Genetic analysis of Mayer-Rokitansky-Kuster-Hauser syndrome in a large cohort of families

Lacey S. Williams, B.S.,[a] Durkadin Demir Eksi, Ph.D.,[b] Yiping Shen, Ph.D.,[c,d,e,f] Amy C. Lossie, Ph.D.,[g] Lynn P. Chorich, M.S.,[a] Megan E. Sullivan, B.S.,[a] John A. Phillips III, M.D.,[h] Munire Erman, M.D.,[i] Hyung-Goo Kim, Ph.D.,[a] Ozgul M. Alper, Ph.D.,[b] and Lawrence C. Layman, M.D.[a,j,k]

[a] Section of Reproductive Endocrinology, Infertility, and Genetics, Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta University, Augusta, Georgia; [b] Department of Medical Biology and Genetics, Akdeniz University Faculty of Medicine, Antalya, Turkey; [c] Department of Laboratory Medicine, Boston Children's Hospital, Boston, Massachusetts; [d] Department of Pathology, Harvard Medical School, Boston, Massachusetts; [e] Guangxi Maternal and Child Health Hospital, Nanning, People's Republic of China; [f] Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, Shanghai, People's Republic of China; [g] Beautiful You MRKH Foundation, Silver Spring, Maryland; [h] Division of Medical Genetics and Genomic Medicine, Department of Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee; [i] Department of Obstetrics and Gynecology, Akdeniz University Faculty of Medicine, Antalya, Turkey; and [j] Department of Neuroscience and Regenerative Medicine and [k]Department of Physiology, Medical College of Georgia, Augusta University, Augusta, Georgia

Objective: To study the genetic cause of Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH). Although a few candidate genes and genomic domains for have been reported for MRKH, the genetic underpinnings remain largely unknown. Some of the top candidate genes are *WNT4*, *HNF1B*, and *LHX1*. The goals of this study were to: 1) determine the prevalence of *WNT4*, *HNF1B*, and *LHX1* point mutations, as well as new copy number variants (CNVs) in people with MRKH; and 2) identify and characterize MRKH cohorts.
Design: Laboratory- and community-based study.
Setting: Academic medical centers.
Patient(s): A total of 147 MRKH probands and available family members.
Interventions(s): DNA sequencing of *WNT4*, *HNF1B*, and *LHX1* in 100 MRKH patients, chromosomal microarray analysis in 31 North American MRKH patients, and characterization and sample collection of 147 North American and Turkish MRKH probands and their families.
Main Outcome Measure(s): DNA sequence variants and CNVs; pedigree structural analysis.
Result(s): We report finding CNVs in 6/31 people (~19%) with MRKH, but no point mutations or small indels in *WNT4*, *HNF1B*, or *LHX1* in 100 MRKH patients. Our MRKH families included 43 quads, 26 trios, and 30 duos. Of our MRKH probands, 87/147 (59%) had MRKH type 1 and 60/147 (41%) had type 2 with additional anomalies.
Conclusion(s): Although the prevalence of *WNT4*, *HNF1B*, and *LHX1* point mutations is low in people with MRKH, the prevalence of CNVs was ~19%. Further analysis of our large familial cohort of patients will facilitate gene discovery to better understand the complex etiology of MRKH. (Fertil Steril® 2017;108:145–151. ©2017 by American Society for Reproductive Medicine.)
Key Words: Müllerian aplasia, MRKH, reproductive genetics, congenital absence of the uterus and vagina, gene mutation

Discuss: You can discuss this article with its authors and with other ASRM members at https://www.fertstertdialog.com/users/16110-fertility-and-sterility/posts/16749-23772

Mayer-Rokitanksy-Kuster-Hauser syndrome (MRKH; MIM #27700), also known as müllerian aplasia, consists of the congenital absence of the uterus and vagina. MRKH, which is the name patients prefer, accounts for 10% of the cases of primary amenorrhea (1), and it affects ~1/5,000 women (2). This anomaly constitutes the most severe malformation of the female reproductive tract, and occurs in isolation in two-thirds of patients, often referred to as type 1 (2). A subset of patients (type 2) presents with associated structural abnormalities such as unilateral renal agenesis (30%), skeletal defects (10%–15%), cardiac anomalies (2%–3%), and deafness (2%–3%). These patients have a normal 46,XX karyotype and typically exhibit normal ovarian

Received January 26, 2017; revised May 10, 2017; accepted May 11, 2017; published online June 7, 2017.
L.S.W. has nothing to disclose. D.D.E. has nothing to disclose. Y.S. has nothing to disclose. A.C.L. has nothing to disclose. L.P.C. has nothing to disclose. M.E.S. has nothing to disclose. J.A.P. has nothing to disclose. M.E. has nothing to disclose. H.-G.K. has nothing to disclose. O.M.A. has nothing to disclose. L.C.L. was supported by National Institutes of Health grant HD33004.
Reprint requests: Lawrence C. Layman, M.D., CA2041, IMMAG, Medical College of Georgia at Augusta University, 1120 15th Street, Augusta, GA 30912 (E-mail: lalayman@augusta.edu).

Fertility and Sterility® Vol. 108, No. 1, July 2017 0015-0282/$36.00
Copyright ©2017 American Society for Reproductive Medicine, Published by Elsevier Inc. All rights reserved
http://dx.doi.org/10.1016/j.fertnstert.2017.05.017

function with normal development of breasts and external genitalia (2, 3).

The molecular pathways of müllerian development have been well studied in nonhuman animal models that document the importance of WNT signaling, because mice with null or mutant alleles for *Wnt9b*, *Wnt4*, *Wnt5a*, and *Wnt7a* manifest varying degrees of müllerian hypoplasia (4). In addition, other genes, such as *Pbx1*, *Pax2*, *Lhx1*, and *Emx2*, have been implicated in normal müllerian development in the mouse (5, 6). However, the genetic underpinnings of human müllerian developmental abnormalities are largely unknown. Although many cases are sporadic, some familial cases have been reported, suggesting a genetic role in the pathogenesis in some patients (7). Genetic transmission is difficult to ascertain, because affected families are often small and affected individuals are unable to have children unless they undergo surrogacy or uterine transplantation (3, 8).

