A novel mutation of HOXA11 in a patient with septate uterus

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Abstract

Background: The etiology of Müllerian duct anomalies (MDAs) is poorly understood at present. The HOXA11 gene is crucial for the development of the Müllerian duct. The objective of this study is to report a unique case of MDAs with a novel mutation in HOXA11.

Results: We identified a potential disease-causing mutation (p. E255K) in a patient with a septate uterus. The mutation was not detected in 169 control subjects or listed in any databases of variations. Bioinformatic predictions and functional studies showed that the mutation reduces the DNA binding affinity and disrupts transactivation ability of HOXA11.

Conclusion: In conclusion, this is the first report to describe a HOXA11 mutation in Chinese women with MDAs. The results demonstrated that mutation in HOXA11 can contribute to the etiology of MDAs, especially the septate uterus, but might not be a common cause.

Keywords: Mutation, HOXA11, Septate uterus patients

Background

During the embryonic development in female eutherian mammals, two paired Müllerian ducts ultimately develop into the oviducts, uterus, cervix and upper portion of the vagina, which constitute most of the female reproductive tract [1]. Interruptions or disturbances at different stages of this process can result in Müllerian duct anomalies (MDAs). The anomalies are estimated to affect about 5.5% of the general population and are often associated with other organ anomalies, such as renal agenesis and skeletal anomalies [2–4]. MDAs can also impair the sexual and reproductive function of women to different extents [5].

The etiology of MDAs remains largely unknown at present, and seems to be multifactorial. Previous studies showed that environmental and iatrogenic factors, such as exposure to ionizing radiation, viral infections and the use of medications such as methotrexate or diethylstilbestrol (DES), might underlie the occurrence of such malformations [6]. Furthermore, familial aggregation of MDAs was observed among first-degree relatives, which strongly implicates linked genetic factors [7]. Studies in animal models have suggested candidate genes potentially involved in human MDAs. However, most of these candidate genes were failed to be proved pathogenic [8].

HOX genes encode evolutionarily conserved transcription factors and exist in almost all metazoans [9]. Humans have 39 HOX genes arranged in four separate clusters, termed HOXA, B, C and D [10]. Each of the HOX genes contains a homeobox sequence encoding a homeodomain, a highly conserved DNA-binding motif. HOX genes act as key regulatory factors in embryonic morphogenesis and cell differentiation [11, 12]. Mutations in HOX genes have been shown to cause synpolydactyly and hand–foot–genital syndrome [13, 14].

The HOXA11 gene located in the HOXA gene cluster is crucial for the development of the Müllerian duct. It is expressed in the mouse Müllerian duct during embryonic development and subsequently expressed in the lower uterus and uterine cervix in neonates [15]. In Hoxa11 null mice, the uterus is smaller than normal and shows some anatomical similarities with the more anterior oviducts [16, 17]. Furthermore, alterations in the expression of Hoxa11 caused by DES exposure resulted in
uterine anomalies [18]. Thus, HOXA11 may contribute to the etiology of MDAs. Here we performed genetic analyses of HOXA11 in Chinese patients with MDAs.

Methods
Subjects
A cohort of 163 Chinese women with MDAs was recruited in our study. Their clinical diagnoses were based on physical examination, ultrasonographic investigations, hysteroscopy and laparoscopy. A group of 169 unrelated healthy women were also screened as controls. Informed written consent was obtained from all individuals. The study protocol was in accordance with the tenets of the Declaration of Helsinki and was approved by the Anhui Medical University ethics committee.

Genetic analysis
Genomic DNA was extracted from peripheral blood samples using standard methods. The two exons and exon–intron boundaries of the HOXA11 gene were amplified by polymerase chain reaction (PCR) using two pairs of gene specific primers (Additional file 1: Table S1). The PCR products were sequenced on an ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems).

To validate the novel mutation discovered in the study, PCR amplifications were repeated three times and the products were sequenced in both directions. The novelty of mutation was verified by consulting the National Center for Biotechnology Information single nucleotide polymorphism database (dbSNP) and 1000 Genome Project database. Conservation analysis was performed by using CLC Main Workbench Software. PMut (http://mmb.pcb.ub.es/PMut/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) were used to predict the effects of mutation on the protein function.

