Nonsense mutation of EMX2 is potential causative for uterus didelphysis: first molecular explanation for isolated incomplete müllerian fusion

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Objective: To investigate the association between human empty spiracles homeobox 2 gene (*EMX2*) and incomplete müllerian fusion (IMF).
Design: Case-control study.
Setting: University-based hospital.
Patient(s): Cohort of 517 clinically well-characterized IMF cases and 563 control women.
Intervention(s): None.
Main Outcome Measure(s): In cases and control women, direct sequencing of EMX2 exons and further functional studies; for functional studies, wild-type and mutant EMX2 expression plasmids constructed; human embryonic kidney cells (HEK293FT) transfected with empty vector, wild-type EMX2, mutant EMX2, and 1:1 combination (wild-type/mutant plasmids) with additional functional studies performed to clarify the deleterious effect of the novel mutation detected.
Result(s): A novel nonsense mutation p.E142X was detected in one woman with a didelphic uterus (1 of 517, 0.19%). The results of Western blot analysis confirmed that the mutation caused a truncated protein as predicted, and functional studies proved that it resulted in a dominant negative effect.
Conclusion(s): The novel nonsense mutation we detected-EMX2, p.E142X- resulted in a dominant negative effect. The functional data were exemplified in HEK293FT cells. This reinforced the likelihood that EMX2 contributed to the pathophysiology of IMF. Although it is uncommon (0.19%), EMX2 is the first gene identified that if perturbed may cause isolated IMF. (Fertil Steril® 2015;103:769–74. ©2015 by American Society for Reproductive Medicine.)
Key Words: Dominant negative effect, EMX2, incomplete müllerian fusion, müllerian duct anomalies, P63

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Müllerian duct anomalies (MDA) are structural malformations resulting from abnormal development of the müllerian ducts. The prevalence is estimated between 0.1% and 3% of live births (1). Anomalies of the müllerian ducts include not only hypoplasia/uterine agenesis (müllerian aplasia or Mayer-Rokitansky-Kuster-Hauser syndrome) but also various forms of incomplete müllerian fusion (IMF): unicornuate uterus, uterus didelphys, bicornuate uterus, septate uterus, and arcuate uterus (2). Women with MDA usually have a normal karyotype (46,XX). Familial aggregates of isolated MDA have been reported, but most cases are sporadic (3). Potential teratogenic exposures evoked include hypoxia during pregnancy, medications such as methotrexate, ionizing radiation, and viral infections (4, 5). However, teratogenic causation is not considered paramount. Incomplete müllerian fusion is presumed to be a polygenic/multifactorial disorder, given familial aggregates and recurrence in first-degree relatives of 2.7% (6).

Many genes have been considered essential for müllerian duct development on the basis of animal models. These include [1] genes essential for the formation of the reproductive ducts, such as *Emx2*, *Wnt* family, *Lhx1*, *Dach1*/*Dach2*, and *Pax2* (7–15); and [2] genes participating in the development of müllerian ducts in females, such as *Hox* genes (1, 16, 17). Relatively few of these genes have been evaluated for their contribution to human MDA, especially IMF. *EMX2* (MIM 600035) is a human homolog of the *Drosophila* empty spiracles gene, a homeodomain-containing transcription factor. In mice, the orthologue *Emx2* is thought to be indispensable for the formation of both müllerian and Wolffian ducts. Mice deficient in *Emx2* lack reproductive tracts, gonads, and kidneys (18). Therefore, *EMX2* is a good candidate gene for human MDA. Incomplete müllerian fusion is distinct from müllerian aplasias such as Mayer-Rokitansky-Kuster-Hauser syndrome and hand-foot-genital syndrome (19). In the present study, we sequenced the *EMX2* gene in a large cohort of Chinese women with IMF, finding one novel nonsense mutation of plausible causability. Functional analysis confirmed its deleterious effect on EMX2 protein and the expression of related genes. To our knowledge, it is the first study that has shown a role for *EMX2* in women with IMF, and the first study to find a causative gene in isolated IMF.

