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ORIGINAL ARTICLE

Polymorphism of 3′ UTR of MAMLD1 gene is also associated with increased risk of isolated hypospadias in Indian children: a preliminary report

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Abstract

Objective To study MAMLD1 gene polymorphisms, serum LH and testosterone levels amongst Indian children with isolated hypospadias (IH) and controls.

Materials and methods Screening of the MAMLD1 gene was performed by PCR sequencing method in 100 Indian children aged 0–12 years presenting with IH and 100 controls. LH and testosterone hormone levels were also assessed (categorized in four age-wise groups).

Results IH subjects had significantly higher incidence of MAMLD1 polymorphism as compared to controls (33 vs 15 %, $p = 0.01$). Of various genomic variants identified in this study, the noteworthy novel ones were missense mutation P299A and single nucleotide polymorphism c.2960C>T in 3′ UTR of Exon 7. While p 299A was found to cause protein structural instability consequent to amino acid change, eighty percent subjects with c.2960C>T in 3′ UTR of Exon 7 (corresponding to newly discovered currently non-validated exon 11) were found to have lower testosterone levels when compared with their age group mean. IH showed statistically higher incidence of c.2960C>T in comparison to controls (22 vs 10 %, p value 0.046) and about 2.5-folds higher risk of this anomaly.

Conclusion Occurrence of MAMDL1 gene polymorphisms, specially of c.2960C>T in 3′ UTR of its exon 7 is associated with a higher risk of IH in Indian children, probably by lowering androgenic levels.

Keywords MAMLD1 · Isolated hypospadias · Polymorphism · India · 3′ UTR

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Introduction

Hypospadias is a common error of the male genital development. It results from an arrest in the normal development of the urethra causing urethral meatus to be present at variable sites on the ventral aspect of the penis during the first trimester [1]. Baskin et al. 2001 reported the incidence of hypospadias as one in 125–1 in 250 male live births [2]. Recently Bianchi et al. and Samtani et al. have reported an increase in incidence of hypospadias in Southern Italy (presently 46.7/10,000) and India (from 7.66 % in 2003 to 9.33 % in 2011), respectively [3, 4].

The etiology of hypospadias is known to be highly heterogeneous involving hormonal, environmental and genetic factors [5], An altered function of genes involved in early penile/genital development [sonic hedgehog (Shh), fibroblast growth factors 8 and 10 (FGF8 and FGF10) and homeoboxA13 and D13 (HOXA13 and HOXD13] [6–8], testicular development and androgenic functions may cause hypospadias. Familial clustering is seen in up to 25 % cases of hypospadias. The incidences of hypospadias have been reported as 27 % in monozygotic twins and 9 % in dizygotic twins [9–11]. Though mutations have been noted for various genes involved in causing androgenic

effects such as SRD5A2 (for 5α-redeuctase) and AR (for androgen receptor), pathologic mutations have been observed only in a very small fraction of patients [5].

Mastermind like domain containing 1 (MAMLD1) gene (earlier known as the Chromosome X open reading frame 6 (CXorf6) was found to be deleted in patients with X-linked myotubular myopathy (MTM1) and the same gene was identified as the first causative gene for hypospadias, and possibly other forms of 46, XY Disorders of Sexual Development (DSD) [12, 13]. MAMLD1 gene contains seven exons and harbors an open reading frame on exons 3–6 which generate two proteins as a result of in-frame alternative splicing with and without exon 4 [14, 15].

As per latest literature, MAMLD1 contains 11 exons (http://www.ncbi.nlm.nih.gov/gene/10046), however only seven exons have been found to be validated till date (http://www.genome.ucsc.edu), further researches is required to validate and to find out exact function of all exons, MAMLD1 gene codes for at least ten different protein coding transcripts which codes for MAMLD1 protein of six different length (708, 733, 749, 774, 998 and 1023 aminoacids). (http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=otherfeatures;g=10046;r=X:150361589-150514178).

MAMLD1 (CXorf6) is expressed in fetal Sertoli and Leydig cells around the critical period of sex development and it is co-expressed with steroidogenic factor (SF-1) which is a modulator of gene transcription involved in testicular differentiation [13, 16]. Thus MAMLD1 gene appears to play a supportive role in testosterone production around the critical period of sex development [17]. It has been observed by earlier studies that mutations in the initial part of Exon 3 are more frequent in patient with hypospadias. Most of these studies targeted mainly children with DSD and only a small fraction of children with IH [13, 15, 17–22]. Since the incidence of IH far exceeds that of hypospadias in association with DSD [5], it was interesting to evaluate if the changes in this gene were also associated in patients with IH as found earlier by few workers [18, 19].

