A missense mutation in TMEM67 causes Meckel-Gruber syndrome type 3 (MKS3): a family from China

Manli Zhang1*, Jing Cheng2*, Aijun Liu3, Longxia Wang4, Lihua Xiong1, Meixia Chen1, Yi Sun5, Jianzhong Li6, Yu Lu2, Huijun Yuan2, Yali Li1, Yanping Lu1

1Department of Obstetrics and Gynecology, Chinese PLA General Hospital, Beijing 100853, China; 2Institute of Otolaryngology, Chinese PLA General Hospital, Beijing 100853, China; 3Department of Pathology, Chinese PLA General Hospital, Beijing 100853, China; 4Department of Ultrasound, Chinese PLA General Hospital, Beijing 100853, China; 5Department of Otolaryngology, Wuhan General Hospital, Wuhan 430070, China; 6Fuzhou General Hospital of Nanjing Command PLA, Fuzhou 350025, China. *Equal contributors.

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Abstract: Meckel-Gruber syndrome (MKS) is a lethal autosomal recessive condition characterized by renal cysts and variably associated features, including developmental anomalies of the central nervous system (typically encephalocele), hepatic ductal dysplasia and cysts, and polydactyly. Genetic heterogeneity has been demonstrated at eleven loci, MKS1-11. Here, we present the clinical and molecular characteristics of a Chinese MKS3 family with occipital encephalocele and kidney enlargement. DNA sequencing of affected fetuses revealed a homozygous c.1645C>T substitution in exon 16 of TMEM67, leading to a p.R549C substitution in meckelin. The R549 residue is highly conserved across human, rat, mouse, zebrafish, chicken, wolf and platypus genomes. Hha I restriction analysis demonstrated that the c.1645C>T mutation was absent in 200 unrelated control chromosomes of Chinese background, supporting the hypothesis that it represents causative mutation, not rare polymorphism. Our data provide additional molecular and clinical information for establishing a better genotype-phenotype understanding of MKS.

Keywords: MKS3, TMEM67, meckelin, mutation

Introduction

Meckel-Gruber syndrome (MKS; MIM 249000) is a lethal autosomal recessive disorder characterized by various severe malformations. The minimal diagnostic criteria are cystic dysplasia of the kidneys, with fibrotic changes in the liver and occipital encephalocele or some other malformations of the central nervous system. Polydactyly is also frequently reported in MKS patients. Patients with classic MKS phenotype usually die in the perinatal period. MKS is genetically heterogenous [1, 2], with eleven causative genes: MKS1 (OMIM 249000), MKS1 (OMIM 609883), 17q23; MKS2 (OMIM 603194), TMEM216 (OMIM 613277), 11q13; MKS3 (OMIM 607361), TMEM67 (OMIM 609884) 8q21.13-q22.1; MKS4 (OMIM 611134), CEP290 (OMIM 610142) 12q21.3; MKS5 (OMIM 611561), RPGRIP1L (OMIM 610937) 16q12.2; MKS6 (OMIM 612284), CC2D2A (OMIM 612013) 4p15; and MKS7 (OMIM 267010), NPHP3 (OMIM 608002), 3q22; MKS8 (OMIM 613885), TCTN2 (OMIM 613846); MKS9 (OMIM 614209), B9D1 (OMIM 614144); MKS10 (OMIM 614175), B9D2 (OMIM 611951); and MKS11 (OMIM 615397), TMEM231 (OMIM 614949). All genes involved in MKS are associated with ciliary functions [4-7].

