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A Novel Hemizygous Mutation of *MAMLD1* in a Patient with 46,XY Complete Gonadal Dysgenesis

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Key Words

Complete gonadal dysgenesis · Disorders of sexual development · *MAMLD1*

Abstract

MAMLD1 is suggested to play a role in the development of 46,XY disorders of sexual development (46,XY DSD). So far, mutations in this gene have been detected in several cases of hypospadias with normal testosterone levels at birth. From in vitro studies it was concluded that *Mamld1* might transiently affect testosterone synthesis during genital development. We describe the first *MAMLD1* mutation in a 46,XY patient with complete gonadal dysgenesis. The novel *MAMLD1* missense mutation (p.P677L) results in a severely reduced transactivation in vitro of the promoter of the *MAMLD1* target gene *HES3/Hes3*. However, as knowledge about the functional role of *MAMLD1* in gonadal development is limited, we suggest that additional factors (digenic or oligogenic cause) play a role in the development of complete gonadal dysgenesis in this patient.

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Disorders of sexual development (DSD) are congenital conditions associated with atypical chromosomal, gonadal, or anatomical sex [Hughes et al., 2006]. Individuals with DSD and a 46,XY karyotype (46,XY DSD) due to disorders of testes development present different degrees of ambiguous genitalia and partial to complete testicular dysgenesis [Houk and Lee, 2008]. To date, various mutations in several genes involved in sexual development, including *SRY*, *NR5A1* (*SF1*), *WT1*, *SOX9*, *DAX1* (*NR0B1*), *DHH*, *WNT4*, *DMRT1/2*, *ATRX*, and *MAP3K1*, have been described in these patients [Biaison-Laubert, 2010; Barthold, 2011; Eggers and Sinclair, 2012], but in ~50% of cases with 46,XY DSD, the underlying genetic cause remains unknown.

Laporte et al. [1997] identified the *MAMLD1* gene (mastermind-like domain-containing 1, previously *CXorf6*) as a candidate gene for 46,XY DSD based on their observations of a contiguous gene syndrome in patients with myotubular myopathy and male hypogonadism harboring deletions on chromosome Xq28 [Hu et al., 1996; Laporte et al., 1997].

In 2006, *MAMLD1* mutations were found in patients with severe hypospadias, a rather mild form of 46,XY DSD. Since this first description, *MAMLD1* mutations

have been described in several patients with hypospadias [Fukami et al., 2006; Kalfa et al., 2008, 2012; Chen et al., 2010]. Moreover, the S-S haplotype, which entails the presence of a double *MAMLD1* gene polymorphism (p.P359S and p.N662S), has been found to be more frequent in patients with hypospadias than in controls [Chen et al., 2010; Kalfa et al., 2011; van der Zanden et al., 2012].

Recently, a cohort of 70 children with severe non-syndromic 46,XY DSD of unknown etiology has been screened for *MAMLD1* mutations. Two new mutations, p.S143X and p.P384L, have been found in 2 patients with hypoplastic phallus and severe hypospadias [Kalfa et al., 2012].

The *MAMLD1* gene codes for a 774 amino acid protein (NP_005482). Two splice variants have been identified: one including and one excluding exon 4. Both proteins are ubiquitously expressed, but the longer protein, containing exon 4, is the major form [Fukami et al., 2006, 2008]. Both *MAMLD1*/*Mamld1* proteins contain the target sequence of the steroidogenic factor 1 (*NR5A1*, *SF1*), which is a key regulator of genes involved in sexual development [Hoivik et al., 2010; Köhler and Achermann, 2010]. The *MAMLD1* protein transactivates the promoter of a non-canonical Notch target gene hairy/enhancer of split 3 (*Hes3*) [Fukami et al., 2008; Ogata et al., 2008, 2009; Nakamura et al., 2011]. *HES/Hes* family genes play a role in repressing/activating transcription factors in multiple tissues and are involved in tissue specific cell differentiation during embryonic development [Kageyama et al., 2007; Katoh and Katoh, 2007, 2009].

