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MAMLD1 GENE MUTATION IN THE INCIDENCE OF HYPOSPADIAS IN THE CHINESE POPULATION

GENSKA MUTACIJA *MAMLD1* GENA I UČESTALOST HIPOSPADIJE U KINESKOJ POPULACIJI

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Summary

Background: This study aimed to investigate the significance of *MAMLD1* mutations in the incidence of hypospadias in a Chinese population.
Methods: The experimental group consisted of 150 domestic children with hypospadias, aged 0.5 to six years and living in different provinces. A total of 120 normal children, aged two to six years, served as the control group. DNA was extracted for the direct sequencing of *MAMLD1* genes.
Results: Twelve cases (8.0%) of the missense mutation p.N589S were found in the experimental group, whereas four cases (3.0%) of the same mutation were found in the control group. No significant difference was observed in the mutation rate between the two groups ($P>0.05$). Four cases (2.7%) had a new missense mutation p.P567S in the experimental group, and three cases (2.5%) possessed the same mutation in the control group. No significant difference was observed between the two groups ($P>0.05$).
Conclusions: In this study, the importance of repeated experiments in mutation-related studies was confirmed, which revealed the difference in predisposing genes among different populations. Although the mutation of the *MAMLD1* gene had no apparent connection with the inci-

Kratak sadržaj

Uvod: Cilj ove studije bio je istražiti u kojoj su meri mutacije *MAMLD1* gena značajne za učestalost hipospadije u kineskoj populaciji.
Metode: Eksperimentalnu grupu činilo je 150 kineske dece sa hipospadijom, uzrasta od šest meseci do šest godina starosti, koja žive u različitim provincijama. Kontrolna grupa obuhvatila je 120 zdrave dece, uzrasta od dve do šest godina. DNK je ekstrahovana radi direktnog sekvenciranja gena *MAMLD1*.
Rezultati: U eksperimentalnoj grupi otkriveno je dvanaest slučajeva (8,0%) mutacije sa pogrešnim kodirajućim značenjem (eng. *missense*) p.N589S, dok su u kontrolnoj grupi pronađena četiri slučaja (3,0%) iste mutacije. Nije uočena značajna razlika u stopi učestalosti mutacije između ove dve grupe ($P>0,05$). Četiri subjekta u eksperimentalnoj grupi (2,7%) nosila su novu mutaciju s pogrešnim kodirajućim značenjem p.P567S, dok su u kontrolnoj grupi istu mutaciju imala tri subjekta (2,5%). Između dve grupe nije uočena značajna razlika ($P>0,05$).
Zaključak: U ovoj studiji potvrđena je važnost ponovljenih eksperimenata u studijama o mutacijama, pomoću kojih je

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dence of hypospadias in a Chinese population, a new mutation site of the *MAMLD1* gene was discovered, which could provide new research topics for future studies.

Keywords: Chinese population, genetics, hypospadias, *MAMLD1* gene, polymorphism

otkrivena razlika u predisponirajućim genima između različitih populacija. Iako nije postojala očigledna veza između mutacije gena *MAMLD1* i učestalosti hipospadije u kineskoj populaciji, otkriveno je novo mesto mutacije na genu *MAMLD1*, što može biti tema za buduće istraživačke studije.

Ključne reči: kineska populacija, genetika, hipospadija, gen *MAMLD1*, polimorfizam

Introduction

Hypospadias is one of the most common congenital malformations among children, and is considered as a mild form of 46,XY disorder of sex differentiation. In 2008, hypospadias affected approximately 0.4% (290/611,730) of living births in China (1). Gene mutations and environmental factors are involved in the occurrence of hypospadias, but the linkages and functions between these two elements remain unknown (2). As of this writing, the exact cause of hypospadias was determined in only 20% of the cases, which were mainly severe hypospadias (3).

The mastermind-like domain-containing 1 gene (*MAMLD1*, formerly CXorf6) is a novel candidate gene for hypospadias. It was discovered while identifying the responsible gene (*MTM1*) for myotubular myopathy (4–7), which maps to proximal Xq28. Individuals with microdeletions of *MTM1* extending to the CXorf6 locus exhibit myopathy and external genital malformations.

A study recently reported that the *MAMLD1* gene mutation was related to the occurrence of hypospadias in a Caucasian population (8, 9). To verify its mutation significance in a Chinese population, direct sequencing of polymerase chain reaction (PCR) products was performed in 150 sporadic cases of hypospadias for exploring the diagnostic values of *MAMLD1* gene mutation and its relationship with the incidence of hypospadias.

