RESEARCH FRONTIER

MAMLD1 (CXorf6) : A New Gene for Hypospadias

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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～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 mutations analyses have been performed for multiple genes involved in androgen effects such as *SRD5A2* for 5alpha-redeuctase 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. In addition, it is likely that many genes for hypospadias remain to be identified.

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 *MAMLD 1* (mastermind-like domain containing, 1), on the basis of its characteristic protein structure (see below) [4]. Here, 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 is normal in patients with intragenic *MTM1* mutations, and invariably abnormal in six patients with microdeletions involving *MTM1* [5–8]. The six patients consist of three sporadic and three familial cases, and five of them have glandular, penile, or penoscrotal hypospadias and 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 a consequence of contiguous gene deletion syndrome.

In 1997, Laporte et al. [9] identified *CXorf6* from a 430-kb region deleted in two sporadic cases with myotubular myopathy and 46, XY DSD [7]. *CXorf6* 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 the loss of *CXorf6* in all patients with myotubular myopathy and 46, XY DSD (Bartsch et al., 1999 ; JL, unpublished observation), and no other candidate gene for 46, XY DSD has been identified within the commonly deleted region. These findings imply that *CXorf6* is an excellent candidate gene for 46, XY DSD, especially hypospadias.

Identification of pathologic *CXorf6* mutations in hypospadiac patients

We performed direct sequencing for the coding exons 3–6 and their flanking splice sites of *CXorf6* in 166 patients with various types of 46, XY DSD (152 sporadic cases and 14 probands of familial cases). The 166 patients consisted of 117 Japanese patients (113 sporadic cases and four probands of familial cases), 45 European patients (39 sporadic cases and six probands of familial cases), and four Chinese patients (four probands of familial cases). The 117 Japa-

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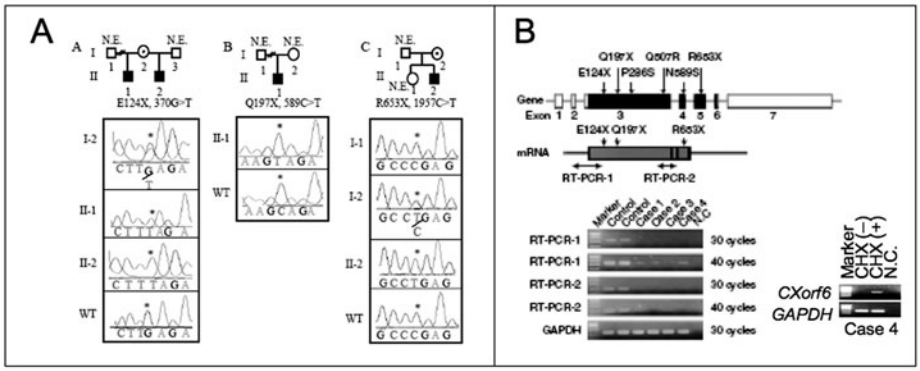

Fig. 1. Molecular findings in patients with nonsense mutations.

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 *CXorf 6*, and the circles with dots represent molecularly confirmed carrier females. The asterisks in the chromatograms indicate the mutant and the corresponding wildtype nucleotides. N.E.: not examined.

B. The 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-1and-2) has produced no bands after 30 cycles and very faint bands after 40 cycles in cases1-4. Lowe part: NMR 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 cycloheximide (CHX) treatment, and a clear band is delineated with CHX treatment.

nese 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 determining genes *SRY, DMRT1, SF1 (alias AD4BP)*, and *LHX9* [2], two cases with 46, XY DSD with feminized genitalia 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 seven with bilateral inguinal type).

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 a patient from family C (case 4) (Fig. 1A) [3]. The mothers of families A and C were heterozygous for the mutations, although the mother of family B was not studied. These mutations were absent in 150 Japanese control males. In addition to the three nonsense mutations, we also found three apparently non-pathologic variants: P286S and Q507R that were not co-segregeated with the 46, XY DSD in affected families, and a previously reported polymorphism N589S (rs2073043). In addition, direct sequencing confirmed lack of a mutation in *AR and SRD5A2* in cases 1-4.