Concerning *MAMLD1* function in sex differentiation, Fukami et al. [2008] suggested a supportive role in testosterone production around the critical period for male sexual development.

Mamld1 mRNA expression levels have been shown to increase gradually between days E8.5 and E12.5 of embryonic development, and *Mamld1* expression has been found from E12.5 to E14.5 in fetal Leydig and Sertoli cells of male mice [Fukami et al., 2008; Miyado et al., 2012]. Transient knockdown of *Mamld1* in mouse Leydig tumor cells has demonstrated a reduction of testosterone production and *Cyp17a1* expression, an enzyme indispensable for testosterone synthesis in Leydig cells [Nakamura et al., 2011].

Recently, a *Mamld1* knock-out (KO) male mouse has been studied. The KO mice display a partial decrease in mRNA levels of genes exclusively expressed in Leydig cells (*Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, and *Ins13*). However, their genital and reproductive development is completely normal. Species differences in sexual development have been suggested for the contradictory findings of gen-

ital phenotypes of *Mamld1* KO mice and patients with *MAMLD1* mutations [Miyado et al., 2012].

A similar constellation could be found for the steroidogenic factor 1 (*NR5A1*, *SF1*). *Nr5a1* KO mice display complete gonadal and adrenal agenesis, whereas almost all humans with *NR5A1* mutations exhibit a wide range of gonadal dysgenesis and mostly normal adrenal development and function [Jameson, 2004].

Mamld1 expression in mice ovaries has been found in granulosa cells between 3 and 8 weeks of age [Ogata et al., 2012]. Deletions in the Xq region (Xq13–Xq28), which contains *MAMLD1*, have been linked with premature ovarian failure [Rossetti et al., 2004; Ferreira et al., 2010]. However, the distinctive role of *MAMLD1*/*Mamld1* in ovarian development and function remains unknown.

We hypothesized that *MAMLD1* mutations might also be involved in more severe forms of 46,XY DSD due to gonadal dysgenesis. In this study, we have performed a mutation screening of the *MAMLD1* gene in a cohort of patients with 46,XY DSD with partial to complete gonadal dysgenesis without known genetic cause.

Patients and Methods

Patients

Thirty-five patients with 46,XY DSD and different degrees of ambiguous genitalia were screened for *MAMLD1* mutations. Twenty-three had partial and 12 had complete gonadal dysgenesis. Mutations of *SRY*, *NR5A1* (*SF1*), and *WT1* were previously excluded in these patients. Before performing genetic screening, informed consent was obtained from each patient or legal guardian.

Mutational Analysis

Genomic DNA was isolated from peripheral white blood cells using the Blood amp kit from Qiagen (Hilden, Germany). Coding exons and their flanking splice sites of the *MAMLD1* gene (NM_005491) were amplified using a standard PCR protocol. Primers for amplification are summarized in table 1. Sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Perkin-Elmer, Weiterstadt, Germany) and run on an ABI 3710xl Automatic Sequencer (Applied Biosystems, Foster City, Calif., USA).

The number of amino acids of the *MAMLD1* protein varies depending on the protein isoform studied. In this study, we used the protein variant of 774 amino acids (NP_005482) for numbering of new and previously described mutations.

Functional Analysis

For functional characterization of the new mutation, its ability to activate the *Hes3* gene promoter was tested. Previously, the *MAMLD1* wild type has already shown a transactivation function of the *Hes3* promoter, while the p.E197X and p.S143X mutants have shown a greatly reduced transactivation capacity [Fukami et al., 2008; Kalfa et al., 2012].

Fig. 1. Chromatogram of the c.2030C>T (p.P677L) mutation. The wild-type sequence is shown on the left, and the hemizygous mutated sequence is shown on the right. Arrows and asterisks indicate the affected nucleotide.

