Mutational analysis of the WNT gene family in women with Mayer-Rokitansky-Kuster-Hauser syndrome

The aim of this study is to determine if Müllerian agenesis has a genetic basis linked to the WNT genes. Genomic DNA analyses for mutations in the coding sequences of four members of this family in a series of 11 women with Mayer-Rokitansky-Kuster-Hauser syndrome found four variants in the coding sequence of these genes, but causal mutations were not observed. This supports the hypothesis that mutations in the coding sequence of WNT4, WNT5A, WNT7A, and WNT9B are not responsible for the Mayer-Rokitansky-Kuster-Hauser syndrome. (Fertil Steril® 2009;91:1604–7. ©2009 by American Society for Reproductive Medicine.)

Müllerian agenesis, also called the Mayer-Rokitansky-Kuster-Hauser syndrome (MRKHS), is a cause of primary amenorrhea with an incidence of approximately 1 in 5,000 newborn girls. It is characterized by the agenesis of the Müllerian structures, including fallopian tubes, uterus, and internal portion of the vagina in women with normal external genitalia. Anomalies of the genital tract range from upper vaginal atresia to total Müllerian agenesis with urinary tract abnormalities (1). Most cases of MRKHS are sporadic, but some familial cases have been described, suggesting a genetic cause. Several candidate genes have been screened during embryogenesis. In mice, a subset of wingless genes are homologous to the Drosophila segment-polarity gene wingless. They control the production of a large family of proteins involved in intercellular signaling by Wnt5a, Wnt5b, and Wnt9b has been shown to be involved in the development of female reproductive organs, but in humans, to date, only the WNT4 gene has been shown to be involved in some particular cases of absence of uterus with no other Müllerian abnormalities but with androgen excess (2). The aim of the present work was therefore to search for mutations in WNT4 and in other members of the WNT gene family (WNT5A, WNT7A, and WNT9B) in a cohort of patients with MRKH.

The WNT genes are homologous to the Drosophila segment-polarity gene wingless. They control the production of a large family of proteins involved in intercellular signaling during embryogenesis. In mice, a subset of Wnt genes including Wnt4, Wnt5a, Wnt7a, and Wnt9b has been shown to be involved in the development of female reproductive organs, but in humans, to date, only the WNT4 gene has been shown to be involved in some particular cases of absence of uterus with no other Müllerian abnormalities but with androgen excess (2). The aim of the present work was therefore to search for mutations in WNT4 and in other members of the WNT gene family (WNT5A, WNT7A, and WNT9B) in a cohort of patients with MRKH.

Eleven unrelated caucasian women were referred to Tenon Hospital in Paris for primary amenorrhea and were diagnosed with MRKH after clinical and ultrasonographic investigations. All women had normal external genitalia and normal ovaries. The karyotype was 46,XX in all cases. None of them had diethylstilbestrol exposition, diabetes, or evidence of hyperandrogenism. In three cases, a renal anomaly was reported. Two patients had unilateral agenesis, and a third case had a familial history of renal agenesis (Table 1). For molecular investigations, a written informed consent, approved by the Ethics Committee, was obtained. Genomic DNA was extracted from peripheral blood leukocytes using standard techniques. Polymerase chain reaction (PCR) using genomic DNA templates was used to amplify the coding sequences of the WNT4A, WNT5A, WNT7A, and WNT9B genes. DNA sequencing was performed with BigDye terminators (Applied Biosystems) and an ABI 377 automated sequencer (primer sequences available on request).

We identified four variants in the coding sequence of these genes (Table 1). The first mutation, c.483C>T, is synonymous and localized on the third exon of WNT4 gene, on chromosome 1, and has not been previously reported. It was present in a patient with a vaginal aplasia and a left pelvic kidney. The second mutation, c.459T>C, was localized on the third exon of WNT7A, on chromosome 3. This reported single-nucleotide polymorphism (SNP) (dbSNP rs3762719) is synonymous pSer153. The third synonymous mutation, c.861G>A, was observed in the fourth exon of the WNT7A gene. The last variant was located in the second exon of the WNT9B gene on chromosome 17, c.317T>C, and was present in 5 of 11 patients. It results in a threonine/methionine substitution (p.M106T). This is a known polymorphism with highest frequencies reported in Asian populations (dbSNP rs4968281). However, this amino acid change was considered to be probably damaging using in silico analysis with two different approaches. Polymorphism phenotyping (polyPhen) is a tool that predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and
<table>
<thead>
<tr>
<th>Patient</th>
<th>Uterus</th>
<th>Symmetry</th>
<th>Kidney</th>
<th>Testosterone&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Height (m)</th>
<th>SNP</th>
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<td>3</td>
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<sup>a</sup> Normal range: 0.2–0.6 ng/mL.

Note: NA = data not available. SNP = single-nucleotide polymorphism.

comparative considerations (http://genetics.bwh.harvard.edu/pph/). Prediction data indicates a position-specific independent counts score difference for the p.M106T variant, considered to be probably damaging. The sorting intolerant from tolerant (SIFT) algorithm predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (http://blocks.fhcrc.org/sift/SIFT.html). The SIFT algorithm also predicts that this amino acid change is not tolerated. Other variants were observed in both the 5' flanking region of the genes and also in the 3'UTR region of the genes (data not shown).