Materials and methods

This study was carried out in Department of Pediatric Surgery in collaboration with the Departments of Pediatrics and of Biochemistry of our tertiary referral centre during the period February 2012–April 2015. The study protocol was approved by the institutional committee for research ethics. Both study subjects and controls were in age range of 0–12 years and were stratified into four age wise subgroups (due to discrete hormonal profile present at different age-points), namely; Group I: Newborn to ≤2 months, Group II: >2 months to ≤4 years, Group III: >4 years to ≤8 years and Group IV: >8 years to ≤12 years.age group. Subjects presenting with genital ambiguity, other obvious dysmorphic features or suspected syndromic association and previous sex steroidal or hormonal treatment for any condition were excluded. The controls were in patients with conditions other than hypospadias, DSD or any other urological condition.

Written informed consents were obtained from the parents of all study subjects and assent was also obtained from all the study subjects with age >7 years. Demographic information and anatomic details of each study subject, as relevant were recorded. Hypospadias was classified based on the anatomical location of the proximally displaced urethral orifice. For both groups (cases and controls), morning fasting blood samples were collected in plain vials (for hormonal estimation) and in EDTA vials (for molecular analysis) and immediately transported to the laboratory. Sample in plain vial was subjected to serum separation and serum was stored at −70 °C until tested. Genomic DNA from patients and controls were isolated from 200 μl whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, GmbH, Germany) according to the manufacturer's instruction. PCR amplification of Exon 3, Exon 4, Exon 5 and Exon 6 of MAMLD1 gene were carried out as per the method of Kalfa et al. 2008. A total of eight primer set were used to amplify the exon 3, 4, 5 and 6 of MAMLD1 gene (Table 1). For designing primers for PCR amplification of Exon 1, Exon 2 and Exon 7 of MAMLD1 gene, complete Human MAMLD1 (CXorf6) gene sequence was retrieved from GenBank. Primer for the amplification of target sequences were designed in Gene Runner version 3.02 software programme observing the following requirements: 20–25 bases in length, with 40–60 % GC content, with no secondary structure and non self complementary (Table 1).

PCR amplification

PCR was carried out in a total volume of 50 ul. The PCR mixture consisted of 18 mM Tris–HCl (pH 8.3), 45 mMKCl, 0.4 mM each of dNTP, 1U of Taq DNA polymerase (Fermentas life sciences) and forward and reverse primer (primer conc. 0.20–0.75 μM), $MgCl_2$ (1.5–2 mM). PCR was carried out in a thermal cycler (C1000 cycler, BIORAD). The PCR conditions were as follows: 94 °C for 3 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 58–60 °C (depending on primer Tm) for 30 s and extension at 72 °C for 30 s; and a final extension at 72 °C for 7 min. All amplified PCR products were subjected to electrophoresis on 1 % agarose gel containing 0.5 μg/ml of ethidium bromide and observed under

Table 1 Primers used for amplification for exons of MAMLD1 gene

	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size
Exon 3			
Part 1	CTCTCTTCTCCTCTTCTCTTCTCT	GGTGAGCTCCTCTAGCAGCTCTTG	416
Part 2	GCCGGCTGTAGACCAGGAGC	GGGCGTGATGCCACTGAGGC	407
Part 3	GTCGCTCTGCCCCCCTTACC	GAATGGAGCTCTGAGGGCCG	412
Part 4	GCT CTC CCA ATG CCT TAC TG	GCT TGG TGT TGC CAA AAG TA	485
Part 5	CATGATCATGCAGCAGGGGATGGCAAGC	CCAAAACAAAAGAACACAGCCAGATATG	401
Exon 4	TAGGACACGGCAGGCCACCTG	AGCCAGCTGATCCAGCTCTGG	282
Exon 5	TTTGTGGCCAAGCAGCTGATG	GACTGTGCCCCGTTGACAGAT	342
Exon 6	CTAGGTCCCCACGCAGCGATC	ACCCCTTGCCCACCCCTTTGG	210
Exon 1	CTCCTCCATCCACCCCTCTACCTTC	ACTCGTCTTCCCTCTCTCATCCTTC	726
Exon 2	GCTACTCTCAGGATTCACTTTGCTA	AATCACACAGGCAAAGGGAGAAGT	518
Exon 7			
Part1	GAGGTGAGAAGTCCGTGTTGGGC	CTGCTGGAGGAACTTGGTGGTGT	787
Part 2	GCTGCGATGGGAACACAAGT	ACTGAAACAAAAGCCGTCGC	792
Part 3	ATGCTTATCCCTCTCTCTACCTGTG	CGGTCTGAATGGTAAAGGGAGGAGG	629
Part 4	ACAGAGACACTTTACCAGGAGGCT	ATCCCCCTCTGTTTCATCCTCTAT	704

ultraviolet light. Specific segment sizes for different exon/exon part of MAMLD1 gene were observed.