Plasmid construction
The human HOXA11 open reading frame (ORF) was PCR-amplified by using HOXA11 cDNA as template. The amplification generated Bam HI and Xba I sites into the 5′ and 3′ ends of the HOXA11 ORF. The PCR product was inserted into pMD18-T simple vector (Takara, USA) following the manufacturer’s instructions. The membrane was then blocked, exposed to streptavidin–horseradish peroxidase, washed four times, and equilibrated. The bands were visualized by chemiluminescence reaction, and images were captured using the Cool II Imager System (Viagene Biotech, Ningbo, P. R. China).

Nuclear extracts of the transfected cells were prepared using a nuclear protein extraction kit (Viagene Biotech, Ningbo, P. R. China). Protein concentration was determined by the BCA method. Decreasing amounts of nuclear protein from each sample were mixed with 1.5 μl 10 × Binding Buffer and 1.5 μl Poly(dI-dC) in a 15 μl reaction volume and were incubated for 20 min at room temperature. Then 0.5 μl of a biotin-labeled OPN5 oligonucleotide probe (5′–TAGTTAATGACATCGTT-CATCAG–3′) containing the Hox binding site was added to the samples [19]. Following a further incubation for 20 min at room temperature, the samples were separated by 5.5% nondenaturing polyacrylamide gel electrophoresis at 180 V for 70 min. Then the products were transferred to a binding membrane in 0.5 × TBE at 390 mA for 40 min. To crosslink DNA, the membrane was placed in a UV StrataLinker 1800 (Stratagene, La Jolla, CA, USA) following the manufacturer’s instructions. The membrane was then blocked, exposed to streptavidin–horseradish peroxidase, washed four times, and equilibrated. The bands were visualized by chemiluminescence reaction, and images were captured using the Cool II Imager System (Viagene Biotech, Ningbo, P. R. China).

Electrophoretic mobility shift assay (EMSA)
293FT cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. For transient transfection, cells were cultured in 10 cm dishes until they reached 70% confluency. 2 μg HOXA11 wild-type and mutant expression constructs were transfected separately into cells using the calcium phosphate method.

Transactivation assay
HeLa cells were cultured in DMEM containing 10% fetal bovine serum. For transient transfection, cells were grown to 70–80% confluency on 24-well plates and transfected using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions.

Aliquots of 150 ng of HOXA11 expression constructs and a pcDNA3.1 empty vector were transfected into the cells with 300 ng of PRL reporter plasmid and 150 ng of FOXO1 expression plasmid, respectively. The pRL-TK plasmid encodes Renilla luciferase (5 ng) was also added in each cotransfection to normalize the transfection efficiency. 48 h after transfection, cells were harvested and assayed for luciferase activity using the Dual Luciferase
 Reporter Assay System (Promega). The experiments were repeated three times in triplicate. Statistical significance was determined using independent-sample t tests.

**Results**

**Phenotypes of analyzed patients**

The clinical phenotypes of all 163 patients are shown in Table 1 according to the vagina/cervix/uterus/adnexa-associated malformation classification [20]. The patients showed a broad spectrum of MDAs. Some patients also had associated malformations of the kidneys, skeletons and hearts.

**Identification of a HOXA11 missense mutation and case report**

We analyzed the coding region of HOXA11 gene in 163 patients and 169 healthy controls. Our analysis identified a novel missense mutation- c.763G > A (p. E255K) in a patient with a complete septate uterus, and it was not detected in 169 healthy controls. This mutation was also observed in her mother, who showed a normal uterus (Fig. 1A). Her mother had a history of spontaneous abortion caused by the natural termination of the fetus growth.

The mutation carrier was 36 years old and had spontaneous abortion four times. She had been diagnosed with secondary infertility. Her menstrual cycle was normal. She had normal body mass index (19.9 kg/m²) and blood pressure (110/76 mmHg) according to a recent physical examination. Her laboratory tests showed normal levels of total testosterone (0.974 nmol/l), follicle-stimulating hormone (FSH) (9.8 U/l), luteinizing hormone (LH) (5.05 U/l), and estradiol (288.25 pmol/l). Abdominal and pelvic ultrasound examinations and hysteroscopy revealed a septum filling >50% of the uterus. The maximum thickness of her endometrium was only 6.5 mm. The patient did not show any anomalies in the limbs, kidneys, ureters, or central nervous system.