The TMEM67 gene encodes a component of the transmembrane protein meckelin, which has 995 amino acids and an extracellular N-terminus containing a signal peptide and a cysteine-rich domain [3]. It also has an intracellular C-terminus with a coiled-coil domain. MKS type 3 (MKS3) is characterized by occipital encephalocele and cystic dysplastic kidneys [4]. Compared with other MKS subtypes, MKS3 patients rarely present with polydactyly and are more likely to have milder central nervous system phenotypes with considerable variability [8-10].
To date, a total of 25 TMEM67 pathogenic mutations have been reported in TMEM67 spectrum disease (http://omim.org/entry/609884) and seven of them identified in MKS3 families. In China, patients with MKS phenotypes have been reported, but no mutation was identified. In this report, we present detailed clinical, genetic and pathological findings of four fetuses in one family who had clinical characteristics of MKS3 and exhibited pathogenic TMEM67 mutations.

Materials and methods

Ethics statement

Written informed consents were obtained from all family participants and normal controls prior to their participation in the study, and all research procedures were approved by the Research Ethics Committee of the Chinese PLA General Hospital. We also have the consent to use the tissues of the aborted normal fetus at 17 gestational week (gw) because the pregnant woman was diagnosed as primary pulmonary hypertension during her first trimester.

Patients

The Chinese family MKS-H01 was from Beijing, China. The proband was a 14 gw fetus (III-3) with occipital encephalocele and kidney enlargement detected by ultrasound. This was the third pregnancy of this couple. The other three pregnancies of this couple carried similar malformed fetuses. Amniotic fluid culture and chro-
**Table 1.** Clinical features observed in four MKS fetuses from the Chinese family

<table>
<thead>
<tr>
<th></th>
<th>Occipital encephalocele</th>
<th>Spina bifida</th>
<th>Renal cystic dysplasia</th>
<th>Ductal plate malformations</th>
<th>Polydactyly</th>
<th>Parietal absence</th>
<th>Cleft palate</th>
<th>Fetal hydrops</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Unknown</td>
</tr>
<tr>
<td>III-2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Unknown</td>
<td>-</td>
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<td>-</td>
<td>Unknown</td>
</tr>
<tr>
<td>III-3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>III-4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

+Present, -absent.
mosome analysis for the first and second fetuses were performed in another hospital and both were normal. Therapeutic termination of pregnancy was performed for the four affected fetuses after ultrasound diagnosis of MKS during the second trimester of pregnancy, followed by autopsies of the first, the third and the fourth affected fetus.

**Isolation of genomic DNA and RNA**

Peripheral venous blood samples (3 ml) were drawn from family participants and 200 healthy controls for genomic DNA extraction using the Genomic DNA isolation kit (Qiagen).

After abortion, fetal tissues of brain, liver and kidney from III-3 and III-4 were stored in liquid nitrogen. DNA was isolated using Gentra Puregene Mouse Tail kit (Qiagen).

The formalin-fixed, paraffin-embedded tissues of brain, liver and spleen from the III-1 fetus were applied to isolate genomic DNA using the QIAamp DNA FFPE Tissue Kit (Qiagen), following the protocol supplied by the manufacturer.

**cDNA preparation**

Total RNA was isolated from aborted proband’s fetal kidney using RNeasy Mini Kit (Qiagen) and cDNA was obtained by RT-PCR amplification using SuperScript™ III Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions.

**DNA sequencing**

After gel purification, each of the amplified PCR fragments was directly sequenced in both for-

**Endonuclease digestion**

In the fast assay of the c.1645C>T mutation in fetal tissues and in 200 normal controls, *Hha I* restriction analysis of PCR fragment covering the mutation site was applied. We designed the primers (Forward: 5’gtttttgaacaccgatgacaga 3’, reverse: 5’agaaggatccagaatggtcaaa 3’) to amplify a 217bp PCR product. *Hha I* digestion distinguishes the homozygote (217 bp) from the wild-type (130, 87 bp) and heterozygote (217, 130, 87 bp) on agarose gel electrophoresis.