Materials and Methods

Subjects

From July 2010 to June 2011, 150 cases of hypospadias, aged 0.5 to six years, underwent surgical treatment. These patients were labelled as the experimental group. Hypospadias is classified based on the anatomical location of the proximally displaced urethral orifice, and the specific sub-types are shown in *Table I*. Simultaneously, 120 healthy patients, aged two to six years, underwent circumcision and were confirmed with normal urethral opening. They were labelled as the control group. Peripheral venous blood was collected from both groups for DNA extraction and sequencing.

Data on the experimental and control groups were obtained from Shanghai Children's Medical Centre, Children's Hospital of Xi'an and Xiamen Maternity and Child Care Hospital. This study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Shanghai Children's Medical Centre affiliated with Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from all participants.

All patients were tested with quantitative fluorescent PCR (10). The results confirm that all the 150 hypospadias cases were of single X chromosome homotopic locus.

DNA extraction

DNA was collected from peripheral venous blood, which was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA concentrations were measured using a UV photometer (Eppendorf, Hilden, Germany).

Mutational analysis

Based on the 25 μL reaction system, the following substances were added to the centrifuge tube: 2.5 μL of 10 × Buffer, 4 μL of dNTP, 0.25 μL of Taq enzyme, 1 μL each of forward and reverse primers and 1 μL of cDNA. Water was then added to reach a total volume of 25 μL. PCR amplification was performed on a 96-well GeneAmp 2700 PCR instrument (Perkin Elmer Co., Ltd., San Francisco, CA, USA). The PCR conditions were as follows: 95 °C for 3 min; 40

Table I Clinical data of 150 patients with hypospadias.

Clinical type of hypospadias	Coronary sulcus type	Scapus penis type	Penoscrotal junction type	Scrotum type	Perineum type
Cases number (n)	21	84	20	14	11
Ratio (%)	14.0%	56.0%	13.3%	9.3%	7.3%

cycles of unlinking at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s; and a final extension at 72 °C for 7 min. The products were preserved at 4 °C. The primers are indicated in *Table II*.

The RT–PCR product (3 μL) underwent 1% agarose gel electrophoresis (95 eV to 100 eV for 20 min). An ImageQuant 100 imaging system was utilised for imaging, and the results were observed under UV. Gel scanning analysis was used for density scanning, and the results were analysed to calculate the OD values of specifically amplified bands. The relative OD values of *MAMLD1* were calculated using a 2000 DNA Marker, and an agarose gel DNA extraction kit (Qiagen, Hilden, Germany) was used for purification.

Table II Primers used for amplification of exons 1–6.

Exons	Forward primer (5′-3′)	Reverse primer (3′-5′)
Exon 1	ctcacaaaaatgcccctcat	tgggaggctatatggaacctt
Exon 2	tgctagtctcctgcactcca	tggctaacaagcccaaaatc
Exon 3 part 1	tcatgcacatttcctgttca	ctcttggactgtgccattga
Exon 3 part 2	ttagatcatccccaggcaac	aggcagagcagacatcaggt
Exon 3 part 3	ccctcagagctccattcttg	tagcctggatttaccctcca
Exon 4	cttgacaccagggagttggt	atgatttgcatgaccccaat
Exon 5	ccaaagcagttggtgggtat	tgggtggacagagctttacc
Exon 6	gtggaattttgctcgagagg	ggggacccctgctatctatc

An ABI PRISM 3700 DNA automatic sequencer (Perkin Elmer Co., Ltd., San Francisco, CA, USA) was used for sequencing, and the dideoxy end-point termination method was adopted for the sequence analysis of PCR products. The sequencing results were analysed using Phred/Phrap software to identify the mutation sites. The discovered mutation sites were verified for reliability and repeatability using repeated processes.

Statistical analysis

SPSS12.0 software was used for data analysis. Ratios were compared using chi-square test. A value of $P>0.05$ was considered statistically significant.

Results

Two missense mutations, namely, c.1766A> G and c.1699C> T were identified among the 150 patients (*Table III*). c.1766A> G changed the 589th amino acid from aspartate to serine (p.N589S). This mutation was already reported by Chen (8) and Fukami et al. (11). Twelve cases of c.1766A> G were found in the experimental group, whereas four cases

Table III Genotype and clinical phenotype of the patients.

Case No	Nucleotide change	Amino acid change	Exon	Hypospadias type
10	c.1766A>G	p.N589S	4	scapus penis type
27	c.1766A>G	p.N589S	4	coronary sulcus type
40	c.1766A>G	p.N589S	4	scapus penis type
63	c.1766A>G	p.N589S	4	scapus penis type
75	c.1766A>G	p.N589S	4	scrotum type
88	c.1766A>G	p.N589S	4	scapus penis type
104	c.1766A>G	p.N589S	4	scapus penis type
117	c.1766A>G	p.N589S	4	scapus penis type
124	c.1766A>G	p.N589S	4	scapus penis type
127	c.1766A>G	p.N589S	4	penoscrotal junction type
135	c.1766A>G	p.N589S	4	penoscrotal junction type
140	c.1766A>G	p.N589S	4	coronary sulcus type
6	c.1699C>T	p.P567S	3	scapus penis type
81	c.1699C>T	p.P567S	3	scrotum type
126	c.1699C>T	p.P567S	3	scapus penis type
131	c.1699C>T	p.P567S	3	perineum type

Table IV Statistical analysis of mutation rates of p.N589S and p.P567S.

