MAMLD1 (CXorf6): A New Gene Involved in Hypospadias

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Key Words

MAMLD1 • CXorf6 • Hypospadias • Testosterone

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 studies have shown that (1) the mouse homolog is transiently expressed in fetal Sertoli and Leydig cells around the critical period of sex development; (2) transient knockdown of *Mamld1* results in significantly reduced testosterone production in murine Leydig tumor cells; (3) MAMLD1 protein shares homology to mastermind-like 2 (MAML2) protein that functions as a co-activator in canonical Notch signaling; (4) MAMLD1 localizes to the nuclear bodies and transactivates the promoter activity of a non-canonical Notch target gene hairy/enhancer of split 3 (*Hes3*), rather than the canonical Notch target genes such as *Hes1* and *Hes5*, without demonstrable DNA-binding capacity, and (5) *MAMLD1* is regulated by steroidogenic factor 1. These findings suggest that the *MAMLD1* mutations cause hypospadias primarily because of compromised testosterone production around the critical period of sex development, and provide useful information for the molecular network involved in fetal testosterone production.

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 ~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 α -reductase and *AR* for androgen receptor, pathologic mutations have been identified only in 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. Indeed, several candidate genes such as *ATF3*, *FKBP52*, *FGFR2*, *FGF8*, *FGF10*, and *BMP7* have been identified, and multiple susceptibility factors for hypospadias have been found in several genes such as *ESR1*, *ESR2*, and *SRD5A2* [3–7].

We have recently shown that *CXorf6* (chromosome X open reading frame 6) is a novel gene for hypospadias [8], and coined a new gene symbol *MAMLD1* (mastermind-like domain containing 1) on the basis of its characteristic protein structure [9]. Here, we review the current knowledge about *MAMLD1*.

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Fig. 1. Identification of *CXorf6* as a candidate DSD gene on Xq28 by deletion mapping. The horizontal bars indicate the deleted segments in each case. Of the 7 male patients with microdeletions around *MTM1* for myotubular myopathy, 5 show 46,XY DSD (primarily hypospadias), whereas 2 patients have normal genitalia as do patients with intragenic *MTM1* mutations. The smallest overlapping deleted region is roughly 208 kb in physical length, and contains *CXorf6* as a sole gene within the critical region.

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 is normal in patients with intragenic *MTM1* mutations, and invariably abnormal in 6 patients with microdeletions involving *MTM1* [10–13]. The 6 patients consist of 3 sporadic and 3 familial cases, and 5 of them have glandular, penile, or penoscrotal hypospadias and the remaining 1 patient exhibits ambiguous genitalia [10–12]. 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 a contiguous gene deletion syndrome.

In 1997, Laporte et al. [14] identified *MAMLD1* (named *CXorf6* at that time) from a 430-kb region deleted in 2 sporadic cases with myotubular myopathy and 46,XY DSD [12]. *MAMLD1* comprises at least 7 exons, and harbors an open reading frame on exons 3–6 that is predicted to produce 2 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 (fig. 1), 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 with various types of DSD or abnormal external genitalia. They consisted of 117 Japanese patients (113 sporadic cases and 4 probands of familial cases), 45 European patients (39 sporadic cases and 6 probands of familial cases), and 4 Chinese patients (4 probands of familial cases). The 117 Japanese patients comprised: 19 cases with gonadal dysgenesis (10 with complete type and 9 with incomplete type) with no demonstrable mutation in the known or candidate sex development genes *SRY*, *DMRT1*, *SF1*, and *LHX9* [2]; 2 cases with 46,XY DSD of unknown cause; 56 cases with hypospadias (16 with glandular type, 16 with penile type, 20 with penoscrotal type, and 4 with perineal type), and 40 cases with isolated cryptorchidism (33 with unilateral inguinal or abdominal type and 7 with bilateral inguinal type). All the Japanese patients had a normal male karyotype and lacked extragenital features except for short stature in 6 cases, mental retardation in 3 cases, and multiple congenital anomalies in 2 cases. Thus, most patients exhibited abnormal external genitalia as the sole recognizable abnormality. The 49 European and Chinese patients had various types of abnormal genitalia, ranging from hypospadias to feminized genitalia (detailed phenotypes are unknown).