A number of chromosomal regions and candidate genes have been studied in humans with MRKH, and several have been observed by different investigators, but conclusive evidence for causation is lacking (3) except for causative mutations in *WNT4* (9) and *HNF1B* (10). An *LHX1* nonsense variant has been reported, but the segregation within families and in vitro confirmation have not been documented (11). However, studies in mice suggest that mutations in *Lhx1* could lead to an MRKH-like phenotype (12–14). Chromosomal microarrays have suggested numerous copy number variants (CNVs) associated with MRKH, with 17q12 and 16p11 being two more commonly affected regions (3, 15). These CNVs contain multiple genes, and it is currently not clear if MRKH is a genomic disorder or if one or a few genes within these regions could be involved in its pathophysiology (3).

The genetic component of MRKH may be complex, and complete understanding is hindered by the lack of large collections of MRKH families. Herlin et al. (16) reported one family with two MRKH probands and reviewed the literature, reporting 67 families with at least two MRKH patients or one MRKH and one with MRKH-associated anomalies. However, these families were identified from multiple publications in the literature, and there has not been any large characterization of unselected MRKH patients from a single research team. In addition, and importantly, the prevalence of gene mutations in *WNT4*, *HNF1B*, and *LHX1* has not been substantiated in a relatively large sample of MRKH women. The purpose of the present study was to: 1) collect and obtain clinical information and blood samples from a large cohort of MRKH families containing at least one MRKH patient; 2) determine the prevalence of variants in two accepted MRKH genes—*WNT4* and *HNF1B*—as well as the candidate gene *LHX1*; and 3) to determine if CNVs are present in a subset of our North American MRKH patients that are absent in their unaffected parents.

MATERIALS AND METHODS

Cohort Characterization

Patients and families were recruited to participate via ascertainment of MRKH probands through our Developmental Gene Discovery Project (DGDP). Many probands were collected by authors L.C.L. and O.M.A., but a substantial number were identified from the Beautiful You MRKH Foundation (author A.C.L.). MRKH was defined as a female with normal breast development, Tanner 5 pubic hair, and an absent vagina and uterus based on physical exam, supported by imaging (ultrasound and/or magnetic resonance imaging) and/or surgery (2, 3). All patients had a 46,XX karyotype except our one previously reported patient with a chromosomal translocation involving chromosomes 3 and 16 (17). MRKH-associated anomalies were identified by reviewing medical records and obtaining family history. Every attempt was made to collect available family members. Peripheral blood was collected for creating lymphoblastoid cell lines and extracting DNA as described previously (18). Lymphoblastoid cells were created so that a long-term supply of DNA, RNA, and protein could be available for in vitro analyses on identified genetic variants for confirmation. This study was approved by the Institutional Review Board at Augusta University, and all participating patients and available family members signed consent forms.

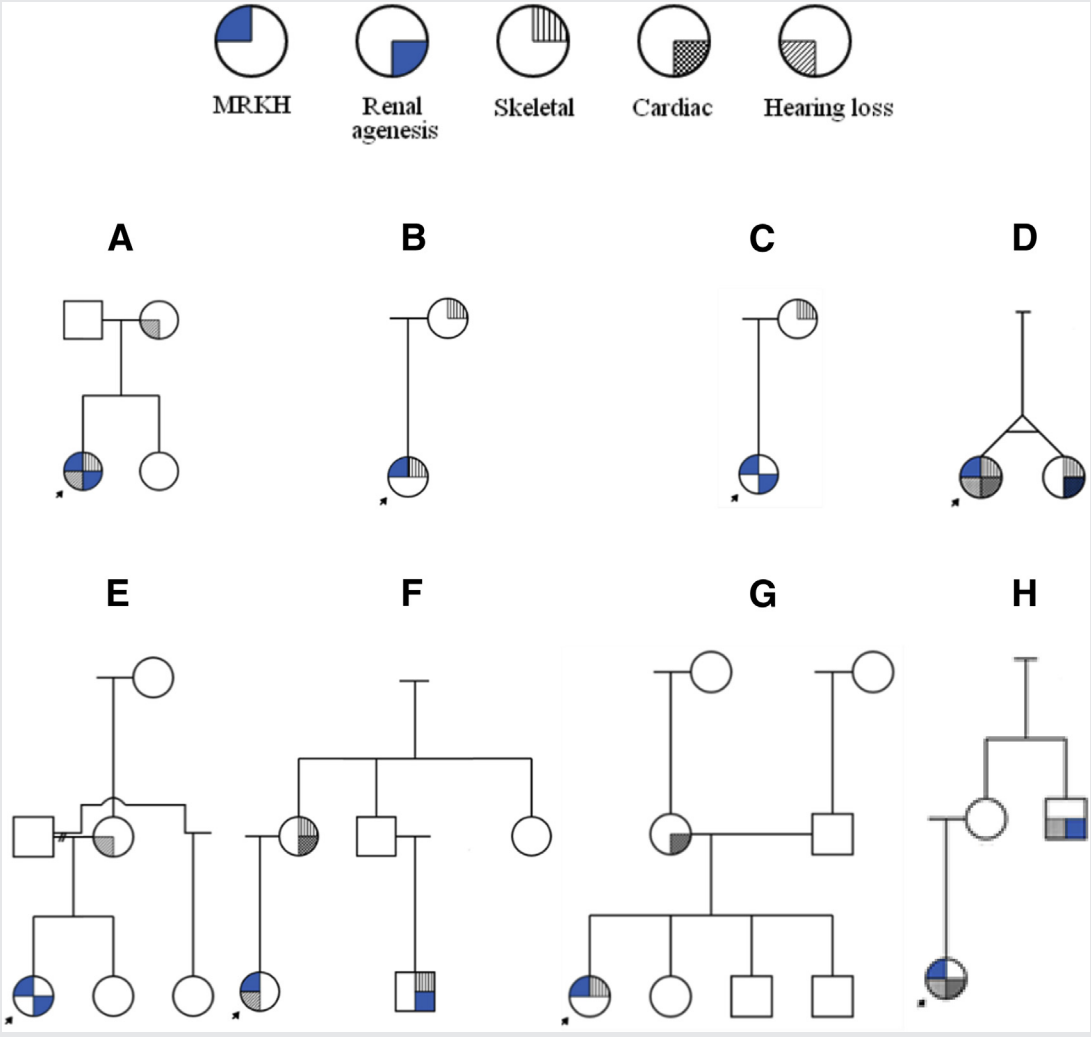
Sanger DNA Sequencing for *WNT4*, *LHX1*, and *HNF1B*