The three nonsense mutations are predicted to cause nonsense mediated mRNA decay (NMD) because of their positions [10]. Consistent with this, RT-PCR for leukocytes indicated drastically reduced transcripts in the cases 1-4 (Fig. 1B). Furthermore, the NMD was protected by an NMD inhibitor cycloheximide, providing further support for the occurrence of NMD in the three nonsense mutations [3, 4].

Clinical findings in mutation positive patients

The cases 1-4 had penoscrotal hypospadias with chordee as the conspicuous genital phenotype, in association with other genital phenotypes (Table1). Pituitary-gonadal serum hormone values remained within the normal range, including the human chorionic gonadotropin (hCG)-stimulated testosterone value in the case 1 at two years and five months of age, and the basal testosterone values in the case 2 at one month of age and in the case 4 at three months of age when serum testosterone is physiologically elevated. Thus, the diagnosis of idiopathic hypospadias was initially made in the 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 human chorionic gonadotropin [2], there was no pregnant episode suggestive of placental dysfunction. The three mothers experienced

Table 1 Clinical findings of the four Japanese cases with *CXorf6* 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	2.5uyr	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)[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)	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)[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)	N.E.	N.E.

Abbreviations. 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 menarchial age is 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 have been based on the literature.

[a]After a human chorionic gonadotropin stimulation (3000 IU/m^2/dose i.m. for three consecutive days ; blood sampling on day 4).

[b]Peak values during a gonadotropin releasing hormone test (100μg/m^2 bolus i.v. ; blood sampling at 0, 30, 60, 90, and 120 min).

menarche at 12–13 years of age and had regular cycles at 35, 37, and 38 years of age, respectively.

Expression pattern

PCR-based human cDNA library screening has revealed ubiquitous expression of *CXorf6* with and without exon 4 (ΔExon 4) [3]. Exon 4 positive splice variant was more strongly expressed than ΔExon 4.

In situ hybridization (ISH) analysis for the murine homologous gene (*G630014P10Rik, here described as m-CXorf6* for convenience) showed cell type-specific expression pattern [3]. Namely, *m-CXorf6* is specifically and transiently expressed in Sertoli and Leydig cells around the critical period for sex development (E12.5 -E14.5) (Fig. 2A). This expression pattern has been confirmed by double staining with antibodies for Ad4 bp/Sf-1 that serves as a marker for Sertoli and Leydig cells. In extragonadal tissues at E12.5, *m-CXorf6* 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.2 B). *m-CXorf6* was also clearly expressed in the Müllerian ducts, forebrain, somite, neural tube, and pancreas. In the postnatal testis, *m-CXorf6* expression was weakly identified within the cords until one week of age and became faint thereafter. In the postnatal ovary, *m-CXorf6* expression was barely detected until two weeks of age and clearly identified in granulosa cells at the perifollicular regions of most Graafian follicles at 3 and 8 weeks of age.

These data imply that nonsense mutations of *CXorf 6* cause hypospadias primarily because of transient testicular dysfunction and resultant compromised testosterone production around the critical period for sex

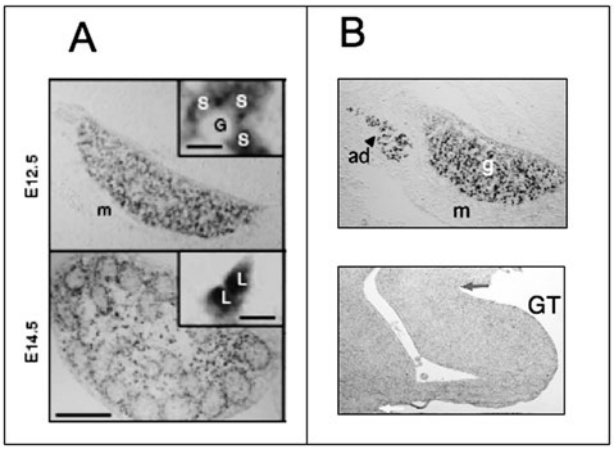

Fig. 2. *In situ* hybridization analysis of the murine homolog for *CXorf6(m-CXorf6)*.