Consequently, 3 nonsense mutations were identified in Japanese patients with hypospadias: E124X in mater-

Fig. 2. Molecular findings in patients with nonsense mutations. **a, b** adapted from Fukami et al. [8, 9]. **a** 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 the corresponding wild-type nucleotides. NE = Not examined. **b** 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. **c** The NMD analysis. 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. In case 4, no band is seen without an NMD inhibitor cycloheximide (CHX), whereas a clear band is delineated with CHX treatment.

nally related half brothers from family A (cases 1 and 2); Q197X in a patient from family B (case 3), and R653X in a patient from family C (case 4; fig. 2a) [3]. The mothers of families A and C were heterozygous for the mutations, although the mother of family B was not studied. In ad-

dition to the 3 nonsense mutations, we also found 3 apparently non-pathologic variants: P286S and Q507R that were not co-segregated with the 46,XY DSD in affected families, and a previously reported polymorphism N589S (*rs2073043*) [3].

Table 1. Clinical findings of the 4 Japanese cases with *MAMLD1* nonsense mutations

	Case 1	Case 2	Case 3	Case 4
<i>Genital findings</i>				
Gestational age, weeks	39	40	40	41
Birth length, cm	51.0 (+1.0 SD)	49.5 (+0.2 SD)	50.5 (+0.7 SD)	47.5 (−0.7 SD)
Birth weight, kg	3.61 (+1.5 SD)	3.40 (+1.0 SD)	3.21 (+0.5 SD)	2.94 (−0.2 SD)
Age at exam	4 months	1 month	2 years	1 month
Clinical diagnosis	Hypospadias with chordee	Hypospadias with chordee	Hypospadias with chordee	Hypospadias with chordee
Urethral meatus	Penoscrotal junction	Penoscrotal junction	Penoscrotal junction	Penoscrotal junction
Age at urethroplasty, years	2.5	3.9	6.0 and 6.6	1.9
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)
Age at orchidopexy, years	6.3	–	–	1.9
Scrotal appearance	Bifid and hypoplastic	Bifid	Bifid	Bifid
Wolffian structures	Normal on MRI	Normal on MRI	NE	NE
Müllerian structures	Absent on MRI	Absent on MRI	NE	NE
Renal structures	Normal on MRI	Normal on MRI	Normal on ultrasounds	NE
<i>Serum hormone values</i>				
Age at exam	4 months	1 month	2 years	3 months
LH, IU/l	1.2 (0.1–4.7)	3.1 (0.1–4.7)	0.2 (<0.2–3.1)	NE
FSH, IU/l	1.5 (0.4–5.7)	2.2 (0.4–5.7)	1.6 (0.2–5.2)	NE
Testosterone, nmol/l	1.4 (0.1–12.0)→9.0 (7.0–15.0) ^a	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 ^a	1.2 (0.2–4.5)	NE	NE
Age at exam, years:months	2:05	2:05	4:00	6:03
LH, IU/l	0.2 (<0.2–3.1)→3.5 (1.4–6.0) ^b	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) ^b	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) ^a	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 ^a	<0.15 (0.05–2.0)	NE	NE

SD = Standard deviation; NE = not examined; B = bilateral; MRI = magnetic resonance imaging; WNR = within the normal range (1–2 ml before puberty); ND = not determined; LH = luteinizing hormone; FSH = follicle-stimulating hormone; DHT = dihydrotestosterone.

Assessments of body sizes (length, height, weight, and head circumference), penile length, testis size, and menarchial age are based on the Japanese reference data. The hormone values in parentheses represent the age- and sex-matched normal range in the Japanese; the reference data for serum hormones are based on the literature.

^a After a human chorionic gonadotropin stimulation (3,000 IU/m²/dose i.m. for 3 consecutive days; blood sampling on day 4).

^b Peak values during a gonadotropin-releasing hormone test (100 µg/m² bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 min).

Fig. 3. External genital findings of cases 1 and 2.