The p. E255K mutation is not reported in dbSNP or the 1000 Genome Project databases. It alters amino acid residues that lie in the homeodomain and is highly conserved among different species (Fig. 1B, C). Furthermore, all three software tools predicted the p. E255K mutation to be possibly damaging. The results of the predictions are shown in Additional file 1: Table S3.

**Functional analyses of HOXA11 mutation**

Because the p.E255K mutation is located in the homeodomain which is considered to relate to DNA binding efficiency we first carried out EMSA using nuclear extracts from 293FT cells transfected with wild-type and mutant expression constructs to investigate whether the mutation would affect the DNA binding properties of HOXA11 (Fig. 2A). For the wild-type protein, the formation of a protein–DNA complex was as efficient as previously reported. In contrast, the p. E255K mutant showed a reduced capacity to bind DNA, indicating that the mutation greatly reduces the normal DNA-binding affinity of HOXA11. Furthermore, we tested the influence of this mutation through a reporter gene transactivation using the PRL luciferase reporter. The results are shown in Fig. 2B. When cotransfected with a FOXO1 expression construct, the wild-type HOXA11 resulted in a 2.8-fold increase in relative luciferase activity compared with the pcDNA3.1 empty vector. Cotransfection of the p. E255K mutant also enhanced the luciferase activity, but it was decreased by 26% when compared with the wild-type (p < 0.001). These results indicate that the p. E255K mutation significantly disrupts the transactivation ability of HOXA11.

**Discussion**

The normal development of the Müllerian duct involves a complex series of events: elongation, fusion, canalization, and septal resorption. Failure to complete any part of this sequence can lead to MDAs. The manifestations of MDAs vary widely among patients, and uterine anomalies are the most common. Although some genetic factors that regulate the development of the

| Vagina                          | Number | Uterus                  | Number | Cervix                  | Number |
|---------------------------------|--------|-------------------------|--------|-------------------------|--------|
| Normal                          | 119    | Normal                  | 2      | Normal                  | 96     |
| Incomplete septate vagina <50%  | 7      | Arcuate                 | 10     | Duplex cervix           | 34     |
| Complete septate vagina         | 8      | Septate <50% of the uterine cavity | 52 | Unilateral aplasia | 16     |
| Hypoplasia                      | 14     | Septate >50% of the uterine cavity | 42 | Associated Malformation | Number |
| Complete atresia                | 15     | Bicornate               | 11     | None                    | 154    |
| Adnexa                          | Number | Hypoplastic uterus      | 30     | Renal system            | 4      |
| Normal                          | 151    | Unilaterally rudimentary or aplastic | 16 | Skeleton                | 2      |
| Bilateral gonadal streak        | 1      |                         |        | Cardiac                 | 1      |
| Other                           | 11     |                         |        | Other                   | 2      |
Fig. 1 A novel variation of HOX11 identified in a patient with septate uterus. a Electropherograms obtained by direct sequencing of PCR products showing the heterozygous G to A substitution at nucleotide 763 of HOXA11 gene in the patient and the patient’s mother, but not in a control subject or in the patient’s father. b The p.E255K mutation situated in the homeodomain of HOXA11. c Sequence alignment of HOXA11 protein among different species showing the mutation located in the highly conserved site of the protein.

Fig. 2 Functional analysis of HOXA11 sequence mutation. a Electrophoretic mobility shift assay of nuclear extracts from transfected 293FT cells. 293FT cells were transfected, respectively, with wild type and mutant HOXA11 expression constructs. Decreasing amounts of nuclear extracts were mixed with labeled DNA probes. Protein-DNA complex is indicated by an arrow and a non-specific band is marked with an arrowhead. b Transactivation assay in cotransfected HeLa cells. Wild type and mutant HOXA11 expression constructs were cotransfected with PRL reporter plasmid, FOXO1 expression plasmid and pRL-TK plasmid. Firefly and Renilla luciferase activities were measured 48 h after transfection. Relative luciferase activity was determined by normalizing the firefly luciferase activity with Renilla luciferase activity. The experiments were repeated three times in triplicate. Representative data shown are expressed as mean ± SD. Statistical significance was determined by the independent-samples T test. **, p < 0.01 versus pcDNA3.1 empty vector. ###, p < 0.01 versus wild-type.
Müllerian duct have been identified, the etiology of MDAs is poorly understood at present.