**Immunohistochemistry**

Immunohistochemistry was conducted with the fetal tissues (liver and kidney) obtained from III-4, as well as a 17 gw fetus as a normal control. Standard procedures were used for staining tissue slides with the antibodies: primary antibodies (Rabbit polyclonal to meckelin ab76786 (Abcam) at 100 dilutions in PBS, matched to appropriate pre-immune negative control sera). The secondary antibody (general two-stage me-

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**Figure 3.** Meckelin expression detected by immunostaining. A. In the liver of the MKS3 fetus (III-4), meckelin was expressed in epithelial cells of enlarged intrahepatic bile ducts around the portal area in the MKS3 fetus. B. In the control, the same location was seen but without bile ducts dilated. Hepatocytes showed slight staining in both affected fetuses and in the control. C. In the kidney of the MKS fetus (III-4), showed intense staining was seen in cysts epithelium but not in the remnant glomerulus. D. In the control, meckelin was expressed in renal tubules but not in the glomeruli. Original magnification, ×400.
Results

Clinical features

The proband fetus (III-3) was found to have occipital encephalocele and kidney enlargement by ultrasound (Figure 1) at 14 gw and was aborted one week later. The III-4 fetus was diagnosed at 13 gw by ultrasound and aborted at 14 gw. The couple had two similarly malformed fetuses (III-1, III-2) before. Four fetuses had similar clinical characteristics, including occipital encephalocele and renal cystic dysplasia, as well as some diverse phenotypes. Table 1 shows the detailed clinical features of the four affected fetuses of this Chinese MKS family. In fetus III-4, we detected parietal absence and polydactyly (Figure 1), which did not show in the first three fetuses. The absence of polydactyly is typical phenotype of MKS3.

Mutation identification

Based on the clinical features of this family, we firstly chose the MKS3 candidate gene, TMEM67, for mutation screening. We performed direct sequencing of full-length cDNA extracted from fetal brain and renal tissues of proband of Chinese family MKS-H01 (III-3). A homozygous C to T transition at position 1645 in exon 16 was identified, resulting in p.R549C substitution in meckelin (Figure 2). Sequencing analysis of the formalin-fixed, paraffin-embedded tissue from III-1 and the fresh tissue from III-4 confirmed this finding (we did not have tissue from III-2). The Arg residue at 549 in meckelin is conserved across human, rat, mouse, zebrafish, chicken, wolf and platypus. Both of the parents were found to be for the carriers of this substitution. Hha I restriction analysis demonstrated that the c.1645C>T mutation in TMEM67 was cosegregated with the MKS phenotype in this family and the parents were heterozygous carriers. Note that the fourth affected fetus, which carried the same TMEM67 mutation, had additional polydactyly which showed phenotypic heterogeneity in the Chinese MKS3 family.

Immunohistochemistry of meckelin

We further visualized the localization of meckelin in paraffin-embedded fetal tissues (liver and kidney from III-4 and the normal control from a 17 gw fetus. In the normal control, moderate to high levels of meckelin were localized at the renal tubule epithelia, but not in the glomeruli. In the MKS3 fetus, intense staining was shown in the cysts epithelium of the kidney but not in the remnant glomeruli. In the liver, meckelin was expressed in the epithelial cell layer of enlarged intrahepatic bile ducts around the portal area in MKS3 fetus. In the control, the same locations were seen, but without bile ducts dilated (Figure 3).

Discussion

In the present study, we report the identification of a TMEM67 mutation in a Chinese MKS3 family with four affected individuals. All affected fetuses displayed renal cystic dysplasia and occipital encephalocele. Ductal plate malformations with proliferation of bile duct were showed in the last three affected fetuses. Comparison of the clinical features of MKS3-linked cases with MKS1-linked cases suggested that polydactyly and possibly encephalocele are less common in MKS3-linked families [9, 10]. The absence of polydactyly in the first three affected fetuses suggested that TMEM67 was the good candidate gene for mutation screening in this family. Sequence analysis and Hha I restriction analysis demonstrated that a homozygous c.1645C>T mutation in TMEM67 was cosegregated with the MKS phenotype in this family and the parents were heterozygous carriers. Note that the fourth affected fetus, which carried the same TMEM67 mutation, had additional polydactyly which showed phenotypic heterogeneity in the Chinese MKS3 family.

