

## Review Article

# *MAMLD1* (*CXorf6*) is a New Gene for Hypospadias

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**Abstract.** *MAMLD1* (mastermind-like domain containing 1), previously known as *CXorf6* (chromosome X open reading frame 6), has been shown to be a causative gene for hypospadias. This is primarily based on the identification of nonsense mutations (E124X, Q197X, and R653X), which undergo nonsense mediated mRNA decay, in patients with penoscrotal hypospadias. Subsequent molecular studies have shown that the mouse homolog is transiently expressed in fetal Sertoli and Leydig cells around the critical period for sex development, and that transient knockdown of *Mamld1* results in significantly reduced testosterone production in murine Leydig tumor cells. These findings suggest that the *MAMLD1* mutations cause hypospadias primarily because of compromised testosterone production around the critical period for sex development.

**Key words:** MAMLD1, CXorf6, hypospadias, testosterone

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### Introduction

Hypospadias is defined by the urethral opening on the ventral side of the penis, and is classified into mild glandular or penile type and severe penoscrotal or perineal type (1). It is a mild form of 46,XY disorders of sex development (DSD), and affects approximately 0.5% of male newborns (2). Hypospadias is primarily caused by compromised androgen effects, and appears as an isolated anomaly or in association with other genital anomalies such as micropenis and cryptorchidism. To date, while mutation analyses have been performed for multiple genes involved in androgen effects such as *SRD5A2* for 5- $\alpha$ -

reductase and *AR* for androgen receptor, pathologic mutations have been identified in only a very small portion of patients (2). This would be consistent with hypospadias being a highly heterogeneous condition subject to multiple genetic and environmental factors.

We have recently shown that *CXorf6* (chromosome X open reading frame 6) is a novel gene for hypospadias (3), and coined a new gene symbol *MAMLD1* (mastermind-like domain containing, 1) on the basis of its characteristic protein structure with homology to mastermind like 2 (*MAML2*) protein (4). Herein, we review the current knowledge about *MAMLD1*.

### Cloning of a Candidate Gene for 46,XY DSD

A gene for 46,XY DSD has been postulated around *MTM1* for myotubular myopathy on Xq28, on the basis of the finding that genital development was normal in patients with intragenic *MTM1* mutations, and invariably abnormal in six patients

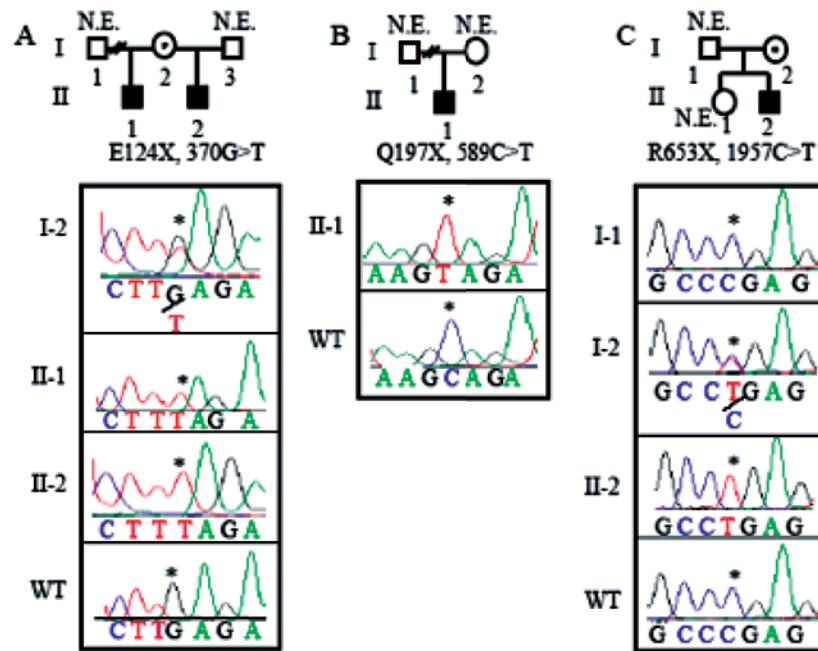
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**Fig. 1.** The pedigrees and electrochromatograms of Japanese patients with nonsense mutations (A–C). The black squares indicate the patients with 46,XY DSD and the mutant *MAMLD1*, and the circles with dots represent molecularly confirmed carrier females. The asterisks in the chromatograms indicate the mutant and corresponding wildtype nucleotides. N.E.: not examined.