DNA was extracted from peripheral blood leukocytes as described previously (18, 19). Sanger sequencing was performed on a cohort of 100 MRKH patients (79 with type 1 and 21 with type 2) for the protein-coding regions and splice junctions for five exons of *WNT4* (NM_030761.4), five exons of *LHX1* (NM_005568.3), and nine exons of *HNF1B* (NM_000458.3). Each fragment was amplified by means of polymerase chain reaction (PCR) for 30 cycles consisting of a 5-minute denaturation step at 95°C followed by 30 cycles of the following: 1 minute at 95°C, 30–60 seconds at 55°C, and 30–60 seconds at 72°C—followed by a 7-minute 72°C extension step. Each fragment was resolved by agarose gel electrophoresis, and then an aliquot of the sample was subjected to dideoxy sequencing on an ABI 310 Automated DNA Sequencer as described previously (20–22). Each fragment was sequenced in the forward and reverse directions. If a variant was identified, two additional sequencing reactions were performed. The obtained sequence was blasted to the wild-type sequence.

Chromosomal Microarrays

DNA from 31 unrelated North American MRKH probands (10 with type 1 and 21 with type 2) was subjected to chromosomal microarrays. CNV analysis was performed at Harvard with the use of an Affymetrix Cytoscan HD array, which consisted of 750,000 single-nucleotide polymorphism probes and 1.9 million copy number probes to detect CNVs. The lower limit of detection for CNVs was 50 kb. One hundred nanograms of genomic DNA was labeled with the use of Cytoscan reagent kit according to the manufacturer's instructions. The array data were analyzed with Chromosome Analysis Suite software as described previously (22). Human genome hg19 assembly was used to map genomic coordinates.

FIGURE 1



Eight Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH) pedigrees from North America with at least one affected family member with an MRKH-associated anomaly. *Arrows* indicate MRKH probands.

Williams. Genetics of müllerian aplasia in MRKH. Fertil Steril 2017.

RESULTS

Cohort Characterization

We acquired DNA (and lymphoblastoid cell lines on most) from a cohort of 147 MRKH female probands. These patients include 80 North American probands, 58 for which we have other family members (Supplemental Fig. 1A; Supplemental Fig. 1 is available online at www.fertstert.org) and 22 singletons (not shown). We also have 67 Turkish probands, 41 with family members (Supplemental Fig. 1B) and 26 singletons (not shown). We collected 43 quads (proband, 1–2 parents, 1–2 siblings), 26 trios (proband, 1–2 parents, 1 sibling), and 30 duos (proband and 1 first-degree relative). We have characterized phenotypes for the patients and family members in pedigrees, including müllerian duct agenesis and associated anomalies (Fig. 1; Table 1). Overall, 87 have type 1 MRKH, and 60 type 2. Of our affected type 2 probands, anomalies include renal (n = 24), skeletal (n = 34), hearing impairment (n = 25), and cardiac (n = 13).

Examination of family members indicates that none of the probands (58 families from North America and 41 from Turkey) in this study has a family member who is also affected by MRKH. However, we observed that 8/58 (14%) of the

TABLE 1

The frequency that an associated anomaly was observed in our cohort of 147 Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH) patients.

MRKH	Renal	Skeletal	Cardiac	Hearing deficits
147	24 (16%)	34 (23%)	13 (9%)	25 (17%)

Williams. Genetics of müllerian aplasia in MRKH. Fertil Steril 2017.

TABLE 2

Copy number variants (CNVs) found in six MRKH patients.

Patient	Location	CNV	Size	Coordinates	Associated anomalies	De novo
DGDP155	2q13	Del	840 kb	chr2:110,520–111,360	Type 2: renal, cardiac, skeletal	NA
DGDP156	17q12	Del	1.9 Mb	chr17:34,455,782–36,307,773	Type 1	De novo
Mother	Normal				Normal	
Father	Normal				Normal	
DGDP273	1q21–1q22	Del	4 Mb	chr1:144,823,069–148,832,359	Type 2: skeletal	NA
DGDP149	2q11	Del	1.5 Mb	chr2:96,712,139–98,249,638	Type 2: renal	
	8p23	Dup	1.9 Mb	chr8:4,322,747–6,214,120	hearing, skeletal	
	12p12–12p13	Dup	1 Mb	chr12:13,938,281–14,954,514		De novo
Mother	2q11	Del	962 kb	chr2:96,766,402–97,728,447	Normal	
	8p23	Del	455 kb	chr8:3,926,021–4,381,311		
Father	8p23	Dup	2 Mb	chr8:4,300,484–6,290,708	Normal	
DGDP284	16p11	Del	746 kb	chr16:29,432,212–30,178,406	Type 2: hearing	NA
	11p11	Dup	456 kb	chr11:48,065,462–48,521,382		
DGDP287	17q12	Del	1.4 Mb	chr17:34,815,077–36,249,799	Type 1	NA

Note: Parents were studied in two (DGDP156 and DGDP149). Human genome hg19 assembly was used for coordinates. Del = deletion; Dup = duplication; kb = kilobases; Mb = megabases; MRKH = Mayer-Rokitansky-Kuster-Hauser syndrome; NS = not studied.