Nucleotide sequencing

PCR product purification was performed with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and nucleotide sequencing was obtained commercially (Sequencing done by ABI 3730x1DNA Analyzer—Applied Biosystems, Foster City, CA, USA). The forward primers were used as sequencing primers. The discovered mutation sites were verified for reliability and repeatability using repeated processes.

The analysis of nucleotide sequences were performed using DNA star software adopted from Institute of Genomic and Integrative Biology (IGIB, CSIR Lab), Chromas v 2.31/Finch TV and Clustal W programme software for the existence of single nucleotide polymorphism in different patient samples and control samples. Nucleotide sequences of study subjects and reference sequence were aligned by using these software. Amino acid sequences were also deduced from nucleotide sequences and compared with amino acid sequence of reference (MAMLD1) protein. Using these software, the novelty of the single nucleotide polymorphism was observed in the sequences when aligned with the reference genome available at National Centre for Biotechnology Information (NCBI) database. The functional consequences of amino acid changes were predicted using four algorithms. Polyphen [23], Sift [24], Provean [25] and mutation tester [26] were used at http://genetics. bwh.harvard.edu/pph2/, http://sift.jcvi.org/, http://provean. jcvi.org/index.php and http://www.mutationtaster.org/ respectively.

Structure prediction

In order to evaluate and understand the structural implications of the newly discovered Pro299Ala mutant and to estimate effect of these changes with the pathogenic effect of the protein, different computational tools were employed. In silico studies involving secondary and tertiary structure prediction of mamld1 protein were carried out using JPred [27] and PHYRE [28] softwares respectively. The consequences of the proline to alanine substitution on protein stability were analysed by I-Mutant2.0, [29] a tool for the prediction of protein stability which predicts changes upon single point mutations. The relative surface accessibility of the amino acids in the wild type and variant was studies by Netsurf [30].

Quantitative estimation of LH and testosterone Hormone

The serum testosterone (ng/ml) and luteinizing hormone level (LH) mIU/mL in isolated hypospadias subjects and controls were measured by using electro chemiluminescence method (ELECYS 2010, Roche), chemiluminescence immunoassay kit (Roche Diagnostics Gmbh, D-68298 Mannheim) as per manufacturer's instruction. The limit of detection for LH and Testosterone were 0.100 mIU/mL and 0.025 ng/mL respectively and specificity was more than 95 %.

Karyotyping

All hundred cases were karyotyped. For karyotyping chromosomal cultures were setup according to GTG banding [31]. For each case, 50 spread metaphases were analyzed.

The results were tabulated both subject-wise for each in IH and controls; and additionally tabulated age group-wise for hormone levels (LH and Testosterone).

Statistical analysis

For the purpose of statistical analysis, the data was tabulated side by sidefor hormone levels and identified exon-specific gene polymorphisms in IH subjects and controls. Two sample Wilcoxon rank sum, Mann–Whitney test and Kruskal–Wallis test were applied to assess separately the statistical significance of mean/median values of hormones for cases and controls. For assessing difference for MAMLD1 gene polymorphism distribution between study and control groups as well as for risk computation, Chi square and the Fisher exact test were used. A p value <0.05 was considered statistically significant. All statistical tests were performed using WHO software Epi Info-6 version 3.3 (CDC, Atlanta, USA).

Results

All subjects had 46XY karyotype. Out of 100 case and 100 controls, MAMLD1 gene variants were detected in 33 (33 %) cases and (15 %) fifteen controls, making the overall incidence of polymorphism among study subjects as twice of that found in controls and this was found to be statistically significant ($p = 0.01$).

During the study, significant mutations of MAMLD1 gene fell in 3′ UTR region as per the validated data of MAMLD1 gene sequence whereas inclusive of all data these fell in exon 11 (Table 2). However since the data

about the latter is awaiting validation, hence we chose to stick to the validated data in the current date and have labelled these mutations in 3′ UTR region of the MAMLD1 gene.

