A novel mutation of HOXA10 in a Chinese woman with a Müllerian duct anomaly

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Submitted on April 7, 2011; resubmitted on August 2, 2011; accepted on August 8, 2011

BACKGROUND: Müllerian duct anomalies consist of a set of congenital structural malformations that occur when the Müllerian ducts do not develop properly during embryonic life. Their molecular genetic basis is poorly understood.

METHODS: In this study, we conducted mutation analysis of the HOXA10 gene in a cohort of 109 Chinese women with Müllerian duct anomalies.

RESULTS: We identified a novel mutation (Y57C) in one patient with a didelphic uterus. The mutation affected the transcriptional regulation capacity of HOXA10.

CONCLUSIONS: Our study showed that mutation of HOXA10 gene may contribute to the development of Müllerian duct anomalies and confirmed that HOXA10 is an important transcription factor in reproductive tract development.

Key words: HOXA10 / mutation / Müllerian duct anomalies

Introduction

Müllerian duct anomalies comprise a mixed group of congenital anomalies that result from failure of organogenesis, fusion or septal resorption of the Müllerian ducts during the development of the female reproductive tract (Chandler et al., 2009). Although these anomalies are relatively uncommon, they can affect reproductive outcomes significantly (Rackow and Arici, 2007). The etiology of Müllerian duct anomalies is likely to be multifactorial, as with most congenital developmental disorders; moreover, the molecular genetic basis is poorly understood.

HOX genes encode highly conserved transcription factors that play important roles in the establishment of the anterior–posterior axis of metazoan body plan (Akam, 1989; McGinnis and Krumlauf, 1992). Each of these genes has a similar 183 bp sequence that encodes a 61-amino acid homeodomain. Homeodomain proteins bind to and activate or repress the expression of downstream target genes (Dessain et al., 1992). There are at least 39 HOX genes in the mammalian genome arranged in four clusters termed HOXA, B, C and D (Krumlauf, 1994). Their temporal and spatial expression patterns during embryonic development precisely parallel their arrangement on the chromosome (McGinnis and Krumlauf, 1992; Krumlauf, 1993).

Genes from the HOXA cluster, HOXA9-13, are involved in the development of the female reproductive tract (DU and Taylor, 2004). They are expressed in a linear fashion along the axis of the developing Müllerian duct (Taylor et al., 1997). Hoxa9 is expressed in the mouse oviduct, Hoxa10 in the uterus, Hoxa11 in the uterus and cervix and Hoxa13 in the cervix and upper vagina. In particular, the Hoxa10 protein is essential for the normal development of the uterus. Hoxa10-deficient female mice exhibit uterine factor infertility and the anterior part of the uterus is transformed into the oviduct (Satokata et al., 1995; Benson et al., 1996). Moreover, altered Hoxa10 expression caused by exposure to diethylstilbestrol leads to uterine malformation (Block et al., 2000).

Therefore, the human HOXA10 gene is a good candidate for involvement in Müllerian duct anomalies. The present study was designed to analyze the complete coding region of HOXA10 in a cohort of Chinese women with Müllerian duct anomalies.
Materials and Methods

Subjects

The study population comprised 109 patients who were diagnosed with Mullerian duct anomalies based on karyotyping, physical examination, ultrasonographic investigations, hysteroscopy and laparoscopy, and who volunteered to participate in the study. A group of 100 unrelated healthy women was used as controls. Informed written consent was obtained from each subject and the study was approved by the local ethics committee.

Mutational analysis and bioinformatics

Genomic DNA was isolated from peripheral blood leukocytes using standard methods. The complete coding regions for HOXA10 along with exon-intron boundaries were amplified by PCR using two pairs of gene-specific primers (Supplementary data, Table S1). PCR products were sequenced using designed PCR primers and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3730XL DNA sequencer (Applied Biosystems).

To confirm the mutation found in sequencing, PCR amplifications were repeated three times and the PCR products were sequenced in both directions. The novelty of mutation was verified by searching in the dbSNP and 1000 Genome Project database (http://browser.1000genomes.org/). Conservation analysis was performed using the ClustalW2 alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). PolyPhen (http://coot.embl.de/PolyPhen/) was used to predict the functional effect of any variant sequence.

Site-directed mutagenesis and plasmid construction

Human HOXA10 open reading frame (ORF) cDNA was obtained from GeneCopoeia (Rockville, MD, USA). Site-directed mutagenesis was performed to generate HOXA10 bearing the p.Tyr57Cys mutation using the QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). DNA sequencing confirmed the introduced mutation.