Williams. Genetics of müllerian aplasia in MRKH. Fertil Steril 2017.

families from North America (Fig. 1) and 0/41 of the families from Turkey had a relative with an associated anomaly (Supplemental Fig. 1).

Sanger DNA Sequencing for *WNT4*, *LHX1*, *HNF1B*, and Chromosomal Microarrays

For the 100 patients with MRKH (79 type 1 and 21 type 2) in our cohort, we performed PCR on all protein-coding exons and splice sites of *WNT4*, *HNF1B*, and *LHX1*. Agarose gel electrophoresis and Sanger sequencing failed to reveal evidence of deletions, small indels, or likely pathogenic variants in *WNT4*, *HNF1B*, or *LHX1*.

Chromosomal microarray studies performed on 31 North American patients revealed that six (~19%) had CNVs of potential clinical significance: Two were found in the ten people with MRKH type 1 and four in the 21 with MRKH type 2 (Table 2). Two MRKH type 1 patients had deletions involving 17q12: DGDP156 had a de novo CNV that was 1.9 Mb, and DGDP287 had a 1.4-Mb deletion. The other four CNVs were found in individuals with MRKH type 2. Another MRKH patient (DGDP284) had a 16p11 deletion (746 kb) and a duplication (456kb) of 11p11. MRKH patient DGDP149 had three different CNVs (a deletion involving 2q11 and two duplications: one at 8p23 and another at 12p12–12p13), but only the 12p CNV was de novo (Table 2). Both parents of DGDP149 had CNVs. involving 8p23 and 2q11. A 4 Mb deletion at 1q21–1q22 was identified in DGDP273, and a 2q13 deletion of 840 kb was found in patient DGDP155. We could only document that one 17q12 deletion (in DGDP156) and the 12p12–12p13 duplication (DGDP149) were de novo; parents were not available for the other four patients with CNVs. Both *HNF1B* and *LHX1* reside within 17q12, whereas *WNT4* is localized to chromosome 1p36.12. Except for the 16p11 and 17q12 deletions, all other CNVs were not found in the DECIPHER and Clinvar databases. Only 14 MRKH patients had both DNA sequencing and chromosomal microarrays.

DISCUSSION

The molecular basis of MRKH remains largely unknown. A number of earlier studies, most of which had small sample sizes, failed to show mutations in reasonable candidate genes, including *WT1*, *CFTR*, *WNT7A*, *GALT*, *HOXA7*, *PBX1*, *HOXA13*, *PAX2*, *HOXA10*, *AMH*, *AMHR*, *RARG*, *RXRA*, *CTNNB1*, *LAMC1*, *DLGH1*, and *SHOX* (3). The best supportive evidence for a causative gene is *WNT4*, which was the first genetic cause identified and corroborated by functional studies (9). Mouse studies also provide additional support for WNT signaling in normal müllerian development (4). The four MRKH patients described have heterozygous *WNT4* mutations that impair WNT4 function in vitro, but there was no evidence of genetic heritability, making inheritance ascertainment difficult (9,23–25). Other affected family members have shown wild-type *WNT4* sequences in some of these families, which suggests de novo autosomal dominant inheritance with variable expressivity. Interestingly, MRKH patients with *WNT4* mutations may also demonstrate hyperandrogenism (9), but hyperandrogenism is common in reproductive-age women (26), so some caution should be exercised. Investigators found that 4/37 (10.8%) of MRKH patients had *WNT4* mutations (9,23–25), so we expected to identify ~10 patients with *WNT4* mutations. However, none of our 100 MRKH patients had *WNT4* mutations detected by means of DNA sequencing. These findings indicate that perhaps the prevalence of *WNT4* mutations is much less than previously reported in an unselected group of MRKH probands.

A second causative MRKH gene is *HNF1B*, mutations of which result in maturity-onset diabetes of the young type 5 (MODY5) with nondiabetic renal disease (10). Interestingly, two affected female patients with heterozygous intragenic *HNF1B* deletions impairing function in vitro also had müllerian aplasia (10). Two studies examined the prevalence of *HNF1B* mutations in small numbers of patients. Bernardini et al. (27) studied 20 MRKH patients and Ledig et al. (28) studied 56 patients, and they found no point mutations. Our DNA

sequencing results also failed to show any *HNF1B* point mutations or small indels in 100 patients, again indicating low prevalence. Interestingly, *HNF1B* is located within the common 17q12 deletion CNV. To date these findings suggest that *HNF1B* mutations are causative, but because the 17q12 CNV has been reported more than 10–15 times, it suggests other genes within this region could play some role (3). Therefore, a different gene within the interval or a contiguous gene deletion syndrome cannot be excluded at this time.

LHX1 is a promising candidate gene for MRKH for several reasons. First, *LHX1* resides within the relatively common 17q12 CNV, which has been associated with MRKH in several studies (3, 15), including ours. Second, conditional deletion of *Lhx1* in wolffian-derived (12) or müllerian-derived (14) tissues recapitulates an MRKH-like phenotype in female mice. One heterozygous human nonsense variant in *LHX1* has been described, but no other family members were available and no in vitro analyses were performed (11). Two other studies found *LHX1* missense variants, but no family studies or in vitro analyses were performed (28, 29). We found neither *LHX1* point mutations nor small intragenic indels in 100 MRKH patients, indicating that these types of mutations in this gene are uncommon in MRKH. This gene also resides within the 17q12 CNV deletion interval, but appears to be uncommonly involved in MRKH by itself. Therefore, there is no conclusive evidence that *LHX1* variants cause MRKH. A variety of other gene variants in *TBX6* (29, 30), *WNT9B* (31, 32), and *RBM8A* (30) have been described, but all reports suffer from a lack of in vitro confirmation and family studies, clouding their true role in MRKH causality at this time.