A total of seven different types of MAMLD1 genomic variants (four missense, two synonymous and one nucleotide change in exon 7 of MAMLD1 gene which corresponds to 3′ UTR of MAMLD1 mRNA) were identified during the study (Table 2) Some other interesting observations were: (1) Two folds higher incidence of polymorphism (single nucleotide change) in 3′ UTR i.e. noncoding region of exon 7 ($n = 22$, 22 %) in comparison to the incidence for coding region ($n = 11$,11 %) of this gene among IH cases and this occurred as single nucleotide polymorphism (SNP, rs2070779). (2) Occurrence of most polymorphisms among coding region in exon 3 in entire study group (75 % i.e. 12/15) and incidence was slightly higher for IH cases (in 81 %, 9/11) than for controls. (3) No polymorphism was detected in exon 1, 2 or 6 of this gene. (4) Polymorphism in exon 5 it was noted exclusively in one control. (5) On application of prediction tools, two polymorphisms in exon 3 (P359S and P299A) emerge out to be possibly damaging and functional status of 3′ UTR variant is not known (Table 3).

The details about the variants identified in our study is given in Tables 2 and 3. In short, four missense variants (c.1075 G>A (P359S), c.895C>G (P299A), c.1985A>G (N662S) and c.2149G>A (G717S);two types of synonymous variants (c.789G>A,c.1131T>G) and one SNP in 3′ UTR (c.2960C>T) were detected in our study. The variants unreported earlier were c.895C>G (P299A) in exon 3 (in one IH only), c.2149G>A (G717S) in exon 5 (in one case only) and c.1131T>G in exon 3 (in one IH only). Even polymorphism c.2960C>T resultant to single nucleotide change in 3′ UTR of MAMLD1 mRNA, though bears rsrs2070779 was not reported in context with this gene for IH subjects and deems to be treated as novel.

We did not find any difference (statistically) in the distribution of individual MAMLD1 variants among

Table 2 Statistical analysis of MAMLD1 gene polymorphism in cases and controls

S. no.	Variant	Amino acid change	Exon	Cases	Controls	Fisher, p value	Test of association Odds ratio (95 % CI)	Z value
1	c.1075 G>A	(P359S)	3	4	3	1.00	1.35 (0.29–6.18)	0.38
2	c.789C>G	–	3	3	0	0.25	7.21 (0.365–141.53)	1.30
3	c.895 C>G	(P299A)	3	1	0	1.00	3.03 (0.12–75.28)	0.67
4	c.1131T>G	–	3	1	0	1.00	3.03 (0.12–75.28)	0.67
5	c.1985 A>G	(N662S)	4	2	1	1.00	1.97 (0.175–22.21)	0.55
6	c.2149G>A	(G717S)	5	0	1	1.00	0.29 (0.011–7.31)	0.747
7	c.2960C>T	–	7	22	10	0.046	2.46 (1.07–5.63)	2.13
Total				33	15	0.01*	2.20 (1.18–4.09)	2.55

Table 3 Details of MAMLD1 gene variants identified in study

S. no	Exon	Variation	Genomic position	Variation type	Type of polymorphism	Nucleotide change/Amino acid change	rs ID	Nucleotide Positions #	Impact of polymorphism on protein function (*By prediction tool)
1	3	G–A	g 109232	Known	Missense	c.1075 (P359S*)	41313406	chrX:149638920	Possibly Damaging
2	3	G–A	g108946	Known	synonymous	c789	150886306	chrX:14938634	Neutral
3	3	C–G	g109052	New	Missense	c.895 (P299A*)	No	chrX:149638740	Possibly Damaging
4	3	T–G	g108826	New	synonymous	c.1131	No	chrX:149638976	Neutral
5	4	A–G	g112331	Known	Missense	c.1985 (N662S*)	2073043	chrX:149642019	Neutral
6	5	G–A	g141964	New	Missense	c.2149 (G717S*)	No	chrX:149671652	Neutral
7	7	C>T	g150866	New	3′ UTR	c.2960	2070779	chrX:149680554	NA

* Amino Acids numbers are according the transcript ID NM_005491.4

* Prediction of affected protein function using four algorithms by prediction softwares (mutation tester, Polyphen, Sift and Provean)

Nucleotide positions are as per the Human genome assembly GRCh37/hg19 available on https://genome.ucsc.edu

isolated hypospadias cases and controls, except for c.2960C>T, identified as rs2070779, which is resultant to a single nucleotide change in 3′ UTR of MAMLD1 mRNA. This was identified in twenty two hypospadias boys and in ten controls and this difference was statistically significant ($p = 0.046$) (Tables 4, 5). The distance between miRNA binding sites and SNP (rs2070779) has been calculated using miRdSNP [32]. The proximal miRNA target sites on 3′ UTRs nearest to this position is hsa-miR-448 which is 1116 nucleotide away from the c.2960 mutation. Overall, the presence of SNP change in exon 7 increased the risk of hypospadias by about 2.5-folds [OR 2.46 (1.07–5.63)] whereas, any polymorphism in this gene increased the risk of this anomaly by two folds [OR 2.20 (1.18–4.09)] (Table 2).