The ORFs of wild-type and mutant HOXA10 sequences were amplified by PCR from cDNAs and inserted into the HindIII- and EcoRI-digested pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) to create the p.Tyr57Cys mutant) or pcDNA3.1 empty vector were cotransfected together with pGL3-ITGB3 or pGL3-EMX2 plasmids, respectively. In each well, a Renilla luciferase plasmid pREP7-Rluc was cotransfected as an internal control. At 30 h after transfection, cells were washed, lysed and assayed for firefly and Renilla luciferase expression using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized to Renilla luciferase activity. The results represent the means of three independent experiments performed in triplicate and the bars in figures denote the SD. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance of differences among the means of three groups.

Transactivation assays

A total of 293T cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100 mg/ml penicillin and 100 mg/ml streptomycin. Cells were seeded in 24-well tissue culture plates 24 h prior to transfection at about 60% confluence. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Equivalent amounts of HOXA10 expression constructs (wild type and mutant) or pcDNA3.1 empty vector were cotransfected together with pGL3-ITGB3 or pGL3-EMX2 plasmids, respectively. In each well, a Renilla luciferase plasmid pREP7-Rluc was cotransfected as an internal control. At 30 h after transfection, cells were washed, lysed and assayed for firefly and Renilla luciferase expression using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activities were normalized to Renilla luciferase activity. The results represent the means of three independent experiments performed in triplicate and the bars in figures denote the SD. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance of differences among the means of three groups.

Results

Case report

Chromosomal analysis of all the patients showed a normal female 46,XX karyotype. All 109 patients were classified according to the vagina cervix uterus adnexa-associated malformation classification (Oppelt et al., 2005; Wottgen et al., 2008) (Table I).

One HOXA10 mutation was found in a woman with a didelphic uterus. This 26.5-year-old woman had been referred for secondary infertility. Thelarche and adrenarche had both occurred at 13 years of age. At physical examination, she had a normal weight (50 kg),

<table>
<thead>
<tr>
<th>Vagina Number</th>
<th>Uterus Number</th>
<th>Adnexa Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 75</td>
<td>Normal 1</td>
<td>Normal 106</td>
</tr>
<tr>
<td>Incomplete septate vagina &lt;50% 3</td>
<td>Arcuate 7</td>
<td>Gonadal streak 1</td>
</tr>
<tr>
<td>Complete septate vagina 7</td>
<td>Septate &lt;50% of the uterine cavity 35</td>
<td>Other 2</td>
</tr>
<tr>
<td>Hypoplasia 11</td>
<td>Septate &gt;50% of the uterine cavity 25</td>
<td>Associated malformation 94</td>
</tr>
<tr>
<td>Complete atresia 13</td>
<td>Bicornate 7</td>
<td>Normal</td>
</tr>
<tr>
<td>Cervix Number</td>
<td>Hypoplastic uterus 24</td>
<td>Renal system 3</td>
</tr>
<tr>
<td>Normal 66</td>
<td>Unilaterally rudimentary or aplastic 10</td>
<td>Skeleton 2</td>
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<tr>
<td>Duplex cervix 18</td>
<td></td>
<td>Cardiac 1</td>
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<tr>
<td>Other 25</td>
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<td>Other 9</td>
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height (155 cm), BMI (20.8 kg/m²) and blood pressure (100/66 mmHg). Breast and pubic hair development had progressed normally (Tanner stage 5). The patient’s clitoral size and vaginal introitus were normal. A vaginal septum was not present. Her laboratory examination showed normal levels of total testosterone (1.42 nmol/l), FSH (5.65 IU/l), LH (7.48 IU/l), estradiol (339.81 pmol/l), progesterone (4.28 nmol/l) and mammotrophic hormone (8.42 ng/ml). Pelvic and abdominal ultrasound examinations and laparoscopy revealed a didelphic uterus; both ovaries and kidneys were of normal size and in their normal positions.

**Genetic and bioinformatics analysis**

Sequence analysis of the HOXA10 gene in this patient’s DNA revealed a novel heterozygous A–G mutation in position 170 of the ORF, resulting in a Y57C substitution in the protein sequence. The mutation was found in her father, who showed a normal phenotype, but not in her mother or in 100 unrelated, unaffected controls (Fig. 1). This substitution was not previously reported in dbSNP or in the 1000 Genome Project database. The mutation resulted in a substitution located at conserved regions among many species (human, mouse, chicken and zebrafish, shown in Fig. 1). PolyPhen analysis predicted it to be possibly damaging.

**Functional analysis**

Transactivation assays were conducted to evaluate whether the mutation affected the ability of HOXA10 protein to regulate transcription. Two luciferase reporter constructs containing HOXA10 responsive elements were tested. The results are shown in Fig. 2. Wild-type HOXA10 effectively enhanced the activities of the pGL3-ITGB3 reporter, as described previously (Daftary et al., 2002). However, the mutant showed significantly diminished stimulation, to about 46% of the wild-type protein (P < 0.05). The wild-type form was also an effective repressor of pGL3-EMX2 reporter transcriptional activation. Again, the mutant displayed a remarkable loss of activity, revealing only about 67% of repressive capacity compared with the wild-type protein (P < 0.05). The results indicated that the mutation affected the capacity of HOXA10 to activate and repress transcription.