Our chromosomal microarray analysis revealed potentially pathogenic CNVs in 6 of 31 patients (~19%), including two patients with 17q12 deletions, both of whom had MRKH type 1. The 17q12 region is one of the most commonly identified CNVs in MRKH, and it has been found in both type 1 and type 2 MRKH (3, 15). We also found one proband with a 16p11 deletion, which has also been reported by multiple different investigators (3, 15). All other CNVs were absent in the DECIPHER and Clinvar databases. Of interest, our proband with the 16p11 deletion also had an 11p11 duplication. We identified a deletion in the 1q21.1–1q21.2 region that includes the region for thrombocytopenia–absent radius syndrome (TAR), marked by absent radii and early-onset thrombocytopenia due to *RBM8A* gene mutations (30). Some patients with TAR have müllerian aplasia, but our patient did not have the TAR phenotype. CNVs of the long arm of chromosome 2 are associated with developmental delay, intellectual disability, microcephaly, skeletal abnormalities, short stature, and cleft lip and palate (33). A patient with type 2 MRKH was found to have a microdeletion at 2q11.2, as we have confirmed here. Of interest, our patient with the 2q13 deletion presented with renal agenesis and cardiac and skeletal defects. The 2q13 region includes *PAX8*, which has been shown to coordinate with *PAX2* to regulate branching morphogenesis and nephron differentiation during kidney development, a process closely linked to müllerian duct development (34).

We could only document two patients with de novo CNVs (we did not have DNA on parents of other probands with CNVs): one with a 17q12 deletion and the other with a 12p12–12p13 duplication. The patient with the 1-Mb duplication at 12p had two other CNVs involving 2q11 and 8p23 that originated from the parents, suggesting that they could be polymorphisms. However, the sizes and breakpoints are different from the proband. This MRKH proband had a 1.5-Mb deletion at 2q11, which encompasses the 962-kb deletion identified in her mother. This same MRKH proband also had a 1.9-Mb duplication at 8p23, which is similar in size to the 2-Mb duplication found in her father. Intriguingly, the proband's mother had an 8p23 deletion (rather than duplication) of 455 kb, which is partially encompassed in the deleted region of the affected daughter and husband (DGDP149 in Table 2). This dynamic change of CNV breakpoints on the same chromosomal regions in this family is worthy of future investigation. Perhaps these parental CNVs of 8p23 and 2q11 deletions in her mother and the 8p23 duplication in the father had an interchromosomal effect contributing to the generation of the 12p12–12p13 duplication in the MRKH proband. Although the CNVs we have identified are possibly pathogenic, they do not provide convincing evidence of causation.

The molecular basis of MRKH is difficult to characterize. The major barrier is the lack of a large familial cohort of MRKH families to systematically study both clinically and at the molecular level. Any identified variant should be subjected to family studies to determine if the variant segregates with the phenotype, which can not be easily done without a large cohort. Then in vitro analysis is required for confirmation. In the present study, we sought to gather a large sample of MRKH families, enlisting the assistance of the patient support foundation Beautiful You MRKH (www.beautifulyoumrkh.org). We collected a sample of 147 MRKH probands, including 99 with available family members (Supplemental Fig. 1). From our large familial MRKH cohort, no proband had an additional affected relative with MRKH. Only 14% of North American and no Turkish probands had a relative with an MRKH-associated anomaly. This, along with unknown phenotypic effects in male individuals, adds to the complexity of MRKH genetics, and it must be considered when studying families. Even in small families, segregation analyses can be performed, and we would not expect unaffected individuals to have the same variant, as exemplified for *WNT4* (9) and *HNF1B* (10) mutations.

Herlin et al. (16) described a familial case of MRKH and identified MRKH probands in the literature who had at least one more person affected with MRKH or MRKH-associated anomalies. They found that 36 of the 67 families had two or more MRKH patients and the remainder had at least one associated anomaly. Our findings are more unselected, because we collect all MRKH patients and families that we can recruit, which perhaps gives a more reasonable estimate of MRKH family structure and avoiding publication bias as in Herlin et al. (16). The family structures of the reported multiplex MRKH families argue against autosomal recessive inheritance, although this seems more possible in our Turkish patients owing to potential consanguinity. Instead de novo, autosomal dominant or polygenic/multifactorial modes of inheritance are more likely, which has been proposed by others (16). As recommended by Schaffer (35), true digenic/

polygenic inheritance is extremely challenging to substantiate, because human families, cellular studies, and mouse models are needed for corroboration.

Clearly, our understanding of the molecular basis of MRKH is in its infancy, unlike other reproductive disorders such as hypogonadotropic hypogonadism and gonadal failure, for which the genetic basis is known for a significant proportion of affected individuals (36–38). Our cohort of MRKH probands and family members, as well as our collection and use of lymphoblastoid cell lines, will allow gene segregation analysis to test for true de novo variants, determine effects on RNA and protein, and examine for inheritance patterns. Because we have a large number of quads and trios, determination of inheritance of identified variants should be more easily accomplished. Future efforts will include whole-exome sequencing and whole-genome sequencing in our cohort to generate comprehensive data via high-throughput next-generation sequencing methods. In vitro analyses will be needed to complement the effect of any genetic variant, particularly for missense variants, which are commonly variants of undetermined significance unless they are studied in vitro (39). However, newer bioinformatic programs, such as pVAAST (pedigree variant annotation, analysis, and search tool), are able to mine next-generation DNA sequencing data from quads, trios, or any family structure. pVAAST incorporates linkage, association, and variant severity. This method has been shown to be superior to other methods in analyzing genome-sequencing data for autosomal dominant, autosomal recessive, and, particularly relevant to the MRKH phenotype, dominant inheritance resulting from de novo mutations (40). Digenic/polygenic disease is also amenable to pVAAST analysis (40).