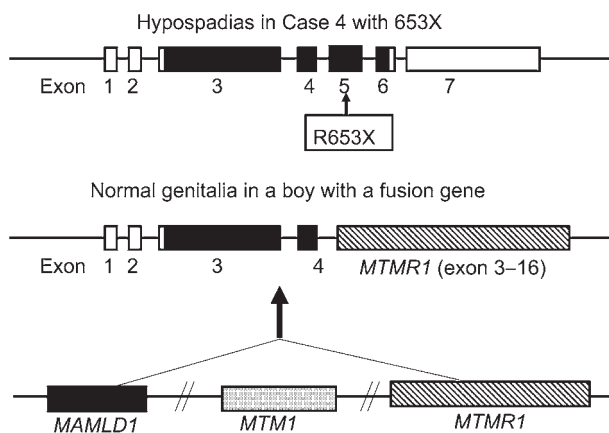
with microdeletions involving *MTM1* (5–8). The six patients consisted of three sporadic and three familial cases, and five of them have glandular, penile, or penoscrotal hypospadias; the remaining one exhibits ambiguous genitalia (5–7). These findings suggest that a gene for 46,XY DSD, especially that for hypospadias, resides in the vicinity of *MTM1*, and that loss or disruption of the gene results in the development of 46,XY DSD as consequence of contiguous gene deletion syndrome.

In 1997, Laporte *et al.* (9) identified *MAMLD1* from a 430-kb region deleted in two sporadic cases with myotubular myopathy and 46,XY DSD (7). *MAMLD1* comprises at least seven exons, and harbors an open reading frame on exons 3–6 that is predicted to produce two proteins of 701 and 660 amino acids as a result of in-frame alternative splicing with and without

exon 4. Furthermore, subsequent studies have shown loss of *MAMLD1* in all patients with myotubular myopathy and 46,XY DSD (our unpublished observation), and no other candidate gene for 46,XY DSD has been identified within the commonly deleted region. These findings imply that *MAMLD1* is an excellent candidate gene for 46,XY DSD, especially hypospadias.

### ***MAMLD1* Mutations in Hypospadiac Patients**

We performed direct sequencing for the coding exons 3–6 and their flanking splice sites of *MAMLD1* in 166 patients including 56 cases with hypospadias. Consequently, three nonsense mutations were identified in Japanese patients with hypospadias: E124X in maternally related half brothers from family A (cases 1 and 2), Q197X in a patient from family B (case 3), and R653X in

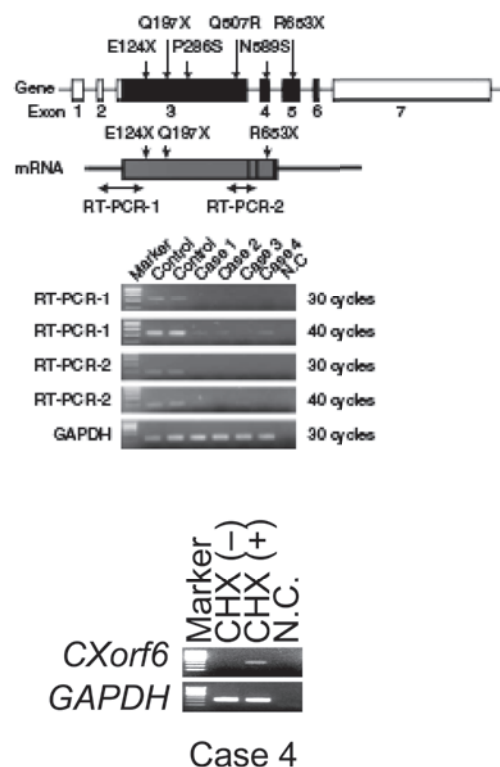


**Fig. 2.** Schematic representation of the R653X mutation in case 4 and the fusion gene between *MAMLD1* and *MTMR1*. The black and the white squares in *MAMLD1* indicate the translated and untranslated regions, respectively.

a patient from family C (case 4) (Fig. 1) (3). The mothers of families A and C were heterozygous for the mutations, although the mother of family B was not studied.