Polymorphisms	Allele		Experimental group		Control group		P
	Nucleotide	Amino acid	Cases	Ratio	Cases	Ratio	
p.N589S	A	N	138	0.92	116	0.97	0.06>0.05
	G	S	12	0.08	4	0.03	
p.P567S	C	P	146	0.98	117	0.99	0.3>0.05
	T	S	4	0.02	3	0.01	

Table V Statistical analysis of genotyping data of polymorphisms p.N589S and p.P567S in proximal type and control group.

Polymorphisms	Proximal type cases	Control group cases	P
p.N589S	42	116	0.20>0.05
	3	4	
p.P567S	43	117	0.32>0.05
	2	3	

Table VI Statistical analysis of genotyping data of polymorphisms p.N589S and p.P567S in distal type and control group.

Polymorphisms	Distal type cases	Control group cases	P
p.N589S	96	116	0.06>0.05
	9	4	
p.P567S	103	117	0.33>0.05
	2	3	

Table VII Statistical analysis of genotyping data of polymorphisms p.P567S in the experimental group and the 1000 Genomes Project data.

Polymorphisms	Experimental group cases	1000 Genomes Project cases	P
p.P567S	146	283	0.14>0.05
	4	3	

were found in the control group. c.1699C> T was a newly discovered missense mutation with no relevant reports. Four and three cases of c.1699C> T were found in the experimental and control groups, respectively. This mutation changed the 567th amino acid from proline to serine (p.P567S).

Taqman genotyping was used to perform a controlled study on the single nucleotide polymorphism (SNP) of p.N589S and p.P567S. The results show no statistical significance ($P>0.05$) (*Table IV*).

In the experimental group, hypospadias was further categorised into two sub-groups, namely, distal and proximal hypospadias. Coronary sulcus type, scapus penis type and radix penis type were defined as distal hypospadias, whereas penoscrotal junction type, scrotum type and perineum type were defined as proximal hypospadias. The mutation rates of the two sub-groups were compared with those of the control group, and the results did not show any statistically significant differences (*Tables V* and *VI*).

According to the detection report on Asian populations from the 1000 Genomes browser (browser.1000genomes.org), three cases of SNP mutations were noted among 286 normal people. These results show that the differences between the *MAMLD1* mutation in the experimental and control groups were not statistically significant (*Table VII*).

Discussion

MAMLD1 gene mutations have been reported in previous studies. Fukami et al. (11) reported three non-synonymous mutations (E124X, Q197X and R653X) in the four non-family cases of hypospadias associated with small penis and scrotum division. Kalfa et al. (9) found a missense mutation p.531ins3Q and a deletion mutation 325delG in proximal hypospadias, and reported polymorphisms of p.P286S, p.N589S and p.P286S + p.N589S in subsequent experiments. Thus, the mutations were considered to be associated with the occurrence of hypospadias, and the protein encoded by this mutation did not inhibit the transcriptional activity of Hes3 gene. Chen et al. (8) have recently reported a non-synonymous mutation Q529K, a synonymous mutation p.D686D and a haplotype p.P286S + p.N589S. However, the current research results show that the aforementioned single polymorphic locus, which was considered as the gene responsible for hypospadias, was insufficient in causing the occurrence of hypospadias. Functional studies on E124X and Q197X showed that they could reduce Hes3 transcriptional activation, whereas p.P286S, p.N589S and the haplotype p.P286S + p.N589S wild-type *MAMLD1* gene all had high expression of transcriptional activation of the Hes promoter. Although *in*