Primary attempts towards secondary and tertiary structure prediction of P299A polymorphism reveal that the region pertaining to the mutation lies mainly in the disordered region of the protein and these regions cannot be meaningfully predicted. However, the PHYRE software generated a protein model with a low confidence level [28]. At the site of mutation, the homology model suggests that there is change in secondary structure of the protein (Fig. 1). Proline, unlike alanine, is an imino acid which not only imparts rigidity to the protein backbone, but is also known to be an α-helix breaker. On the other hand, the amino acid alanine, is comparatively more hydrophobic and flexible. The substitution of the rigid proline with the flexible alanine results in local structural rearrangements and induces the extension of the α-helix formation. Hence, in the wild type protein, the presence of proline tends to induce a specific backbone conformation which is probably required at this position for its optimal functioning. The substitution of proline with alanine affects the local secondary structure of the mastermind domain and thus might influence the overall global structure of protein leading to an alteration or disruption in the manner it interacts with neighbouring protein residues. The Netsurf software [30] predicted that there is change in the relative surface accessibility indexes from 0.31 for proline to 0.43 for alanine and therefore the presence of mutation changes the accessibility status of the amino acid signifying that the tertiary structure of the protein might be affected due to this replacement. The protein stability checked through the I-mutant 2.0 server indicated that the Pro299Ala mutation also reduces the stability of the protein.

LH and testosterone level was not significantly different when composite IH and control group was compared (Table 5). We found significantly low LH levels in isolated hypospadiacs, only in group III (Table 6). The testosterone level was not significantly different between IH and controls on age wise group. It was interesting to note that testosterone level in 80 % of subjects with c.2960C>T polymorphism was lower when compared with mean testosterone level of respective age groups (Table 4).

Discussion

Mutations in MAMLD1 gene has been identified in hypospadias cases in several studies [13, 18, 33]. In our study, MAMLD1 gene variants were detected in thirty three cases (33 %) and fifteen in controls (15 %) The incidence of MAMLD1 gene polymorphism in IH subjects in our study from India was higher than that reported earlier by Kalfa et al. [18] who reported polymorphism of

Table 4 Distribution of MAMLD1 polymorphism among study subjects and controls along with their identification numbers and hormone levels (IH isolated hypospadias, IC = controls)

Nucleotide change	Amino acid change	Exon	Case/control	Hypospadias type	LH level compared with mean of age gp)	Testo level (compared with mean of age gp)
c.1075 G>A	P359S	Exon 3	Case (IH4)	Distal	↓	↓
c.1075 G>A	P359S	Exon 3	Case (IH33)	Proximal	↓	↓
c.1075 G>A	P359S	Exon 3	Case (IH46)	Distal	↓	↑
c.1075 G>A	P359S	Exon 3	Case (IH93)	Mid-penile	↓	↓
c.1075 G>A	P359S	Exon 3	Control (IC37)	–	↓	↓
c.1075 G>A	P359S	Exon 3	Control (IC54)	–	↓	↑
c.1075 G>A	P359S	Exon 3	Control (IC60)	–	Normal	↓
c.789G>A	–	Exon 3	Case (IH14)	Distal	↓	↓
c.789G>A	–	Exon 3	Case (IH52)	Distal	↓	↓
c.789G>A	–	Exon 3	Case (IH59)	Mid-penile	↓	↓
c.895C>G	P299A	Exon 3	Case (IH41)	Mid-penile	↓	↓
c.1131T>G	–	Exon 3	Case (IH89)	Mid-penile	↑	↓
c.1985A>G	N662S	Exon 4	*Case (IH21)	Distal	↑	↑
c.1985A>G	N662S	Exon 4	Case (IH86)	Distal	Normal	↓
c.1985A>G	N662S	Exon 4	Control (IC25)	–	↓	↓
c.2149G>A	G717S	Exon 5	Control (IC74)	–	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH2)	Distal	↑	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH5)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH8)	Distal	↑	↑
c.2960C>T	–	Nt change in 3′ UTR	Case (IH13)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH15)	Distal	↑	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH16)	Distal	↑	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH 19)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH20)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	*Case (IH21)	Distal	↑	↑
c.2960C>T	–	Nt change in 3′ UTR	Case (IH23)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH25)	proximal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH26)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH28)	Mid-penile	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH39)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH61)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH64)	Proximal	↓	↑
c.2960C>T	–	Nt change in 3′ UTR	Case (IH68)	Proximal	↓	↑
c.2960C>T	–	Nt change in 3′ UTR	Case (IH69)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH72)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH74)	Distal	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH77)	Distal	Normal	↓
c.2960C>T	–	Nt change in 3′ UTR	Case (IH97)	Mid-penile	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Control (IC5)	–	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Control (IC10)	–	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Control (IC14)	–	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Control (IC18)	–	Normal	↓
c.2960C>T	–	Nt change in 3′ UTR	*Control (IC25)	–	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Control (IC34)	–	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	*Control (IC60)	–	Normal	↓
c.2960C>T	–	Nt change in 3′ UTR	Control (IC63)	–	↓	↑