### Nonsense Mediated mRNA Decay (NMD)

One may argue that the hypospadias in case 4 with R653X on exon 5 is inconsistent with the apparently normal genital development in a previously reported boy with a microdeletion involving *MTM1* that has resulted in generation of a fusion gene between exons 1–4 of *CXorf6* and exons 3–16 of *MTMR1* (locus order: *CXorf6-MTM1-MTMR1*), because the coding exons 3 and 4 are preserved in both case 4 and the boy with the fusion gene (10) (Fig. 2). However, in contrast to the positive expression of the fusion gene confirmed in the biopsied muscle tissue (10), the three nonsense mutations are predicted to cause NMD because of their positions (11). Consistent with this, RT-PCR for leukocytes indicated drastically reduced transcripts in cases 1–4 (Fig. 3). Furthermore, NMD was protected by an NMD inhibitor cycloheximide, providing further support for the occurrence of NMD in the three



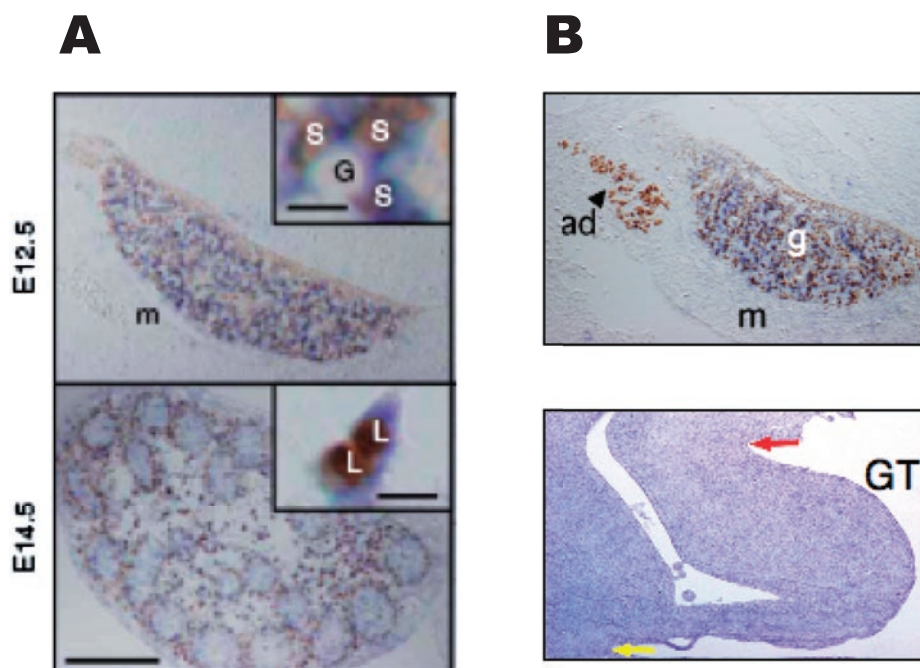
**Fig. 3.** NMD analysis. Upper part: The black and gray boxes represent the coding regions, and the open boxes denote the untranslated regions. The positions of the mutations and variations are shown. RT-PCR for the two regions (RT-PCR-1 and -2) has produced no bands after 30 cycles and very faint bands after 40 cycles in cases 1–4. Lower part: NMD analysis in case 4 with and without an NMD inhibitor cycloheximide (CHX). After 40 cycles of RT-PCR for the region 1, no band is seen without CHX treatment, and a clear band is delineated with CHX treatment.

nonsense mutations (3, 4). Thus, although NMD has not been confirmed in the testicular tissue, the results explain the apparent discordance in the genital development between case 4 and the boy described by Tsai *et al.* (10), and indicate that the three nonsense mutations including R653X are pathologic mutations.