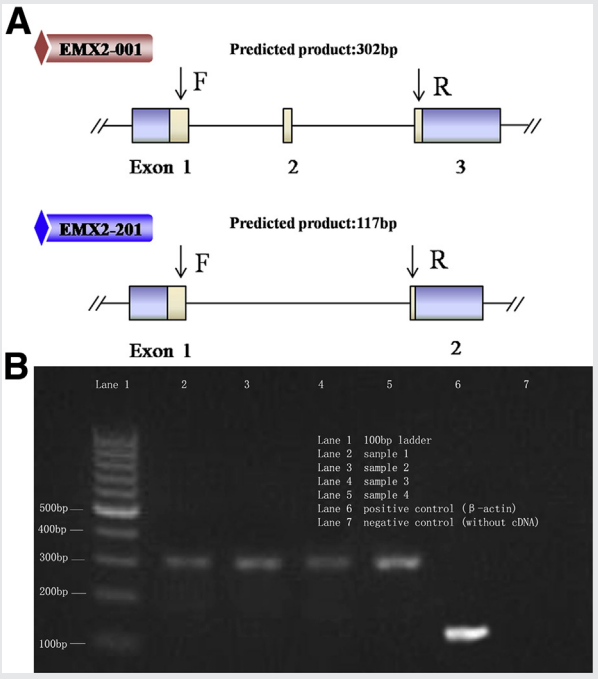
MATERIALS AND METHODS

Bioinformatics

The *EMX2* gene has two transcripts, according to the National Center for Biotechnology Information (NCBI) Gene database and Ensemble database (http://www.ensembl.org/). Compared with the longer transcript (GenBank NM_004098.3, EMX2-001 in Ensemble), the shorter one (GenBank NM_001165924.1, EMX2-201 in Ensemble) lacks an exon in the coding region, which results in a frameshift and a protein lacking the C-terminal homeodomain (Fig. 1A).

First, reverse-transcription polymerase chain reaction (RT-PCR) was performed to determine which transcript variant is expressed in the human uterus. Total RNA was extracted from uterine tissues with QiaShredder and the RNeasy kit (Qiagen) following the manufacturer's protocol. We generated cDNA using M-MLV Reverse Transcriptase (Promega) and random primers. We performed RT-PCR to amplify and identify the product (primers provided in Supplemental Table 1, available online).

FIGURE 1



Identification of the transcript variant expressed in human uterus. (**A**) Schematic diagram of the two transcripts of the *EMX2* gene. GenBank NM_004098.3 (EMX2-001) has three exons and encodes a protein with 252 amino acids. GenBank NM_001165924.1 (EMX2-201) lacks an exon in the coding region, encoding a protein with 169 amino acids. Specific primers were designed to distinguish the transcript variant expressed in the human uterus. *Arrows* indicate the positions of the forward primer (F) and reverse primer (R). The predicted PCR products are 302 bp and 117 bp, respectively. (**B**) Results of agarose gel electrophoresis show PCR products of 302 bp. Four cDNA samples were collected from myometrial tissue obtained from women undergoing myomectomy.

Liu. Mutation of EMX2 in isolated IMF patient. Fertil Steril 2015.

Patients

Direct sequencing of the *EMX2* exons was performed in 517 clinically well-characterized women who had IMF diagnosed at the Center for Reproductive Medicine, Provincial Hospital Affiliated to Shandong University. Diagnosis and classification of this anomaly was made using ultrasound, hysterosalpingography and hysteroscopy. All 517 had a normal female karyotype (46,XX). Of the 517 women, 106 patients had a unicornuate uterus, 92 had uterus didelphys, 144 had a bicornuate uterus, and 175 had a septate uterus. Recruited as controls were 563 women with no MDA as shown by ultrasonography or hysterosalpingogram. The control group came from infertile women who had requested infertility treatment because of fallopian tubal obstruction or male factor infertility. Informed consent for genetic studies was obtained