Table 4 continued

Nucleotide change	Amino acid change	Exon	Case/control	Hypospadias type	LH level compared with mean of age gp)	Testo level (compared with mean of age gp)
c.2960C>T	–	Nt change in 3′ UTR	Control (IC64)	–	↓	↓
c.2960C>T	–	Nt change in 3′ UTR	Control (IC87)	–	↑	↑

Table 5 LH and Testosterone levels in isolated hypospadias cases and controls

Parameter	Cases $N = 100$		Controls $N = 100$		p value
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	
LH mIU/ml	1.07 ± 2.25	0.17 (0.01–13.88)	1.41 ± 3.37	0.70 (0.1–8.65)	NS
Testosterone (ng/ml)	0.28 ± 0.68	0.025 (0.02–3.94)	0.46 ± 0.99	0.032 (0.01–4.36)	NS

LH mIU/ml luteinizing hormone, *NS* nonsignificant
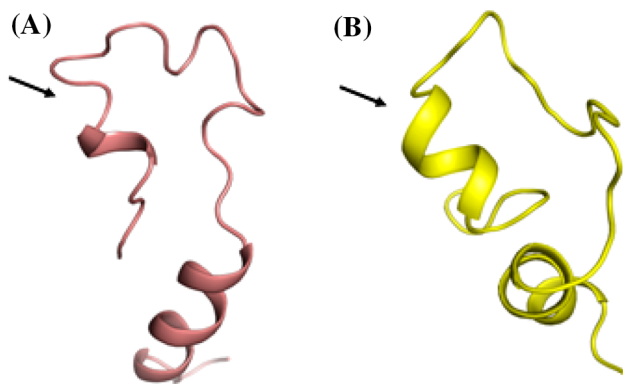
Fig. 1 The predicted change in the secondary structure surrounding the mutation P299A is marked by the *arrow* (**a**) *Cartoon* representation of native protein and (**b**) mutant protein

this gene among 9.7 % in their population hailing from Japan. In study by Chen et al. [19], comparable (29.29 %) incidence of polymorphism of this gene were found among hypospadiacs in Chinese population. Therefore, we found that a fairly good incidence of this gene polymorphism among Indian children too and the incidence among hypospadiacs was found to be about twice of that found in controls ($p = 0.01$).

One-third of the variants of MAMLD1 gene were found in coding exons (Exon 3, 4, 5 and 6) while majority i,e. two-thirds were found in non-coding exons (Exon 1, 2 and 7) and this difference was statistically significant. The polymorphisms in 3′ UTR (non-coding region) of exon 7 has not been reported in context with hypospadias and interestingly, its incidence was significantly higher among the cases as compared to controls ($p = 0.046$).