**Table 1** Clinical findings of the four Japanese cases with *MAMLD1* nonsense mutations

Patient	Case 1	Case 2	Case 3	Case 4
<Genital findings>				
Age at exam. (yr:mo)	0:04	0:01	2:00	0:01
Clinical diagnosis	Hypospadias with chordee	Hypospadias with chordee	Hypospadias with chordee	Hypospadias with chordee
Urethral meatus	Penoscrotal junction	Penoscrotal junction	Penoscrotal junction	Penoscrotal junction
Urethroplasty	2.5 yr	3.9 yr	6.0 and 6.6 yr	1.9 yr
Penile length (cm)	2.5 (-1.5 SD)	2.5 (-1.5 SD)	2.0 (-3.4 SD)	1.2 (-3.5 SD)
Testis size (mL)	1-2 (B) (WNR)	1-2 (B) (WNR)	1 (B) (WNR)	1-2 (B) (WNR)
Testis position	Inguinal (B)	Scrotal	Scrotal	Retractile (B)
Orchidopexy	6.3 yr	...	...	1.9 yr
Scrotal appearance	Bifid and hypoplastic	Bifid	Bifid	Bifid
Wolffian structures	Normal on MRI	Normal on MRI	N.E.	N.E.
Müllerian structures	Absent on MRI	Absent on MRI	N.E.	N.E.
Renal structures	Normal on MRI	Normal on MRI	Normal on ultrasounds	N.E.
<Serum hormone values>				
Age at exam. (yr:mo)	0:04	0:01	2:00	0:03
LH (IU/L)	1.2 (0.1-4.7)	3.1 (0.1-4.7)	0.2 (<0.2-3.1)	N.E.
FSH (IU/L)	1.5 (0.4-5.7)	2.2 (0.4-5.7)	1.6 (0.2-5.2)	N.E.
Testosterone (nmol/L)	1.4 (0.1-12.0)→9.0 (7.0-15.0) <sup>a</sup>	9.0 (4.0-14.0)	0.1 (0.1-1.0)	9.4 (4.0-14.0)
DHT (nmol/L)	0.8 (0.2-4.5)→3.7 <sup>a</sup>	1.2 (0.2-4.5)	N.E.	N.E.
Age at exam. (yr:mo)	2:05	2:05	4:00	6:03
LH (IU/L)	0.2 (<0.2-3.1)→3.5 (1.4-6.0) <sup>b</sup>	0.2 (<0.2-3.1)	<0.2 (<0.2-1.2)	0.2 (<0.2-1.4)
FSH (IU/L)	<0.2 (0.2-5.2)→1.5 (2.3-6.9) <sup>b</sup>	0.8 (0.2-5.2)	1.6 (0.7-3.0)	1.2 (0.3-4.0)
Testosterone (nmol/L)	<0.3 (0.1-1.0)→10.1 (7.0-15.0) <sup>a</sup>	0.7 (0.1-1.0)	<0.3 (<0.5)	0.3 (<0.5)
DHT (nmol/L)	0.07 (0.05-2.0)→2.84 <sup>a</sup>	<0.15 (0.05-2.0)	N.E.	N.E.

SD: standard deviation; N.E.: not examined; B: bilateral; MRI: magnetic resonance imaging; WNR: within the normal range (1-2 mL before puberty); N.D.: not determined; LH: luteinizing hormone; FSH: follicle stimulating hormone; and DHT: dihydrotestosterone. Assessment of body sizes (length, height, weight, and head circumference), penile length, testis size, and menarcheal age is based on Japanese reference data. The hormone values in parentheses represent the age- and sex-matched normal range for the Japanese; the reference data for serum hormones are based on the literature. <sup>a</sup>After a human chorionic gonadotropin stimulation (3000 IU/m<sup>2</sup>/dose i.m. for three consecutive days; blood sampling on day 4). <sup>b</sup>Peak values during a gonadotropin releasing hormone test (100 µg/m<sup>2</sup> bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 min).