Here we present the evidence that mutation in HoXA11 can contribute to the etiology of MDAs, especially the septate uterus. Our study started with a genetic analysis of HoXA11 gene in Chinese women with MDAs and detected a novel non-synonymous mutation. The mutation was not observed in controls or listed in current variation databases.

The p.E255K mutation is located in the homeodomain of HoXA11, which is highly conserved among many different species. It results in a glutamic acid-to-lysine substitution. The glutamic acid is an acid residue with negative charge, while the lysine is a positively charged residue. This substitution might damage the structure of the protein and impair its DNA-binding ability. Bioinformatic predictions and functional analyses both showed that the mutation impairs the function of HOXA11. Therefore, the p. E255K mutation is a potential disease-causing mutation.

There are increasing evidences indicate that HOXA11 plays an essential role in the uterus development and human fertility. In the development of uterus, Hoxa11 promotes proper proliferation and survival of stromal cells. The Hoxa11 adult mutant uterus displayed little to no stromal tissue and exhibited a reduced diameter [17, 21]. During pregnancy, Hoxa11 was critical for endometrial receptivity and embryonic implantation [22]. Hoxa11 mutated female mice showed high embryo absorption rates because of implantation failure [17]. In another murine model, knockdown of Hoxa11 led to significantly reduced implantation rates [23]. Furthermore, Hoxa11 expression dramatically increased at the time of implantation and remained high in pregnancy [24]. And the reduced expression of Hoxa11 was reported in women with defective implantation [25]. The patient’s endometrium was as thin as only 6.5 mm. She also had spontaneous abortion four times. These phenotypes may owe to the defective uterine development and impaired endometrial receptivity caused by HOXA11 mutation.

It was noteworthy that the patient’s mother also carried the same mutation, but showed normal uterus. The main reason behind this may due to low penetrance. The similar phenomenon has also been reported in previous studies of synpolydactyly, in which some members of the families who carry the HOXD13 mutation showed normal phenotypes in the hands and feet [26, 27]. Furthermore, the patient’s mother also had a history of spontaneous abortion. It was quite possible that she also had defects in the endometrium as her daughter.

The phenotype of the p. E255K mutation carrier is a complete septate uterus, which results from the failed resorption of the medial septum after the fusion of Müllerian ducts [28]. And our previous research identified a novel mutation of HOXA10 in a patient with didelphic uterus [29]. Moreover, several nonsense mutations (p.W369X, p.S136X, p.Q196X, and p.Q365X), missense mutations within the homeodomain (p.I368F, p.N372H and p.V375F), and in-frame polyalanine expansions of HOXA13 gene have been found to cause Müllerian duct fusion defects in patients with hand-foot-genital syndrome [14, 30–32]. Taken together, these results suggest that HOX genes might play important roles in the fusion of the Müllerian duct and in the resorption of the uterine septum. Further investigations such as transgenic mice studies are required to elucidate the precise molecular mechanisms.

In the present study, mutation of HoXA11 was only detected in 0.6% (1/163) of the patients. A previous study that screened HOXA11 gene in 192 patients with MDAs identified no mutation [33]. These results demonstrated that mutation in HOXA11 might not be a common cause for MDAs. Other studies regarding Wnt9b, Pax2, Lhx1, Hoxa7 and Hoxa9 also got similar results. Mutation screening of these genes had been performed in Chinese patients with MDAs, but no mutation was detected [34–38]. These results suggested that non-genetic factors might contribute to the pathogenesis of MDAs.

Conclusion

In conclusion, this is the first report to describe a HOXA11 mutation in Chinese women with MDAs. The results demonstrated that mutation in HOXA11 can contribute to the etiology of MDAs, especially the septate uterus, but might not be a common cause.

Additional file

Additional file 1: Table S1. Primers used for PCR. Table S2 Primers used for site-directed mutagenesis and plasmids construction. Table S3 Functional significance of HOXA11 mutation by bioinformatic prediction. (DOC 96 kb)

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors’ contributions

ZY carried out the clinical sample collection and clinical diagnosis. CZ provided the manuscript preforming and carried out experiments preforming. WJ carried out the manuscript revision and the data analysis. LBH and CLF participated in the experiments preforming. CBL participated in...
the clinical sample collection. CXY and WBB conceived the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study protocol was in accordance with the tenets of the Declaration of Helsinki and was approved by the Anhui Medical University ethics committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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