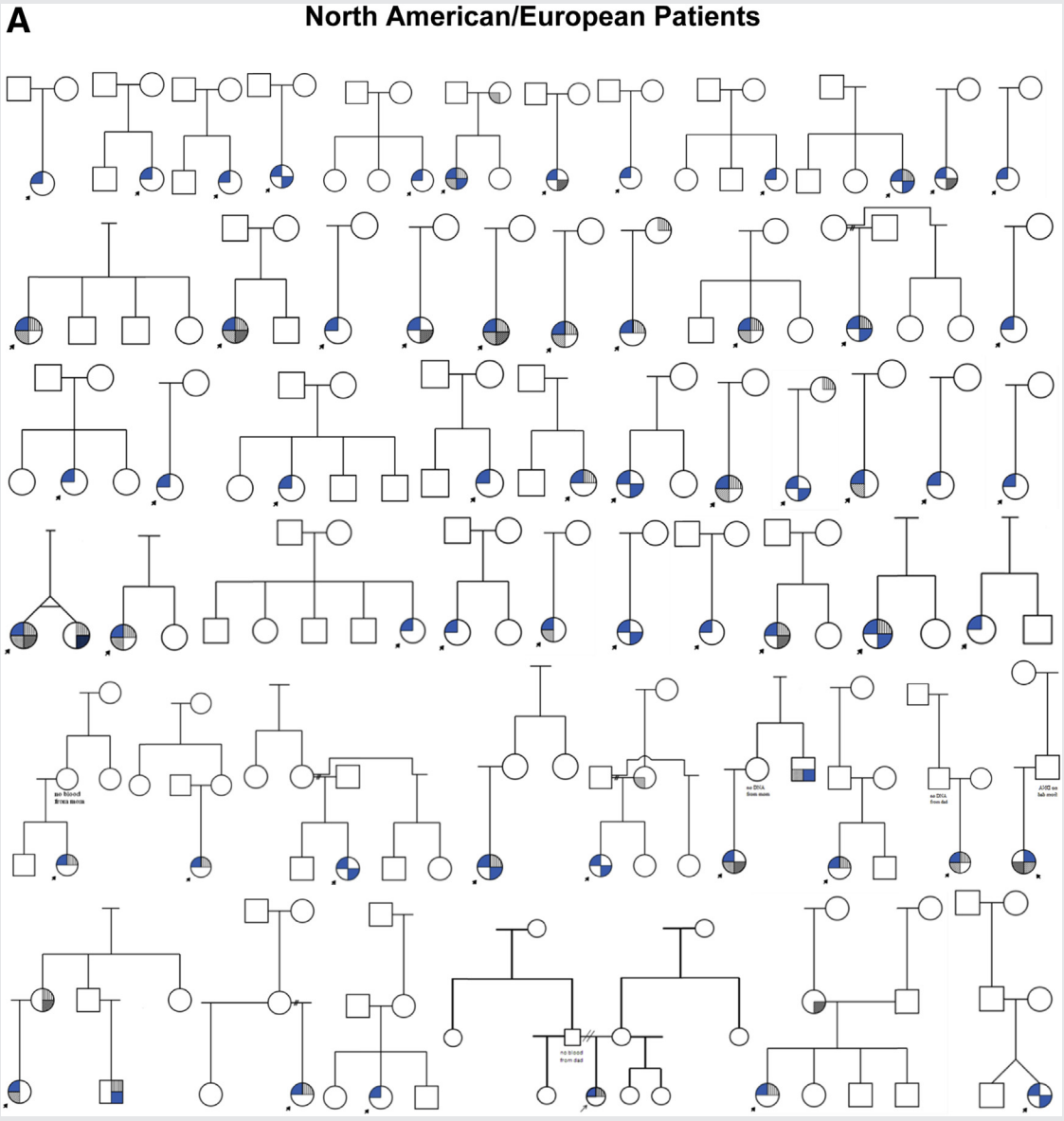
In summary, the genetic basis for MRKH remains largely unknown, and nongenetic causes, such as epigenetic changes and somatic anomalies, could play a role in some patients. The prevalence of mutations in the two known genes *WNT4* (n = 4) and *HNF1B* (n = 1 family) is exceedingly low. There is no solid evidence at this time for causation in other genes, although there are promising candidate genes, such as *LHX1*, *TBX6*, *WNT9B*, and *RMB8A*, that require future study. The repetitive identification of CNVs in regions such as 17q12, 16p11, and 1q21 suggests their involvement, as well as a potential explanation for the etiology of multiple cases that would be missed by DNA sequencing of exons. However, further work is needed to prove causation. Large well characterized MRKH families with lymphoblastoid cell lines, such as our large international cohort, along with significant public support from the MRKH community, will be essential to unraveling its complex genetics.