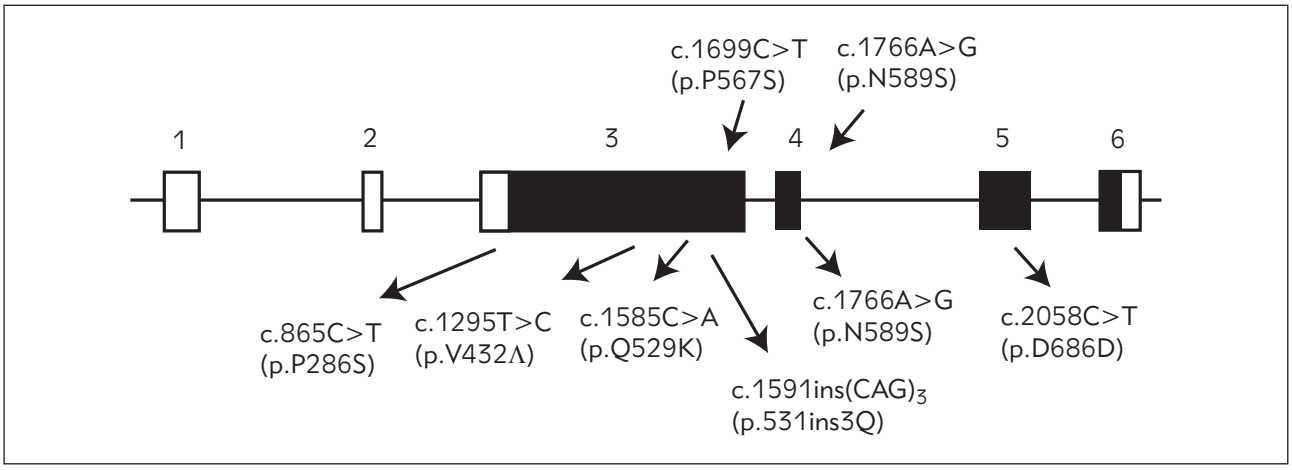

Figure 1 Locations of the genetic variants found in the MAMLD1 gene.
The boxes represented the exons, and the exon numbers were indicated in turn; the black boxes represented the protein-coding regions, and grey boxes denoted the untranslated regions.

vitro experiments showed that *MAMLD1* has transcription functions, whether the mutations may also affect the *in vivo* synthesis of testosterone cannot be proven (12–15). Thus, these reported mutations lacked reproducible results and multicentre validation, and were based on single-centre studies. The samples were more concentrated in the Caucasian population because a large sample study in a Chinese population was unavailable. This experiment aimed to study *MAMLD1* gene mutations in a Chinese population, and determine its diagnostic values in hypospadias.

The majority of the associations reported in genetic studies cannot be replicated across subsequent studies (16, 17). This study had the largest sample population for the investigation of hypospadias cases and controls for associated SNPs in a Caucasian population. We were unable to replicate the results of three earlier studies on Caucasian populations and two earlier studies on non-Caucasian populations. Previous findings could not be generalised to a Chinese population in our study. Large Chinese sample sizes for *MAMLD1* in Chinese populations are currently absent (18), and the frequency of *MAMLD1* mutations in a larger sample from a Chinese population should be investigated.

The results reveal two mutations, which were located in the *MAMLD1* coding region. Mutation p.P567S, a newly discovered mutation, was located in the third exon (*Figure 1*). Three cases in the control group and four cases in the experimental group (i.e., two cases of proximal scapus penis type, one case of penoscrotal junction type and one case of perineum type) were isolated. Statistical analysis of the SNPs between the experimental and control groups showed no statistical significance in the mutation rate ($P>0.05$). Compared with the SNP locus of an Asian population in the 1000 Genomes Project (3/286, 1%), the mutation p.P567S was considered benign and acceptable. Therefore, the SNP locus did not affect the occurrence of hypospadias.

SNP locus is located in the fourth exon of p.N589S. Chen et al. (8) showed a weak link between p.N589S and hypospadias. Kalfa showed a higher incidence of p.N589S in a large sample experiment among Caucasians, but this rate was not statistically significant (9). In the present study, statistical analyses on SNP showed that the p.N589S mutation rate was not statistically different from that of hypospadias among a Chinese population ($P>0.05$). When the mutation rates of the distal and proximal sub-groups were compared with those of the control group, no significant differences were also found ($P>0.05$). These results differed from those of other studies (10) possibly because of the following reasons:

1) Sampling population: The samples in this study were Chinese people, whereas Chen et al. (8) focused on a North Caucasus Swedish population. Population differences possibly led to different results.
2) Individual gene susceptibility: Hypospadias is a polygenic disease. The specific susceptibility genes possibly did not have key functions in the different genetic background populations. The experimental results still need further confirmation from a multicentre trial.

Conclusion

In this study, the importance of repeated mutant correlation experiments was proven. The results may determine the differences in susceptible genes among different populations. The experimental results did not reveal obvious associations between the incidence of hypospadias in a Chinese population and the *MAMLD1* gene mutation. p.P567S was discov-

ered as a new gene mutation of *MAMLD1* in hypospadias. Our study can provide information on the generalisability of the findings and contribute to the determination of realistic estimates of effect sizes, which confirms the importance of replication studies for validating the results of genetic association approaches. From an epigenetic perspective, such as DNA methylation and histone modification, this study will contribute to a more comprehensive understanding of the occurrence of this multi-genetic disease.

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Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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