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

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

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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 [Biason-Lauber, 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 hypogonitalism 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 Insl3). However, their genital and reproductive development is completely normal. Species differences in sexual development have been suggested for the contradictory findings of genital 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].
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 × 10⁵ cells/well) and were transiently transfected using Metafectene (Biontex, Planegg, Germany) with 2 μg of luciferase reporter vector, 4 μg of pCMV-Myc expression vector containing either wild-type MAMLD1, p.E197X or p.P677L mutant, and 2 μg 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.

### 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

### 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].

### Table 1. Primers used for MAMLD1 mutational screening

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCCTGTGTCTAGGTCGTTTGG</td>
<td>CCTGCCACCAAGTTTGCACAG</td>
<td>263</td>
</tr>
<tr>
<td>2</td>
<td>GTAAACGATGACTCTGTCGCTC</td>
<td>GACCTCTTTGAGGTCCTTTTC</td>
<td>261</td>
</tr>
<tr>
<td>3, start</td>
<td>GTGTTGTCACCTCTCTTCC</td>
<td>GGTTGACATCTCAAGGCAAG</td>
<td>172</td>
</tr>
<tr>
<td>3, middle</td>
<td>TGATGCGGGGCTACCCCTATA</td>
<td>GGTCCCCGCTGCTCGGCTGT</td>
<td>1,300</td>
</tr>
<tr>
<td>3, end</td>
<td>GTCTCGTCTGCCCCCTTTACA</td>
<td>CAAAAACAAAGACACAGGAGAATG</td>
<td>1,050</td>
</tr>
<tr>
<td>4</td>
<td>CGTACACAGGAGGATGTGG</td>
<td>ATGATTGTGAAGACCCAAT</td>
<td>385</td>
</tr>
<tr>
<td>5</td>
<td>CACTCAGGGCAGTGCAAGA</td>
<td>GCATACAGCTGAGGAGAG</td>
<td>594</td>
</tr>
<tr>
<td>6</td>
<td>GCCAGTGTATGCTGCTGTGT</td>
<td>TGGGAACACTGCCTTAC</td>
<td>207</td>
</tr>
<tr>
<td>3, sequencing</td>
<td>CCAAACATGGTTGCGTAC</td>
<td>GAGCCTGATTCTGGCAACCTC</td>
<td>–</td>
</tr>
</tbody>
</table>

Additional primers were used for exon 3 sequencing.
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].

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/
**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.

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

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

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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 still has to be elucidated.

References


MAMLD1 Mutation in a Patient with Gonadal Dysgenesis

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