**Discussion**

The role of the HOX family of genes in embryogenesis of the female reproductive tract is well established (DU and Taylor, 2004). In this study, we carried out mutation analysis of HOXA10 in 109 unrelated Chinese individuals with Müllerian duct anomalies. We found a novel mutation of HOXA10 in a patient with a didelphic uterus. This is the first identified HOXA10 gene mutation in an individual with a reproductive tract abnormality.

The Y57C mutation is located at a highly conserved position among several species (human, mouse, chicken and zebrafish) and was not identified in 100 healthy control subjects. Moreover, it has not been reported to be a polymorphism in dbSNP or the 1000 Genome Project database. Furthermore, PolyPhen predicted it to be possibly damaging. Therefore, we speculate that it is a disease-causing mutation.

ITGB3, the gene encoding β3-integrin, and EMX2 are downstream targets for HOXA10 (Daftary et al., 2002; Troy et al., 2003). HOXA10 upregulates ITGB3 gene expression through directly binding to a 41-bp regulatory element. The expression of EMX2 is repressed by HOXA10 through a 150-bp regulatory element. Luciferase reporter assays were conducted to determine the functional consequence of this mutation. We found that wild-type HOXA10 could activate ITGB3 transcription and down-regulate EMX2 expression in

![Figure 1](image-url) Heterozygous mutation in the patient resulting in a Y57C substitution. (A) DNA sequence chromatograms obtained by direct sequencing of PCR products showing the heterozygous A–G substitution in HOXA10 gene in the patient and the patient’s father, but not in a representative control subject or in the patient’s mother. (B) Sequence alignment of HOXA10 protein among many species, showing the conserved Y57 mutated in the patient.
293T cells. However, the mutant HOXA10 displayed significantly reduced transcriptional activation and repression compared with the wild-type. Thus, the mutation affected the transcription-regulating functions of HOXA10.

EMX2 encodes a homeobox-containing transcription factor and is orthologous to the Drosophila empty spiracles gene (Dalton et al., 1989; Grossniklaus et al., 1994). It is expressed in the epithelial components of the developing urogenital system and Emx2 is thought to be indispensable for the formation of both Müllerian and Wolffian ducts in mice. Emx2-deficient mice lack reproductive tracts, gonads and kidneys (Miyamoto et al., 1997). HOXA10 negatively regulates EMX2 expression in the human reproductive tract. The Y57C mutation affects the function of HOXA10 in regulation and may alter the regulatory network involved in EMX2 during the normal development of reproductive tract and lead to Müllerian duct anomalies.

The phenotype of this heterozygous patient with a HOXA10 mutation seems to be different from that of homozygous Hoxa10 knockout mice. A didelphic uterus results from the complete non-fusion of both Müllerian ducts during embryonic development (Chandler et al., 2009). This phenotype might suggest the involvement of HOXA10 in the fusion of the Müllerian duct in humans. Alternatively, the mutant HOXA10 could have dominant negative properties and interfere with the normal function of other HOX transcription factors in female reproductive tract development.

Mutations in several HOX genes have been shown to cause human developmental defects (Muragaki et al., 1996; Thompson and Nguyen, 2000; Tischfield et al., 2005). Different types of mutations in the HOXA13 have been identified in patients with hand-foot-genital syndrome (HFGS), a condition characterized by distal limb malformations and urogenital defects (Goodman and Scambler, 2001). The female patients with HFGS display varying degrees of incomplete Müllerian fusion. Our results and previous studies suggest that mutations in the HOXA10 and HOXA13 may be culprits for Müllerian fusion defects.

In conclusion, we carried out mutation analysis of HOXA10 among patients with Müllerian duct anomalies and identified a novel disease-causing mutation. This confirms that HOXA10 is an important transcription factor in reproductive tract development and provides new insights into the molecular mechanisms of Müllerian duct anomalies.

### Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

### Authors’ roles

The authors’ roles are as follows: Z.C. and Y.Z. drafted the manuscript and carried out the molecular genetic studies. D.S. participated in functional analysis. J.W., L.C., B.C., Z.W. and P.Z. participated in manuscript revision and samples collection. B.W., X.M. and Y.C. conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

### Acknowledgements

The authors thank the patients and the family members for their cooperation during the study. B.W. (wbbahu@163.com), X.M. (nicgr@263.net) and Y.C. (caoyunxia6@126.com) contributed equally to the research project and should be considered as co-corresponding authors.

### Funding

This work was supported by the National Basic Research Program of China (2010CB529505), the National Basic Research Program of China.
China (2007CB947403), the National Natural Science Foundation of China (30973197), the National Science & Technology Pillar Program of China (No.2008BAH22B05), the National Infrastructure Program of Chinese Genetic Resources (2006DKA21300).

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