- GDVSPSNITHVDKACKLG *Homo sapiens* (NP_005482)
- GHVPPSNLTHVDKACKLG *Mus musculus* (XP_006528172.1)
- GDVSPSNITHVDKACKLG *Pan troglodytes* (XP_521302.3)
- GDVSPSNITHVDKACKLG *Equus caballus* (NP_001166929.1)
- GAVSPSNITHVDKACKLG *Physeter catodon* (XP_007129627.1)

Fig. 2. MAMLD1 homologous protein sequence alignments around the mutation site (red) in different mammals.

Table 1. Primers used for *MAMLD1* mutational screening

	Forward	Reverse	Product size, bp
Exon 1	CCCTGTGTCTAGGTCGTTTGG	CCTGCCACCAAGTTTGCACAG	263
Exon 2	GTAACAGTCTGATCTGTGCCTC	GACCTCTTGTAGGTCCTTTC	261
Exon 3, start	GTGTGTGTACCTCTCTTCCC	GGGTGACATCTTCAAGGCAAGG	172
Exon 3, middle	TGATGGGGGCTACCTAATA	GGCTCCGTGGCTCCTGGGCTG	1,300
Exon 3, end	GTCGCTCTGCCCCCTTACC	CCAAAACAAAAGAACACAGCCAGATATG	1,050
Exon 4	CTTGACACCAGGGAGTTGGT	ATGATTTGCATGACCCCAAT	385
Exon 5	CACTCAGGGCAGTGACAAGA	GGCATAACAGCTGAGGGAGAG	594
Exon 6	GCGAGTGTATGCTGGTGTGT	TTGGGAACACTGGCCTTAAC	207
Exon 3, sequencing	CCAAGCCTTCGGTTCAGATGTC		–
	GAGCTCCATTCTTGCCAACCTC		

Additional primers were used for exon 3 sequencing.

A pCMV-Myc plasmid vector containing the *MAMLD1* full-length cDNA, a pCMV-Myc vector containing the nonsense p.E197X mutation, and a reporter vector with the *Hes3* response element and a firefly luciferase gene were used for functional analysis.

The p.P677L mutation was obtained by site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis kit, Stratagene, La Jolla, Calif., USA) of the pCMV-Myc vector containing the wild-type *MAMLD1* sequence.

For transactivation analysis of the p.P677L mutation, a Dual-Luciferase Reporter Assay System (Promega, Fitchburg, Wis., USA) was used. COS-7 cells were seeded in 6-well dishes (2.5×10^5 cells/well) and were transiently transfected using Metafectene (Biontex, Planegg, Germany) with 2 μ g of luciferase reporter vector, 4 μ g of pCMV-Myc expression vector containing either wild-type *MAMLD1*, p.E197X or p.P677L mutant, and 2 μ g of Renilla luciferase as an internal control. Transactivations of the wild-type and mutant proteins were compared, and an empty pCMV-Myc vector was used as a negative control.

The cells were lysed 48 h after transfection, and the mouse *Hes3* promoter transactivation activity was measured. The assays were repeated 3 times in duplicates.

Statistical data analysis was performed using GraphPad Prism 5 (Graph Pad Software, La Jolla, Calif., USA). Results were considered significant if $p < 0.05$ using a one-way ANOVA and Tukey-Kramer test.

In silico Analysis

For in silico analysis of the mutation, the program Mutation Taster (www.mutationtaster.org), which employs a Bayes classifier to predict the disease potential of an alteration, was used [Schwarz et al., 2010].

Results

A novel hemizygous c.2030C>T (p.P677L) mutation was found in a patient with 46,XY complete gonadal dysgenesis (fig. 1). The patient presented with primary amenorrhea at the age of 13 years. The external genitalia were completely female. No gonads could be detected, but the uterus was present. At diagnosis, the gonadotropins were elevated (FSH 76 U/l, LH 17.3 U/l). Estrogen level was low, and testosterone level was in the normal range for females.

The mother was found to be a heterozygous carrier of the mutation, while the father displayed the wild-type allele. The 41-year-old mother has not shown any impairment of gonadal function, and her sexual hormone levels

are in the normal range. There is no family history of 46,XY DSD or 46,XX DSD.