A. Expression patterns in the fetal testes at E12.5 and E 14.5. The blue signals are derived from *in situ* hybridization for *m-CXorf6*, and the brown signals from immunohistochemical staining with Sf1 (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 μm and 20 μm, 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). *CXorf6* is not expressed in the adrenal, and weakly and diffusely expressed in the external genitalia as in other non-genital skin tissues.

development. The data also explain why postnatal endocrine data were normal in the cases 1–4. Furthermore, from the expression patterns in the adult testes and ovaries, it is worth examining whether spermatogenic function can be preserved in *CXorf6* mutation positive patients, and whether *CXorf6* is involved in the adult ovarian function.

SF-1 target sequence in *CXorf6*

Mouse *CXorf6* is co-expressed with *Ad4bp/Sf-1*, and *SF-1* is known to regulate multiple genes involved in sex development, by binding to specific DNA sequences [11–13]. This implies that *CXorf6* is also controlled by *Ad4bp/Sf-1*. Consistent with this notion, *CXorf6* harbors a putative SF-1 binding sequence "CCAAGGTCA" at intron 2 in the upstream of the coding region. This binding site also resides at intron 1 in the upstream of the coding region of the *m-CXorf6*. Furthermore, we performed DNA binding and luciferase assays, showing that SF-1 binds to the putative target sequence and exert a transactivation function [4]. These findings suggest that *CXorf6* is regulated by *Ad4bp/Sf-1*.

Function of *m-CXorf6* in testosterone production

We performed knockdown analysis with siRNAs for *m-CXorf6*, using mouse Leydig tumor (MLT) cells that retain the capability of testosterone production and the responsiveness to hCG [4]. When the mRNA level of endogenous *m-CXorf6* was severely reduced in the mouse Leydig tumor cells (25–30%), testosterone production was decreased to 50–60% after 48 hours of incubation and one hour after hCG stimulation. This implies that *CXorf6* is involved in the testosterone biosynthesis. Furthermore, since testosterone production would probably be attenuated rather than abolished in the absence of *CXorf6*, this is consistent with the hypospadias phenotype in the affected patients [2].

Functional studies of CXorf6 protein

We found that CXorf6 protein has a unique structure with homology to that of mastermind like 2 (MAML2) (alias, Mam-3) protein (Fig. 3A) [4]. A unique amino acid sequence, which we designate mastermind -like (MAML) motif, was inferred from sequence alignment with MAML1, MAML2, and MAML3 proteins [14, 15]. The MAML motif was well conserved among CXorf6 orthologs identified in frog, bird, and mammals. In addition, a glutamine-rich, a proline-rich, and a serine-rich domains were identified in *CXorf6*.

MAML2 is a non-DNA binding transcriptional co-activator in Notch signaling [14, 15] 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 [16]. Upon ligand-receptor interaction, 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* [17]. In this canonical Notch signaling process, MAML2 forms a ternary complex with N -ICD and RBP-J at nuclear bodies, enhancing the tran-