The effect of 3′ UTR polymorphism as was found in our study on MAMLD1 gene function needs to be investigated. Three prime untranslated region (3′ UTR) is the region of messenger RNA (mRNA) that immediately follows the

translation termination codon and is not translated into protein besides few other regions as 5′cap, 5′UTR and the poly(A) tail. The 3′ UTR often contains regulatory regions that post transcriptionally influence gene expression apart from influencing polyadenylation, translation efficiency, localization and stability of m RNA [34, 35]. This is because it binding sites for regulatory proteins as well as for mi RNA. The latter are short non coding RNA molecules that can decrease gene expressions of various mRNA by either inhibiting translation or causing degradation of transcript. Another way 3′ UTR acts is by possessing 'silencer region' (which binds to repressor protein and inhibit expression of m RNA) and AU rich elements (by affect mRNA stability/decay, rate of transcription or affecting translation initiation). Not only the sequences in 3′ UTR, but also its length affects gene expression with longer 3′ UTR corresponds to lower expression rates. Mutated UTR can cause disease due to translation deregulation. In fact, 3′ UTR mutations can be very consequential as one alteration can affect function of many genes (including unrelated genes) as UTR binding proteins also function in processing and nuclear export of RNA [36]. Few of 3′ UTR regulatory elements could also function at DNA level as distal enhancers to modulate transcription and in fact dysregulation of ARE binding protein due to mutation in AU rich region of 3′ UTR has been implicated in genesis of a number of diseases including tumorigenesis, hemotopoietic malignancies, myotonic dystrophy, acute myeloid leukemia, neuroblastoma, congenital heart disease etc. [36, 37]. In a study by Meritt et al. [38] on C. elegans germline 3′ UTR was found to be the main source of regulation throughout germ cell development, proving thereby that this region is a rich source of regulatory diversity that can bypass need for most promoter specificity. The importance of 3′ UTR has also been highlighted in a study by Beleza-Meireles [39] aiming to find expression of Activating

Table 6 LH and testosterone level in age groups of isolated hypospadias cases

Age group	Parameter	Cases N = 100		Controls N = 100		p value
		Mean ± SD	Median (range)	Mean ± SD	Median (range)	
Age group I (Newborn to ≤2 months)	LH mIU/ml	6.60 ± 3.30	6.18 (1.82–13.88)	6.52 ± 5.93	4.65 (0.29–0.35)	NS
	Testosterone (ng/ml)	1.53 ± 1.03	1.25 (0.53–3.94)	2.18 ± 1.40	1.52 (0.63–4.36)	NS
Age group II (>2 months to ≤4 years)	LH mIU/ml	0.57 ± 1.27	0.1 (0.01–6.64)	0.54 ± 1.50	0.1 (0.05–9.12)	NS
	Testosterone (ng/ml)	0.07 ± 0.2	0.02 (0.02–1.13)	0.09 ± 0.32	0.02 (0.02–2.04)	NS
Age group III (>4 years to ≤8 years)	LH mIU/ml	0.21 ± 0.26	0.11 (0.01–1.29)	0.28 ± 0.76	0.09 (0.045–3.25)	0.013
	Testosterone (ng/ml)	0.15 ± 0.56	0.025 (0.02–2.9)	0.26 ± 0.66	0.02 (0.02–2.4)	NS
Age group IV (>8 years to ≤12 years)	LH mIU/ml	0.90 ± 1.30	0.28 (0.1–4.78)	0.39 ± 0.44	0.20 (0.057–2.19)	NS
	Testosterone (ng/ml)	0.52 ± 0.91	0.15 (0.02–3.03)	0.10 ± 0.09	0.07 (0.01–0.38)	NS

Transcription Factor3, an estrogen responsive gene, which is considered to have a role in sexual development and is said to be a candidate gene for hypospadias. These authors found gene variants at 3′ UTR in three patients and postulated that though hormonal responsiveness may be the main underlying mechanism, the other aspects as epithelial mesenchymal interactions may also be important. In our study, a decrease in androgen levels in 80 % subjects possessing variants of MAMLD1 gene in 3′ UTR points to the role of this region in hormonal production but manifestation of this anomaly only among study subjects lead us to deduce that there must be involvement of multiple factors in causing this anomaly in vulnerable subjects.

Of all instances of polymorphism found in coding region (75 %, n = 12/15) were found in exon 3 proving this region to be labile region for gene polymorphism, especially in association with conditions of sexual development. This observation matches with observations by earlier workers [13, 18, 19]. But we did not get any nonsense mutation in this region probably because we studied only IH where we expect far fewer hormonal aberrations than what is expected in DSD conditions. The described nonsense mutation or frame shifts have been reported by earlier workers were found usually in severe variants of hypospadias and in those with 46 XY, DSD [13, 18].