The novel mutation p.P677L is located in a highly conserved region of *MAMLD1*/*Mamld1* in mammals (fig. 2). So far, the mutation is not described in the National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (dbSNP) database, Exome Variant Server (<http://evs.gs.washington.edu/EVS/>), 1000 Genomes (<http://browser.1000genomes.org/index.html>), and ALFRED database (<http://alfred.med.yale.edu/alfred/index.asp>), and it was absent in 120 healthy controls.

The in silico analysis of the mutation predicted it to be disease causing with a probability of 0.53.

Accordingly, we tested the ability of the resulting mutant protein to activate the promoter of its downstream partner *Hes3* in vitro. Functional characterization of the mutant p.P677L showed a significant loss of the transactivation function on the mouse *Hes3* promoter (fig. 3) compared to the wild-type protein ($p < 0.05$).

Discussion

This is the first report of a *MAMLD1* mutation in a patient with 46,XY DSD and complete gonadal dysgenesis. The novel hemizygous mutation (p.P677L) is located in a highly conserved region of *MAMLD1* and results in a nearly complete loss of transactivation of the promoter of *Hes3*, a target gene of *MAMLD1*. So far, *MAMLD1* mutations have only been described in patients with hypospadias, a less severe phenotype of 46,XY DSD. Most patients display normal testosterone production after birth [Gaspari et al., 2011], and it is hypothesized that *MAMLD1* has only a transient effect on Leydig cell function and testosterone synthesis during embryonic development. So far, *MAMLD1* mutations have been identified in 11 out of 250 cases with hypospadias (4.4%), and 5 of them have been functionally characterized: 3 nonsense mutations in exon 3 (p.E197X, p.Q270X, and p.S143X) have shown a complete loss of transactivation function, 1 missense mutation (p.P384L in exon 3) has shown a significant reduction in transactivation function, and 1 nonsense mutation (p.R726X in exon 5) retains normal transactivating activity but seems to exert a deleterious effect through early degradation by nonsense-mediated mRNA decay. To date, no correlation has been found between the severity of the phenotype and complete or partial loss of transactivation activity (table 2; fig. 4) [Fukami et al., 2006; Kalfa et al., 2008, 2011].

Fig. 3. Transactivation of the promoter of the *MAMLD1* target gene *Hes3* by *MAMLD1* WT, p.E124X, and p.P677L cloned into the pCMV-Myc vector. Data are shown as mean (columns) and standard error of the mean (bars). p.P677L, p.E124X, and the empty vector showed a statistically significant reduction of transactivation function compared to *MAMLD1* WT ($p < 0.05$). WT = Wild type.

So far, all studies investigating the function of *MAMLD1* mutations in vitro have used the mouse *Hes3* promoter as a target gene, as no other target genes of *MAMLD1* are known.

The *Hes3* gene belongs to the *Hes/Hey* gene family involved in processes of maintenance of stem cells, binary cell fate decisions, and timing of developmental events in the embryo [Kageyama et al., 2007; Katoh and Katoh, 2007]. Therefore, factors influencing a correct expression of these regulators could hypothetically also affect early sexual development. *HES3* has been found to be expressed in human fetal testes and adult ovaries [Fukami et al., 2008]. Furthermore, *Hes3* is a target of a non-canonical Notch signaling pathway, and Notch receptors have been proven to be expressed in mouse testes and regulate their development [Tang et al., 2008].

The presence of a functionally relevant *MAMLD1* mutation in our patient suggests that *MAMLD1* might be involved not only in testosterone synthesis of the Leydig cells but also in gonadal development. However, we only hypothesize that the *MAMLD1* mutation contributes to the severe phenotype of complete gonadal dysgenesis in our patient as knowledge about the role of *MAMLD1*/

Fig. 4. Location of previously described *MAMLD1* mutations and the new p.P677L mutation. An impairment of transactivation function was demonstrated in all mutations shown (except for p.R726X). The boxes represent the exons. Black colored areas denote protein-coding regions and white areas are untranslated regions.