Nonsense-Mediated mRNA Decay

When the 3 nonsense mutations were identified, one problem was that hypospadias in case 4 with R653X on exon 5 may be inconsistent with apparently normal genital development in a previously reported boy with a microdeletion involving *MTM1* that resulted in the generation of a fusion gene between exons 1–4 of *MAMLD1* and exons 3–16 of *MTM1* (locus order: *MAMLD1*–*MTM1*–*MTM1*), because the coding exons 3 and 4 are preserved in both case 4 and the boy with the fusion gene [15] (fig. 2b). However, in contrast to the positive expression of the fusion gene [15], the 3 nonsense mutations are predicted to cause nonsense-mediated mRNA decay (NMD) because of their positions [16]. Consistent with this, RT-

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; L = Leydig cell. The scale bars in the low and high power fields represent 200 and 20 μ m, respectively. Adapted from Fukami et al. [8]. **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; GT = genital tubercle (the region 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.

PCR from leukocytes indicated drastically reduced transcripts in cases 1–4 (fig. 2c) [3, 4]. Furthermore, the NMD was prevented by the NMD inhibitor cycloheximide, providing further support for the occurrence of NMD in the 3 nonsense mutations. The occurrence of NMD was also demonstrated in the carrier mothers [4]. Thus, although the 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. [15], and indicate that the 3 nonsense mutations including R653X are pathologic mutations.

Phenotypes in Mutation-Positive Patients

Cases 1–4 had penoscrotal hypospadias with chordee as the conspicuous genital phenotype, in association with other genital phenotypes (fig. 3, 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 2 years and 5 months of age, and the basal testosterone values in case 2 at 1 month of age and in case 4 at 3 months of age when serum testosterone is physiologically elevated. Thus, the diagnosis of idiopathic hypospadias was initially made in cases 1–4.

In situ Hybridization Analysis for Mouse *Mamld1*

In situ hybridization analysis for mouse *Mamld1* showed a cell type-specific expression pattern [3]. Namely, *Mamld1* is specifically and transiently expressed in Sertoli and Leydig cells around the critical period of sex development (E12.5–E14.5; fig. 4a). 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 [17–19]. 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. 4b). *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 of sex development, and explain why postnatal endocrine data were normal in cases 1–4.

Fig. 5. Effects of siRNA on testosterone production in the mouse Leydig tumor (MLT) cells. Adapted from Fukami et al. [9]. Relative mouse *CXorf6* and *Sf-1* mRNA levels have been reduced to 25–30% in the MLT cells after 48 h of incubation with two siRNAs. NC = Negative control transfected with non-targeting RNA. **a** Testosterone concentration in the medium after 48 h of incubation with siRNAs. **b** Testosterone concentration in the medium after 1 h of incubation with hCG using the MLT cells cultured with siRNA for 48 h.

Function of *Mamld1* in Testosterone Production

We performed knockdown analysis with siRNAs for *Mamld1* using mouse Leydig tumor 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% after 48 h of incubation and 1 h after hCG stimulation (fig. 5). However, the testosterone reduction was much milder than that caused by siRNAs for *Sf-1* (fig. 5; our unpublished observation). The results were confirmed with 2 different siRNAs. This implies that *MAMLD1* is involved in 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].

Sf-1 Controls *Mamld1*

Mouse *Mamld1* is co-expressed with *Ad4bp/Sf-1*, and *SF-1* is known to regulate the transcription of a vast array of genes involved in sex development by binding to specific DNA sequences [17–19]. This implies that *Mamld1*

is also controlled by *Sf-1*. Consistent with this notion, human *MAMLD1* harbors a putative SF-1-binding sequence ‘CCAAGGTCA’ at intron 2 upstream of the coding region [4]. This binding site also resides at intron 1 upstream of the coding region of the mouse *Mamld1*. Furthermore, we performed DNA binding and luciferase assays, showing that SF-1 protein binds to the putative target sequence and exerts a transactivation function [4]. These findings argue for the possibility that *Mamld1* expression is regulated by *Sf-1*.

Functional Studies of MAMLD1 Protein

We found that MAMLD1 protein has a unique structure with homology to that of mastermind like 2 (MAML2) protein (fig. 6a) [4]. A unique amino acid sequence, which we designate mastermind-like (MAML) motif, was inferred from sequence alignment with MAML1, MAML2, and MAML3 proteins. The MAML motif was well conserved among MAMLD1 orthologs identified in frogs, birds, and mammals. In addition, glutamine-rich, proline-rich, and serine-rich domains were identified in MAMLD1.

