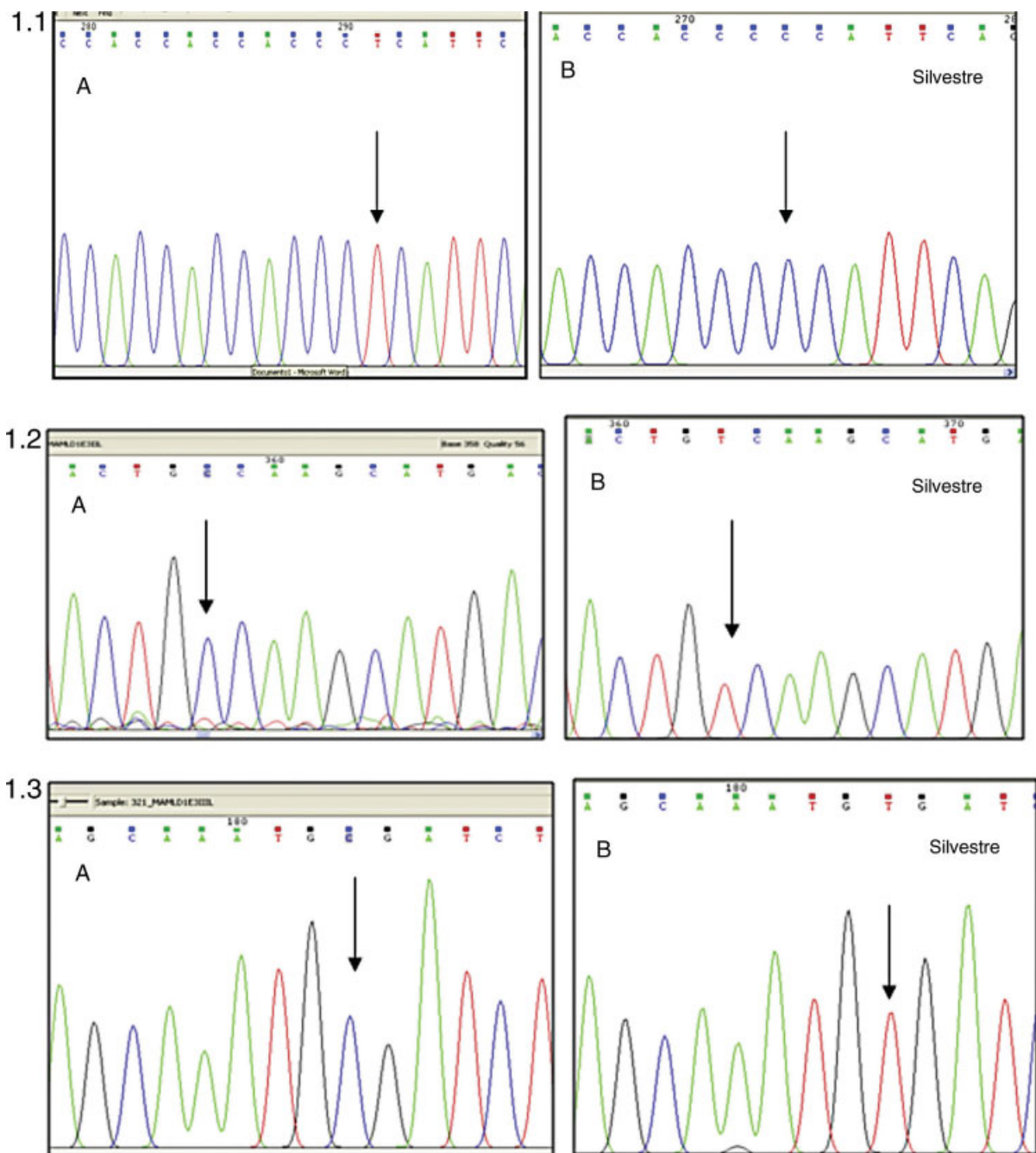
(rs61740566), present in patients number 21 and number 130, was identified as benign in 5 of the 6 algorithms. This change has been previously described (**►Table 1**). Allele frequency analysis showed that the population is in balance for each variant according to Hardy–Weinberg model ( $p = 1.000$ ). The SNP rs41313406 presented genotype frequencies for our study of CC (0.960), CT (0.039) and TT (0). There are two populations reported in the literature reporting genotype frequencies similar to those found in the present study: the U.S. population and the African American and Caucasian mix. People with African American and Caucasian mix present the following frequencies: CC (0.842), CT (0.158) in 38 analyzed individuals. This variant was found in the Latin American population. The SNP rs61740566 presented genotype frequencies in our study of TT (0.960), TC (0.039) and CC (0). There are also two populations reported in the literature with frequencies similar to ours. The North American population presents the following sequences: TT (0.884), CT (0.092) and CC (0.024), in 2272 individuals analyzed.