Table 2. Reported *MAMLD1* mutations in 46,XY DSD patients

Reference	Mutation	Age	Genital phenotype	Testosterone, nmol/l	DHT, nmol/l	AMH, ng/ml	Inhibin B, ng/ml	Transactivation function
Fukami et al. [2006]	p.E197X (prev. p.E124X)	4 months	hypospadias with chordee, inguinal testis	<0.3	0.07	—	—	abolished
Fukami et al. [2006]	p.E197X (prev. p.E124X)	1 month	hypospadias with chordee, scrotal testis	0.7	<0.15	—	—	abolished
Fukami et al. [2006]	p.Q270X (prev. p.Q197X)	2 years	hypospadias with chordee, scrotal testis	0.1	—	—	—	abolished
Fukami et al. [2006]	p.R726X (prev. p.R653X)	1 month	hypospadias with chordee, retractil testis	0.3	—	—	—	normal
Kalfa et al. [2008]	p.V505A (prev. p.V432A)	5.5 years	hypospadias with chordee, scrotal testis	—	—	—	—	normal, Ogata et al. [2012]
Kalfa et al. [2008]	c.544delG (prev. c.325delG)	1 year	hypospadias with chordee	<0.06	<2	—	—	n.d.
Kalfa et al. [2008]	c.544delG (prev. c.325delG)	1 year 2 months	hypospadias with chordee	—	—	—	n.d.	n.d.
Kalfa et al. [2008]	p.Q604_Q605insQQQ (prev. p.Q531_Q532insQQQ)	1 month	isolated hypospadias, coronal meatus	—	—	—	—	n.d., present in normal individuals, Chen et al. [2010]
Chen et al. [2010]	p.Q602K (prev. p.Q529K)	—	severe hypospadias, bilateral cryptorchidism	—	—	—	—	n.d.
Kalfa et al. [2012]	p.S143X	neonate	scrotal hypospadias, microphallus, scrotal testis	5.9	—	336	—	abolished
Kalfa et al. [2012]	p.P384L	neonate	posterior hypospadias with chordee, microphallus, scrotal testis	<0.23	—	19	<15	reduced
Present case	p.P677L	13 years	female external genitalia	undetectable	—	—	—	abolished

Mamld1 in gonadal development and function is still limited.

The phenotype of our patient might be caused by a digenic or oligogenic pathogenic mechanism where the interaction of 2 or more loci is needed for the expression of a certain phenotype. One well-known example of this complex genetic background is congenital hypogonadotropic hypogonadism where new genes involved in its oligogenic inheritance have been recently discovered [Miraoui et al., 2013]. In the case of Kallman syndrome, consisting of hypogonadism and anosmia, a digenic mode of inheritance has been proven in a few patients. These patients carry heterozygous mutations in 2 different

genes (*PROKR2* and *PROK2* or *PROK2* and *KAL1*) [Dodé and Rondard, 2013].

Digenic mutations have also been described in some rare diseases, such as retinitis pigmentosa, long QT syndrome, Bardet-Biedl syndrome, or deafness [Dodé and Rondard, 2013; Schäffer, 2013].

Subsequently, we suggest that until further insight is gained into *MAMLD1* function, other factors or mutations in so far unknown genes are very likely to be involved in the development of the complete gonadal dysgenesis in our patient. In particular, digenic or oligogenic mutations should be considered being a possible underlying cause of disorders of gonadal development.

Notably, in our patient the mutation was transmitted by the mother. Maternal transmission of *MAMLD1* mutations has been described in 3 more cases [Ogata et al., 2012]. Subsequently, maternal transmission of *MAMLD1* mutations should be considered in a familial occurrence of 46,XY DSD.

In conclusion, *MAMLD1* mutations are involved in the development of 46,XY DSD. It is hypothesized that *MAMLD1* mutations might contribute to the development of severe forms of 46,XY DSD through a multiple loci interaction manner. However, the distinct function of *MAMLD1* in gonadal development and function in humans has still to be elucidated.

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