MAML2 is a non-DNA-binding transcriptional co-activator in Notch signaling that plays an important role in cell differentiation in multiple tissues by exerting either inductive or inhibiting effects according to the context of the cells [20–22]. Upon ligand-receptor interaction, the Notch intracellular domain (N-ICD) is translocated from the cell surface to the nucleus and interacts with a DNA-binding transcription factor, recombination signal binding protein-J (RBP-J), to activate target genes like hairy/enhancer of split 1 (*Hes1*) and *Hes5* [23]. In this canonical Notch signaling process, MAML2 forms a ternary complex with N-ICD and RBP-J at nuclear bodies, enhancing the transcription of the Notch target genes [20, 21, 24–26]. In addition to such canonical Notch target genes, recent studies have shown that *Hes3* can be induced by stimulation with a Notch ligand, via a STAT3 (signal transducer and activator of transcription 3)-mediated pathway [27]. This finding, together with lack of *Hes3* induction by N-ICD [22], implies that *Hes3* represents a target gene of a non-canonical Notch signaling.

Thus, we first examined whether MAMLD1 localizes to the nuclear bodies, as observed for MAML2 [4]. Since PCR-based human cDNA library screening has revealed that the exon 4-positive splice variant is more strongly expressed than the exon 4-negative splice variant (Δ Exon 4) [3], functional studies were performed primarily with

Fig. 6. Functional studies of the wild-type MAMLD1 protein. Adapted from Fukami et al. [9]. **a** Protein structure analysis. The structure of human CXorf6 (MAMLD1) and MAML2 proteins. The identified domains are shown, together with the positions of the three nonsense mutations. **b** Subcellular localization analysis, showing co-localization of the wild-type MAMLD1 and MAML2

in the nuclear bodies. **c** Transactivation functions for the promoter of *Hes3*. + = Presence of expression vectors with cDNAs for *MAMLD1*, *MAML2*, *N1-ICD* (Notch 1 intracellular domain), and *N2-ICD* (Notch 2 intracellular domain); – = presence of expression vector only (empty).

the exon 4-positive splice variant (thereafter, this variant is simply described as *MAMLD1*). *MAMLD1* was distributed in a speckled pattern and co-localized with the *MAML2* protein (fig. 6b). Furthermore, while the E124X and Q197X fusion proteins resided in the nucleus, they were incapable of localizing to the nuclear bodies. The R653X and apparently non-pathologic missense proteins showed a punctate pattern, and co-localized with the wild-type *MAMLD1*.

Next, we studied whether *MAMLD1* has a transactivation function for Notch targets using luciferase reporter assays [4]. Although *MAMLD1* was incapable of enhancing the promoter activities of the canonical Notch target genes *Hes1* and *Hes5* with the RBP-J-binding site [22], *MAMLD1* transactivated the promoter activity of the non-canonical Notch target gene *Hes3* without the RBP-J-binding site (fig. 6c) [28]. These results argue that *MAMLD1* exerts its transactivation activity independent

of RBP-J-binding sites. Thus, while it was predicted that *MAMLD1* protein has a DNA-binding capacity, after extensive analysis, no evidence has been obtained for a positive DNA binding of *MAMLD1* [4].

Furthermore, the E124X and Q197X proteins had no transactivation function, whereas the R653X protein as well as the 3 variant (P286S, Q507R, and N589S) proteins retained a nearly normal transactivating activity [4]. In addition, the transactivation function was significantly reduced in the L103P protein (an artificially constructed variant affecting the MAML motif) and normal in the Δ Exon 4 [4]. These findings suggest that the E124X and Q197X proteins have no transactivation function, consistent with the inability of localizing to the nuclear bodies. However, the R653X protein, when it is artificially produced, has a normal transactivating activity, although R653X as well as E124X and Q197X have been demonstrated to undergo NMD in vivo [3, 4].

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 of sex development. MAMLD1 protein lo-

calizes to the nuclear bodies and has a transactivation function for *Hes3* at least in vitro. Further studies including knockout mouse experiments will enable clarification of the *MAMLD1*-dependent molecular network involved in testosterone production.

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