The WNT genes and products (derived from “Wingless” and “INT”) constitute the WNT signaling pathway, which controls developmental processes. The Wnt family consists of at least 19 members with functions that contribute to the regulation of a wide range of cellular processes, including proliferation and differentiation. The Wnt proteins activate many signaling cascades, which can be divided broadly into two general categories: the canonic β-catenin–dependent pathway and the noncanonic β-catenin–independent pathway (3). It is a large family of secreted signaling proteins, binding to the Frizzled Lrp and/or other receptors and causes a stabilization of intracellular β-catenin, which is then translocated into the nucleus, where it interacts with the transcription factors to regulate gene expression. The central player in the Wnt pathway is β-catenin, whose stability is regulated by anaphase-promoting complex (APC). When Wnt receptors are inactive, β-catenin localizes with the membrane protein E-cadherin (CDH1) and kinases in the APC phosphorylate cytoplasmic β-catenin for its rapid degradation. Findings from mouse knockout studies involve several genes of this Wnt family in various stages of sexual duct formation and development of female reproductive tract.

In Wnt4 mouse knockout, mutant females fail to develop Müllerian ducts and present with sex determination defects. The Wnt4 protein appears to be necessary to suppress the development of Leydig cells in the developing ovary and consequently ectopic testosterone synthesis by repression of the migration of steroidogenic adrenal precursors into the gonad (4). In human, WNT4 plays a role in sex-determination cascade as well as the development and maintenance of the female phenotype by regulation of Müllerian duct formation and control of ovarian steroidogenesis. Female patients carrying a heterozygous mutation in the WNT4 gene were recently identified. These mutations were shown to alter the regulation of steroidogenic enzymes in ovarian and adrenal cell lines (5). Indeed, it seems that complete loss of function of Wnt4 leads to an MRKH-like syndrome associated with hyperandrogenism which might be a clinical entity distinct from the classic MRKH (2, 6). In the present study, although hormonal levels were available in only 3 of 11 patients, none of them showed clinical signs of androgen excess. Women with polycystic ovary syndrome (PCOS) also have hyperandrogenism. Extensive gene expression analysis in ovaries from PCOS patients has shown disturbances in the Wnt signaling pathway, with an abnormal level of expression of the WNT5A gene (7). In mice, Wnt5a provides a specific signal derived from stromal cells permitting the luminal epithelium to form uterine glands (8). The Wnt5a protein is also required for normal mammary development (9). To date, no mutation in WNT5A has been reported in the human, and, in our study, the WNT5A coding sequences of all patients were normal. These observations are in keeping with those of other studies excluding the role of WNT4 and WNT5A as major genes in MRKH without virilization (10, 11).

Mutations in the WNT7A gene were not observed in our series of 11 patients with MRKH. However, this gene has been shown to influence Müllerian duct regression in mammals (12). Mutations of WNT7A in human have been related to a range of limb hypoplasias, including the Fuhrmann syndrome and the Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome (13). In this latter study (13), the female patients had no menses, but unfortunately neither endocrine studies nor ultrasound examinations for detecting malformations of the internal genitalia were conducted. In mouse, Wnt7a-deficient females are infertile because of an abnormal development of Müllerian derivatives including small uterus, thin muscular development in uterine walls, and incompletely coiled or absent oviducts, whereas, in males, a normal function of the Wnt7a gene is required for Müllerian duct regression (14). Our data are consistent with those of Timmreck et al. (15) who failed to find any mutation in this gene in a series of 40 patients with congenital genital abnormalities, including 31 women with MRKH.

Analyses of the Wnt7a and Wnt5a mutants in mouse demonstrate the requirement of both genes in glandular genesis of the uterus (16). The fact that Wnt7a is expressed in uterine epithelium and that Wnt5a is expressed in uterine stroma support that cytodifferentiation of the uterus requires epithelial-mesenchymal paracrine interactions. The Wnt5a gene is expressed throughout the uterine mesenchyme and Wnt7a is down-regulated specifically in the invaginating epithelium that gives rise to the glands during postnatal development. Mericskay et al. (8) proposed that repression of Wnt7a is required to allow luminal epithelium to change fate, invaginate, and form glands and that Wnt5a is required for this down-regulation. Moreover, it seems that Wnt5a is required for down-regulation of Wnt7a in response to diethylstilbestrol (16).

Previous studies have demonstrated that Wnt9b plays a primary role in induction of the mammalian kidney and reproductive system. The Wnt9b protein in mouse is essential for the development of mesonephric and metanephric tubules and caudal extension of the Müllerian duct, acting upstream of Wnt4, in this pathway. Mutant females present with normal ovaries but are devoid of uterus and upper
vagina, and mutant males present an absence of the epididymis (17). Here, for the first time, we screened the WNT9B gene for mutations but did not identify any mutation associated with MRKHS in the present study.

This is the first study to have evaluated a series of genes involved in the Wnt signaling pathway in a large cohort of patients with the rare MRKHS. Our study failed to find pathogenic mutations in these genes but only either a normal sequence (WNT5A), synonymous SNPs (WNT4, WNT7A) or a T>C change leading to a threonine/methionine substitution (WNT9B) which is known to be present in more than 30% of individuals of caucasian origin (HapMap data; www.hapmap.org). Although coding sequences for these genes were normal, we cannot exclude the possibility that mutations disrupting the normal regulation of these genes may be associated with the phenotype.

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REFERENCES