We observed three missense variants of MAMLD1 in IH children as well as in controls. P299A was observed in only in one IH case and has not been reported previously. In vitro studies have shown that MAMLD1 proteins containing missense polymorphisms have the potential to activate Hes3 promoter [20]. Kalfa et al. [20] observed that P359S (previously known as p.P286S), p.N662S (previously known as N589S) variants have persistent trans activating potential for Hes3 promoter as compared with wild type MAMLD1 . On using prediction tools, the former emerged out to be possible damaging whereas latter was neutral. It is possible that these missense polymorphisms had compromised the testosterone production during critical time of sex differentiation in these subjects so as to

cause hypospadias. Functional studies with site directed mutagenesis are required to confirm the exact role of missense polymorphism in transactivational function of MAMLD1 protein. c.1985A>G (N662S) polymorphism was detected in both group (two case and one control) suggesting its nonpathological nature. This particular polymorphism was also previously reported by Kalfa et al. and 2011, Chen et al. both in subjects and control populations [19–21]. The c.2149G>A (G717S) was only identified in control population suggesting its non-pathological nature. The effect of newly identified P299A polymorphism was also found to be damaging using prediction tools and may be due structural instability of the resultant protein and affects the surface accessibility of the mutant protein as compared to its wild counterpart.

Previously reported c.1075 G>A (P359S) (previously known as P286S) polymorphism in our study subjects (four with isolated hypospadias and three controls) The P359S has been identified by Fukami et al. in Swedish hypospadias patients but was also observed in control population; while Chen et al. have found this polymorphism in 42 (out of 360) cases with Swedish origin and 28 (out of 379) in control population in their study [19]. Further invitro studies by Fukami et al. have observed that p.P286S variant of MAMLD1 protein is functional and p.P286S variant of MAMLD1 protein showed similar type of nuclear staining patterns as the wild type protein [33]. The distribution of this polymorphism seems related to ethnicity and may benon-pathological (OR 1.35).

In our study we found that risk of IH increases with polymorphism of MAMLD1 gene by more than two folds (OR 2.20). When calculated for coding region of MAMLD1, the OR was 2.34, for exon 3 it was 3.19 and for noncoding region it was 2.46. This shows that the polymorphisms in any of these regions of MAMLD1 gene have a comparable role for genesis of hypospadias and is a new finding requiring more research. It also invites into the need to relook the earlier speculation that exon 3 of MAMLD1 is the 'hotspot' for mutational changes in

patients with DSD and hypospadias as in our study importance of 3′ UTR region of non coding exon 7 also emerges but its functional mechanism awaits deduction through further studies. It is postulated that SNP in this region may affect affect MAMLD1 mRNA stability leads to changes in its gene expression profile. However, with evolution of knowledge about MAMLD1 gene few new facts have surfaced and few more are awaited. During our study duration itself, few more exons of this gene (making total number till 11) have been discovered though awaiting validation. If that data is considered then the mutations described by us in 3′ UTR region of exon 7 would correspond to exon 11. But the same may prove to be the case with other genes too when with evolving knowledge the hitherto described 3′ UTR considered region may correspond to some newly discovered coding exons and gives fresh insight into the functioning of these genes. However, our scientific writing is based on validated data at the time-point of study duration.

LH and testosterone levels did not significantly differ between IH cases and controls. However, we found significantly lower LH levels in IH in age-group III (>4–8yrs). While few workers have also reported normal gonado-trophins levels in hypospadias patients. Shima et. al. 1986, found low basal LH level, in not only in hypospadiacs with DSD but also among IH [40, 41]. Similarly workers differ on their observations of testosterone levels among hypospadiacs in comparison to controls though we did not find significant difference in testosterone levels between IH and controls, even in age-wise group comparison. However testosterone levels were also found to be low in 80 % subjects with exon 7 polymorphisms (in non coding region) making field for further research on its pathogenesis. These findings support the speculation fact that low androgenic levels/action at critical time of fetal development resultant to this polymorphism might have lead to development in hypospadias in the affected individuals. However, it cannot explain the absence of hypospadias among controls with this polymorphism who also had this polymorphism. It may therefore be conclude that formation of genitalia is a complex process involving multiple gene interactions, hormonal influence and role of receptors and of course epigenetic modulators.

In conclusion, besides providing a base line data about the occurrence of MAMLD1 gene polymorphisms and their association with LH and testosterone levels in Indian IH children, this study has highlighted the importance of polymorphism in 3′ UTR of exon 7 of MAMLD1 gene in IH children which has never been reported earlier. Apart from previously reported MAMLD1 variants, some novel MAMLD1 polymorphism have also been identified but their impact on the development of hypospadias needs to be confirmed by further gene functional studies. Overall,

this study concluded that occurrence of MAMDL1 gene polymorphisms is likely to increase the risk of isolated hypospadias in Indian children.

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