REFERENCES

1. Reindollar RH, Byrd JR, McDonough PG. Delayed sexual development:study of 252 patients. Am J Obstet Gynecol 1981;140:371–80.
2. Oppelt PG, Lermann J, Strick R, Dittrich R, Strissel P, Rettig I, et al. Malformations in a cohort of 284 women with Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH). Reprod Biol Endocrinol 2012;10:57.
3. Layman LC. The genetics of müllerian aplasia. Expert Rev Endocrinol Metab 2014;9:411–9.
4. Mericskay M, Kitajewski J, Sassoon D. Wnt5a is required for proper epithelial-mesenchymal interactions in the uterus. Development 2004; 131:2061–72.
5. Masse J, Watrin T, Laurent A, Deschamps S, Guerrier D, Pellerin I. The developing female genital tract: from genetics to epigenetics. Int J Dev Biol 2009; 53:411–24.
6. Kurita T. Normal and abnormal epithelial differentiation in the female reproductive tract. Differentiation 2011;82:117–26.
7. Simpson JL. Genetics of female infertility due to anomalies of the ovary and mullerian ducts. Methods Mol Biol 2014;1154:39–73.
8. Petrozza JC, Gray MR, Davis AJ, Reindollar RH. Congenital absence of the uterus and vagina is not commonly transmitted as a dominant genetic trait: outcomes of surrogate pregnancies. Fertil Steril 1997;67:387–9.
9. Biason-Lauber A, Konrad D, Navratil F, Schoenle EJ. A WNT4 mutation associated with müllerian-duct regression and virilization in a 46,XX woman. N Engl J Med 2004;351:792–8.
10. Lindner TH, Njolstad PR, Horikawa Y, Bostad L, Bell GI, Sovik O. A novel syndrome of diabetes mellitus, renal dysfunction and genital malformation associated with a partial deletion of the pseudo-POU domain of hepatocyte nuclear factor-1beta. Hum Mol Genet 1999;8:2001–8.
11. Ledig S, Brucker S, Barresi G, Schomburg J, Rall K, Wieacker P. Frame shift mutation of LHX1 is associated with Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome. Hum Reprod 2012;27:2872–5.
12. Kobayashi A, Kwan KM, Carroll TJ, McMahon AP, Mendelsohn CL, Behringer RR. Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. Development 2005;132:2809–23.
13. Kobayashi A, Shawlot W, Kania A, Behringer RR. Requirement of Lim1 for female reproductive tract development. Development 2004;131:539–49.
14. Huang CC, Orvis GD, Kwan KM, Behringer RR. Lhx1 is required in müllerian duct epithelium for uterine development. Dev Biol 2014;389:124–36.
15. Nik-Zainal S, Strick R, Storer M, Huang N, Rad R, Willatt L, et al. High incidence of recurrent copy number variants in patients with isolated and syndromic müllerian aplasia. J Med Genet 2011;48:197–204.
16. Herlin M, Hojland AT, Petersen MB. Familial occurrence of Mayer-Rokitansky-Kuster-Hauser syndrome: a case report and review of the literature. Am J Med Genet A 2014;164A:2276–86.
17. Williams LS, Kim HG, Kalscheuer VM, Tuck JM, Chorich LP, Sullivan ME, et al. A balanced chromosomal translocation involving chromosomes 3 and 16 in a patient with Mayer-Rokitansky-Kuster-Hauser syndrome reveals new candidate genes at 3p22.3 and 16p13.3. Mol Cytogenet 2016;9:57.
18. Bhagavath B, Podolsky RH, Ozata M, Bolu E, Bick DP, Kulharya A, et al. Clinical and molecular characterization of a large sample of patients with hypogonadotropic hypogonadism. Fertil Steril 2006;85:706–13.
19. Layman LC, Roach DJ, Plouffe L Jr, McDonough PG, Wilson JT. The detection of Hind III restriction fragment length polymorphisms (RFLP's) using a DNA probe for the β-subunit of follicle-stimulating hormone (FSH). Fertil Steril 1990;53:261–5.
20. Layman LC, Cohen DP, Jin M, Xie J, Li Z, Reindollar RH, et al. Mutations in gonadotropin-releasing hormone receptor gene cause hypogonadotropic hypogonadism. Nat Genet 1998;18:14–5.
21. Layman LC, Lee EJ, Peak DB, Namnoum AB, Vu KV, van Lingen BL, et al. Delayed puberty and hypogonadism caused by mutations in the follicle-stimulating hormone beta-subunit gene. N Engl J Med 1997;337:607–11.
22. Quaynor SD, Stradtman EW Jr, Kim HG, Shen Y, Chorich LP, Schreihofer DA, et al. Delayed puberty and estrogen resistance in a woman with estrogen receptor alpha variant. N Engl J Med 2013;369:164–71.
23. Biason-Lauber A, De Filippo G, Konrad D, Scarano G, Nazzaro A, Schoenle EJ. WNT4 deficiency—a clinical phenotype distinct from the classic Mayer-Rokitansky-Kuster-Hauser syndrome: a case report. Hum Reprod 2007;22:224–9.
24. Philibert P, Biason-Lauber A, Gueorguieva I, Stuckens C, Pienkowski C, Lebon-Labich B, et al. Molecular analysis of WNT4 gene in four adolescent girls with müllerian duct abnormality and hyperandrogenism (atypical Mayer-Rokitansky-Kuster-Hauser syndrome). Fertil Steril 2011;95:2683–6.
25. Philibert P, Biason-Lauber A, Rouzier R, Pienkowski C, Paris F, Konrad D, et al. Identification and functional analysis of a new WNT4 gene mutation among

28 adolescent girls with primary amenorrhea and mullerian duct abnormalities: a French collaborative study. J Clin Endocrinol Metab 2008;93:895–900.
26. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. J Clin Endocrinol Metab 2006;91:4237–45.
27. Bernardini L, Gimelli S, Gervasini C, Carella M, Baban A, Frontino G, et al. Recurrent microdeletion at 17q12 as a cause of Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome: two case reports. Orphanet J Rare Dis 2009;4:25.
28. Ledig S, Schippert C, Strick R, Beckmann MW, Oppelt PG, Wieacker P. Recurrent aberrations identified by array-CGH in patients with Mayer-Rokitansky-Kuster-Hauser syndrome. Fertil Steril 2011;95:1589–94.
29. Sandbacka M, Laivuori H, Freitas E, Halttunen M, Jokimaa V, Morin-Papunen L, et al. TBX6, LHX1 and copy number variations in the complex genetics of müllerian aplasia. Orphanet J Rare Dis 2013;8:125.
30. Tewes AC, Rall KK, Romer T, Hucke J, Kapczuk K, Brucker S, et al. Variations in RBM8A and TBX6 are associated with disorders of the müllerian ducts. Fertil Steril 2015;103:1313–8.
31. Waschk DE, Tewes AC, Romer T, Hucke J, Kapczuk K, Schippert C, et al. Mutations in WNT9B are associated with Mayer-Rokitansky-Kuster-Hauser syndrome. Clin Genet 2016;89:590–6.
32. Ma W, Li Y, Wang M, Li H, Su T, Li Y, et al. Associations of polymorphisms in WNT9B and PBX1 with Mayer-Rokitansky-Kuster-Hauser Syndrome in Chinese Han. PLoS One 2015;10:e0130202.
33. Riley KN, Catalano LM, Bernat JA, Adams SD, Martin DM, Lalani SR, et al. Recurrent deletions and duplications of chromosome 2q11.2 and 2q13 are associated with variable outcomes. Am J Med Genet A 2015;167A:2664–73.
34. Narlis M, Grote D, Gaitan Y, Boualia SK, Bouchard M. Pax2 and Pax8 regulate branching morphogenesis and nephron differentiation in the developing kidney. J Am Soc Nephrol 2007;18:1121–9.
35. Schaffer AA. Digenic inheritance in medical genetics. J Med Genet 2013;50:641–52.
36. Layman LC. The genetic basis of female reproductive disorders: etiology and clinical testing. Mol Cell Endocrinol 2013;370:138–48.
37. Layman LC. Clinical genetic testing for Kallmann syndrome. J Clin Endocrinol Metab 2013;98:1860–2.
38. AlAsiri S, Basit S, Wood-Trageser MA, Yatsenko SA, Jeffries EP, Surti U, et al. Exome sequencing reveals MCM8 mutation underlies ovarian failure and chromosomal instability. J Clin Invest 2015;125:258–62.
39. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405–24.
40. Hu H, Roach JC, Coon H, Guthery SL, Voelkerding KV, Margraf RL, et al. A unified test of linkage analysis and rare-variant association for analysis of pedigree sequence data. Nat Biotechnol 2014;32:663–9.