## Discussion

This is the first study in our population and in Latin America that analyzes the presence of variations in the *MAMLD1* gene. Three nucleotide changes were found in four of the 51 analyzed patients (**►Table 1**). The p.S364P change has not been reported previously. As with other variants of the *MAMLD1* associated with hypospadias, the correlation of the genotype–phenotype changes from patient to patient.<sup>7,8</sup> Analysis *in silico* of this change showed a deleterious effect on

**Table 1** Demographic description of patients with detected variants and their impact on the *MAMLD1* protein

Patient	Age	Meatus	rs#	Variant in proteine	Genetic variant		
PTE 14	39	Scrotal	rs41313406	P334S	c.1000C > T cDNA.1175C > T g.109232C > T		
PTE 21	10	Glanular	ND	S364P	c.1090T > C c.1265T > C g.109322T > C		
PTE 21	10	Glanular	rs61740566	V432A	c.1439T > C cDNA.1614T > C g.109671T > C		
PTE 130	6	Glanular	rs61740566	V432A	c.1439T > C cDNA.1614T > C g.109671T > C		
PTE 64	8	Penile	rs41313406	P334S	c.1000C > T cDNA.1175C > T g.109232C > T		
Patient	In silico algorithms						
	Polyphen	SIFT	Provean	SNP&GO	Mutation taster	Mustab	Described
PTE 14	Probably damaging	Damaging	Neutral	Neutral	Polymorphism	Diminishes stability	Yes 1000 genomas Medellín C = 94% T = 6% MAF = 4%
PTE 21	ND	Damaging	Deleterious	Neutral	Polymorphism	Diminishes stability	No 1000G ExAC 0.0002148 en latinos MAF = 2.291e - 05 No NCBI
PTE 21	Benigne	Tolerable	Neutral	Neutral	Polymorphism	Diminishes stability	1000G Medellín T = 99% C = 1% MAF = 0.05 ExAC Latinos = 0.007632 Total = 0.01835
PTE 130	Benigne	Tolerable	Neutral	Neutral	Polymorphism	Diminishes stability	1000G Medellín T = 99% C = 1% MAF = 0.05 ExAC Latinos = 0.007632 Total = 0.01835
	Probably damaging	Damaging	Neutral	Neutral	Polymorphism	Diminished stability	1000 genomas Medellín C = 94% T = 6% MAF = 4%



**Fig. 1** Electroferograms of the three identified variants. The arrows show the variants in each electroferogram. (1.1) (A) Patients number 14 and number 64 with the p.P334S variant (rs41313406). (B) Wild-type sequence. (1.2) (A) Patient number 21 with the p.S364P variant. (B) Wild-type sequence. (1.3) (A) Patients number 21 and number 130 with the p.V432A variant (rs61740566). (B) Wild-type sequence.

the protein. In addition, the amino-acid change that occurs with this variant changes the polarity of the protein and must therefore have a greater impact on the normal functioning of the protein.

Two of the SNPs identified, rs41313406 and rs61740566, have already been reported in other studies. The SNP rs41313406 was found in two of the 51 patients with isolated hypospadias, and when analyzing the change in the protein, it is observed that it is likely to damage, contrary to some previous reports.<sup>2</sup> Chen et al, described eleven patients with the SNP,

Fukami in 2008 and Kalfa in 2012 reported 14 patients. They state that the amino-acid change generates no effect on the protein. They argue this because the variant is not located on a highly conserved region and for that reason, it might not be deleterious. With respect Kalfa's findings, their results differ from other authors and ours because the change in the protein reported does not match the position we identified (p.P359S vs. p.P334S), but yet, it has the same SNP number.<sup>3,5</sup> Kalfa's work, this change is defined as a mutation. Other authors report this effect as a polymorphism. Our *in silico* analysis suggests a

negative effect on the protein. Interestingly, cases reported by other authors, and ours have the same phenotype of proximal hypospadias.

On the other hand, the SNP rs61740566 found in two of the 51 patients with isolated hypospadias, has no effect on the protein. Our results are consistent with other authors who have found it even in healthy controls.<sup>9,10</sup> The phenotype of our two individuals with the variant is distal hypospadias, compared with those reported by Kalfa et al. that had proximal hypospadias.<sup>11,12</sup>

Our results as well as other authors, demonstrate that the phenotype varies in different degrees of severity of the hypospadias, with the variations of the *MAMLD1*.

We acknowledge that the majority of cases are proximal due to the fact that our hospital is a reference center. We believe the average age of surgery is influenced by the administrative difficulties in our health system, that makes it harder for patients to consult a specialist. Nonetheless this limitations, they do not affect the results of our study.

## Conclusions

For the first time in our country and our region, we assess on a molecular level patients with isolated hypospadias, looking for changes in the *MAMLD1* gene. This new mutation gives more information about this recently described gene and the development of hypospadias. The variability of the phenotype in patients with the same genotype predicts a complex pathophysiology of the *MAMLD1* gene. Future studies must focus on the co-expression with other genes and possible epigenetic and environmental factors associated with genetic variants in patients with hypospadias.

## Ethical Responsibilities

### Protection of people and animals

The authors state that the procedures conformed to the ethical standards of human experimentation committee responsible and according to the World Medical Association and the Declaration of Helsinki.

### Confidentiality of data

The authors declare that they have followed the protocols of the workplace on the publication of patient data.

### Right to privacy and informed consent

The authors have obtained the informed consent of patients and/or subjects referred to in Article consent. This document is held by the corresponding author.

## Conflict of interest

The authors declare that they have no conflicts of interests.

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