TMEM67 spectrum diseases range from embryonically lethal Meckel syndrome, to less severe multisystem disorders, such as Bardet-Biedl syndrome (MIM 209900), COACH syndrome (MIM 216360), Joubert syndrome 6 (MIM 610688) and nephronophthisis 11 (MIM 613550). A systematic analysis of the phenotypic burden of TMEM67 mutations has been reported [9]. A differential distribution of mutations along the TMEM67 gene in lethal (MKS) versus non-lethal (JSRD, NPH and ARPKD-like) phenotypes is observed, particularly with regard to missense mutations. In MKS patients, most missense mutations cluster in exons 8 to 15, encoding the extracellular region of meckelin that follows the cysteine-rich domain [11]. We report a lethal missense mutation in exon...
16 of the TMEM67 gene in Chinese family, which encodes the extracellular region of meckelin. This is the first report of TMEM67 mutation in Chinese population. The same substitution was reported by Katharina Hopp as a highly likely mutation [7]. It is possible that, within the range of exons 8 to 16, all encoding extracellular fragments are located just before the corresponding transmembrane fragments. Except for lethal MKS, primary cilia-related disease, including Joubert syndrome, Bardet-Biedl syndrome and COACH syndrome, were accompanied with some structural abnormality at the fetal stage, such as ‘molar tooth sign’ (MTS)- cerebellar vermis hypo-dysplasia, thickening and horizontalization of superior cerebellar peduncles and deepening of the interpelucular fossa on MRI, Dandy-Walker malformation and polydactyly [12, 13]. Because many genes are involved in these disorders, traditional methods of mutation screening are difficult to identify the causative genes. With the development of a new generation of sequencing strategies, we can test all ciliopathy-related genes simultaneously following ultrasound identification of features associated with ciliopathy by prenatal diagnosis [14].

We also visualized the localization of meckelin in the MKS fetus and normal controls. As previously reported there was strong staining in the epithelial cell layer of the renal tubule but not in the glomerulus [6]. Our results showed intense staining of renal cysts in the MKS3 fetus. Dawe et al. reported that meckelin immunostaining was absent in the renal tissues of the MKS3 fetus who carried a homozygous c.1127A>C (p.Q376P) mutation in TMEM67 [6]. In our study, meckelin staining was present in both kidney tissues of affected fetus with homozygous c.1645C>T (p.R549C) mutation in TMEM67 and normal control.

In summary, we have identified a homozygous TMEM67 mutation in a Chinese family exhibiting clinical characteristics of MKS3. Statistically, this couple would be predicted to have a 25% chance of producing an affected embryo. However, four previous natural pregnancies of this couple all turned to be affected MKS3 fetuses detected by ultrasound, and the couple repeatedly opted to terminate the pregnancies by artificial abortions. The identification of the causative mutation of TMEM67 in this family provided a ground for PGD procedure for this family. Further efforts will be focused on developing a PGD protocol to the couples at risk of conceiving a pregnancy affected with MKS3 and other known monogenic diseases.

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Disclosure of conflict of interest

None.

Address correspondence to: Huijun Yuan, Institute of Otolaryngology, Chinese PLA General Hospital, Beijing 100853, China. Tel: +86 10 6693 8147; Fax: 86 10 6815 6974; E-mail: yuanhj301@163.com; Yali Li or Yanping Lu, Department of Obstetrics and Gynecology, Chinese PLA General Hospital, Beijing 100853, China. E-mail: li_Yali@hotmail.com (YLL); yanpinglu569@163.com (YPL)

References

The transmembrane protein meckelin (MKS3) is mutated in Meckel-Gruber syndrome and the wpk rat. Nat Genet 2006; 38: 191-196.