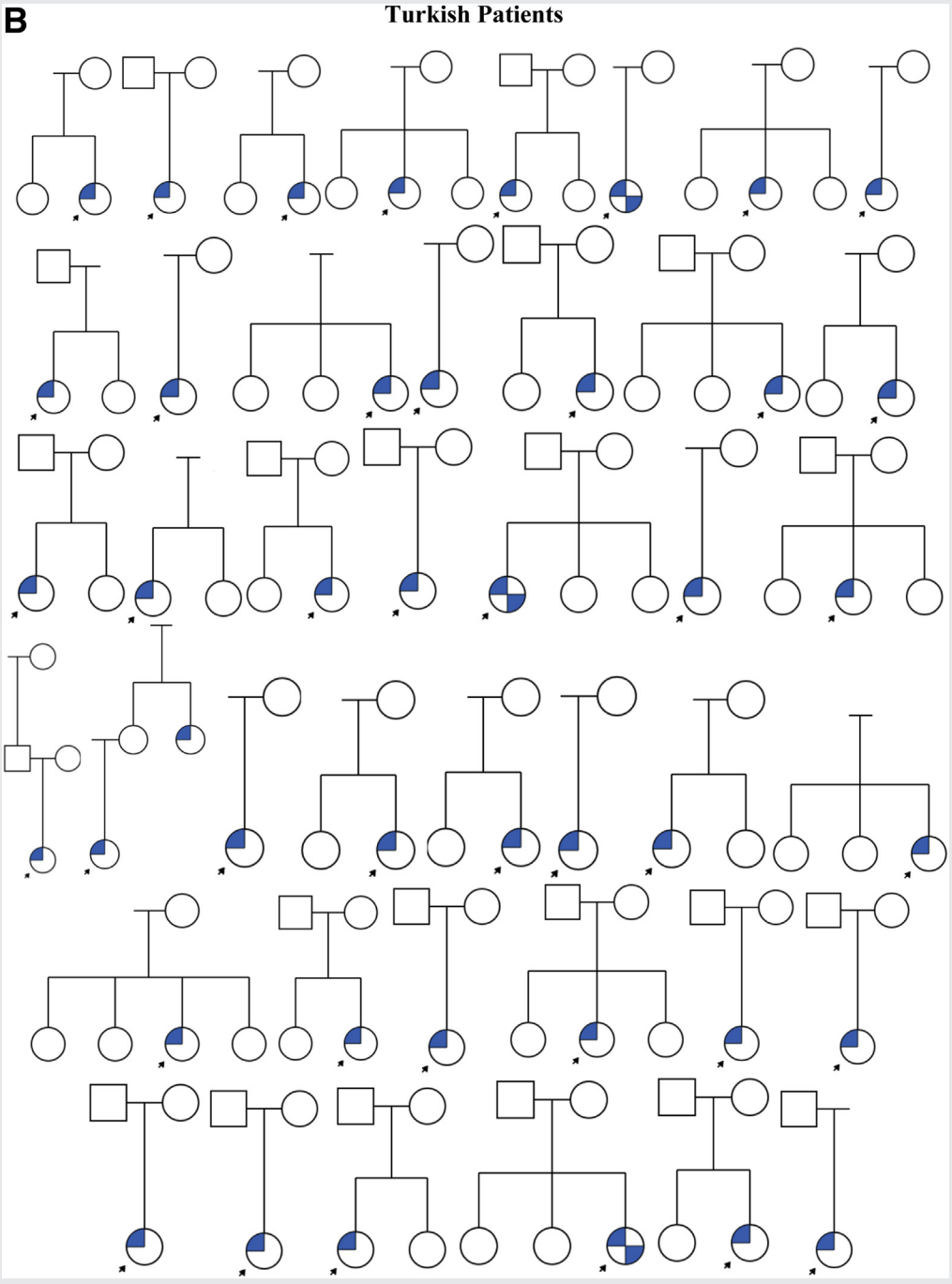
SUPPLEMENTAL FIGURE 1



Pedigrees of (**A**) North American and (**B**) Turkish Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH) patients are shown. Arrows indicate MRKH probands. The key is shown in Figure 1.

Williams. Genetics of müllerian aplasia in MRKH. Fertil Steril 2017.

SUPPLEMENTAL FIGURE 1 Continued



Williams. Genetics of müllerian aplasia in MRKH. Fertil Steril 2017.