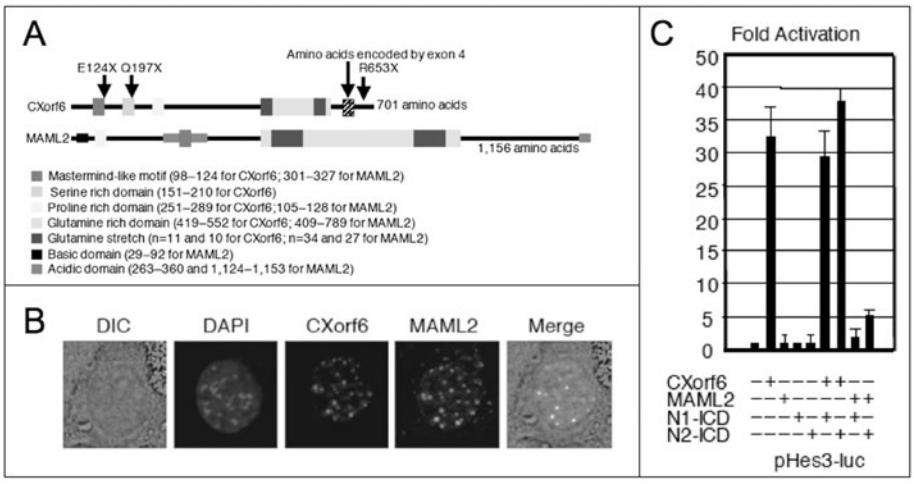

Fig. 3. Functional studies of the wildtype CXorf6 protein.

A. Protein structure analysis. The structure of human CXorf6 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 wildtype CXorf6 and MAML2 in the nuclear bodies.

C. Transactivation functions for the promoter of *Hes3*. The (+) symbols indicate the presence of expression vectors with cDNAs for *CXorf6*, *MAML2*, N1-ICD (Notch 1 intracellular domain), and N2-ICD (Notch 2 intracellular domain), whereas the (−) symbols denote the presence of expression vector only (empty).

scription of the Notch target genes [14, 15, 18–20]. 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 transcription3) mediated pathway [21]. This finding, together with lack of *Hes 3* induction by N-ICD [22], implies that *Hes3* represents a target gene of a non-canonical Notch signaling.

Thus, we first examined whether CXorf6 localizes to the nuclear bodies, as observed for MAML2 [4]. CXorf6 (wildtype with exon 4) was distributed in a speckled pattern and co-localized with the MAML2 protein (Fig. 3B). 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 wildtype CXorf6.

Next, we studied whether *CXorf6* has a transactivation function for Notch targets using luciferase reporter assays [4]. Although *CXorf6* was incapable of enhancing the promoter activities of the canonical Notch target genes *Hes1* and *Hes5* with the RBP-J binding site (16), *CXorf6* transactivated the promoter activity of the non-canonical Notch target gene *Hes3* without the RBP-J binding site (Fig. 3C) [22]. These results argue that *CXorf6* exerts its transactivation activity independently of RBP-J binding sites.

Furthermore, the E124X and Q197X proteins had no transactivation function, whereas the R653X protein as well as the three variant (P286S, Q507R, and N589 S) proteins retained a nearly normal transactivating activity. In addition, the transactivation function was significantly reduced in the L103P protein (an artificially constructed variant affecting the MAML motif) and normal in the ΔExon 4. These findings suggest that the E124X and Q197X proteins have no transactivation function, and that R653X protein, when it is artificially produced, has a normal transactivating activity, although R653X as well as E124X and Q197X undergoes NMD *in vivo*.

However, *CXorf6* is unlikely to have DNA-binding activity [4], so that it remains to be clarified how *CXorf6* transactivates *Hes3*. It also remains to be determined whether transactivation for *Hes3* also takes place *in vivo*, and is involved in testosterone production.

Conclusions and perspectives

MAMLD1 (CXorf6) is a causative gene for hypospadias, and possibly other forms of 46, XY DSD. *MAMLD1 (CXorf6)* appears to play a supportive role in

the testosterone production around the critical period for sex development. MAMLD1 (CXorf6) protein localizes to the nuclear bodies and has a transactivation function for *Hes3* at least *in vitro*.

However, several matters remain to be clarified. They include : (1) the prevalence of *MAMLD1(CXorf6)* mutations in hypospadias and other 46, XY DSD ; (2) the involvement of *MAMLD1(CXorf6)* in spermatogenic function and ovarian function ; (3) *MAMLD1(CXorf6)* dependent molecular network involved in testosterone production ; and (4) the molecular relevance of *MAMLD 1(CXorf6)* to Notch signaling. Further studies including knockout mouse experiments, are necessary to resolve these matters.

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