**Fig. 4.** *In situ* hybridization analysis of the murine *Mamld1*.

- A. Expression patterns in the fetal testes at E12.5 and E14.5. The blue signals are derived from *in situ* hybridization for *Mamld1*, and the brown signals from immunohistochemical staining with Sf-1 (Ad4bp) antibodies. m: mesonephros; G: germ cell; S: Sertoli cell; and L: Leydig cell. The scale bars in the low and high power fields represent 200  $\mu\text{m}$  and 20  $\mu\text{m}$ , respectively.
- B. Expression patterns in the fetal adrenal (upper part) and external genitalia (lower part) of male mouse at E12.5. m: mesonephros; g: gonad; ad: adrenal; and GT: genital tubercle (the between two arrows). *MAMLD1* is not expressed in the adrenal, and weakly and diffusely expressed in the external genitalia as in other non-genital skin tissues.

### Phenotypes in Mutation Positive Patients

Cases 1–4 had penoscrotal hypospadias with chordee as the conspicuous genital phenotype, in association with other genital phenotypes (Table 1). Pituitary-gonadal serum hormone values remained within the normal range, including the human chorionic gonadotropin (hCG)-stimulated testosterone value in case 1 at two years and five mo of age, and the basal testosterone values in case 2 at one mo of age and in case 4 at three mo of age when serum testosterone is physiologically elevated. Thus, the diagnosis of idiopathic hypospadias was

initially made in cases 1–4. It was suspected that testosterone production was compromised only during fetal life, or that external genitalia had defective development of anlagen or impaired responsiveness to testosterone. While placental dysfunction could also affect male genital development by attenuating the production of hCG (2), there was no pregnant episode suggestive of placental dysfunction.

### *In situ* Hybridization (ISH) Analysis for Mouse *Mamld1*

ISH analysis for mouse *Mamld1* showed cell

type-specific expression pattern (3). Namely, *Mamld1* is specifically and transiently expressed in Sertoli and Leydig cells around the critical period for sex development (E12.5–E14.5) (Fig. 4). This expression pattern has been confirmed by double staining with antibodies for Ad4bp/Sf-1 that serves as a marker for Sertoli and Leydig cells. In extragonadal tissues at E12.5, *Mamld1* expression was absent in the adrenals and weakly and diffusely identified in the external genital region including the genital tubercle at a level similar to that detected in the neighboring extragenital tissues (Fig. 2B). *Mamld1* was also clearly expressed in the Müllerian ducts, forebrain, somite, neural tube, and pancreas. By contrast, *Mamld1* expression was absent in the postnatal testes. These data imply that nonsense mutations of *MAMLD1* cause hypospadias primarily because of transient testicular dysfunction and resultant compromised testosterone production around the critical period for sex development, and explain why postnatal endocrine data were normal in cases 1–4.

#### Function of *Mamld1* in Testosterone Production

We performed knockdown analysis with siRNAs for *Mamld1*, using mouse Leydig tumor (MLT) cells that retain the capability of testosterone production and the responsiveness to hCG stimulation (4). When the mRNA level of endogenous *Mamld1* was severely reduced in the mouse Leydig tumor cells (25–30%), testosterone production was decreased to 50–60% of the previous level after 48 h of incubation and one h after hCG stimulation. This implies that *MAMLD1* is involved in the testosterone biosynthesis. Furthermore, since testosterone production would probably be attenuated rather than abolished in the absence of *MAMLD1*, this is consistent with the hypospadias phenotype in the affected patients (2).

#### Conclusions

*MAMLD1* is a causative gene for hypospadias, and possibly other forms of 46,XY DSD. It appears to play a supportive role in the testosterone production around the critical period for sex development. Further studies including knockout mouse experiments will permit to clarify *MAMLD1* dependent molecular network involved in testosterone production.

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