Reproductive BioMedicine Online (2014) **28**, 80–85

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ARTICLE

Analysis of *WNT9B* mutations in Chinese women with Mayer–Rokitansky–Küster–Hauser syndrome

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Abstract Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome is a rare congenital female genital anomaly, which is caused by aplasia of the caudal portion of the Müllerian duct. The *WNT9B* gene encodes a secretory glycoprotein essential for the caudal extension of the Müllerian duct during embryonic development in mice. Coding regions and exon/intron boundaries of the *WNT9B* gene were amplified and sequenced in 42 Chinese women with MRKH syndrome and 42 controls. Two novel heterozygous mutations were identified, which were absent in controls. One was a missense mutation in exon 1, and the other was located in the 3′-untranslated region. Both variants were detected in one out of 42 patients. The two novel mutations may be pathogenic variants in MRKH patients and warrant further functional study.

KEYWORDS: Mayer–Rokitansky–Küster–Hauser syndrome, Müllerian duct, mutation, WNT9B, Wolffian duct, Single-nucleotide polymorphism

Introduction

Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome is a female genital anomaly caused by aplasia of the caudal portion of the Müllerian duct. It is characterized by the congenital absence of the uterus and upper vagina with normally functioning ovaries and a female karyotype (46,XX). Isolated uterovaginal aplasia/hypoplasia is referred to as typical or type I MRKH syndrome, while atypical or type II

1472-6483/$ - see front matter
http://dx.doi.org/10.1016/j.rbmo.2013.09.022

MRKH syndrome is accompanied by associated malformations (Morcel et al., 2007).

The Müllerian duct is the anlagen of the oviduct, uterus, cervix and upper portion of the vagina. The *WNT* genes, which encode cysteine-rich proteins that are involved in intercellular signalling, are involved in Müllerian duct development. Based on mouse models, *Wnt9b* is essential for caudal extension of the Müllerian duct (Carroll et al., 2005). *Wnt9b*$^{-/-}$ female mice possess normal ovaries but lack the uterus and upper vagina, which is similar to the clinical syndrome of uterovaginal aplasia noted in MRKH women. In addition, the kidneys of the mutant mice are absent, which is also seen in a subset of MRKH patients, as renal agenesis is the most common anomaly associated with MRKH.

Carroll et al. (2005) demonstrated that *Wnt9b* is expressed throughout the Wolffian duct epithelium during embryogenesis in mice. The Wolffian duct is essential for Müllerian duct extension, as the Wolffian duct not only has close contact with the Müllerian duct but also delivers critical signals that guide the Müllerian duct to extend caudally (Orvis and Behringer, 2007).

Based on the above data, this study postulated that *WNT9B* mutations may be relevant to the aetiology of MRKH syndrome in humans. Studies on MRKH syndrome in China are limited, and there are no studies on *WNT9B*. This study investigated whether *WNT9B* mutations could contribute to the aetiology of MRKH syndrome in Chinese women.

Materials and methods

A sample of 42 unrelated female patients (age 17–36 years), all of whom were of Han ethnicity, complaining of primary amenorrhoea and diagnosed with MRKH syndrome, were included in this study. The patients were enrolled in the study when they were admitted for surgery to Tongji Hospital in Wuhan, Hubei from March 2011 to April 2012. The clinical diagnosis was confirmed by medical history, gynaecological examination, detection of serum hormone concentrations, karyotype analysis, ultrasound and laparoscopy or laparotomy. The majority of patients had typical MRKH, while four had atypical MRKH; of these four patients, two had left renal agenesis, one had an ectopic kidney in the pelvic cavity and one had rachischisis. All patients presented with normal secondary sexual characteristics. Detection of serum hormone concentrations in all patients indicated that none had signs of hyperandrogenism. Karyotype analysis showed a karyotype of 46,XX for all participants. As controls, 42 women who were of Han ethnicity were recruited during the corresponding period, who were aged 17–36 years of age, had at least one pregnancy and were without genital malformations. The study was approved by the Medical Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (reference no. S463, approved 26 July 2011), and informed consent was obtained from each participant.

Peripheral blood samples from each subject were collected. Genomic DNA was extracted using the Quick Gene DNA whole blood kit S (Fujifilm, Japan) and identified by agarose gel electrophoresis and a NanoDrop2000 spectrophotometer. All exons and exon–intron junctions of *WNT9B* were amplified by PCR. Four pairs of primers were designed for four exons of this gene (Table 1). PCR products were verified by agarose gel electrophoresis, purified with EZgene Gel Extraction Kit (Biomiga, USA) and sequenced on an ABI 3730XL automated DNA sequencer (Applied Biosystems, USA).

Analyses of sequence variation were performed using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/). This study used Polymorphism Phenotyping v2 (PolyPhen-2; http://genetics.bwh.harvard.edu/pph2/index.shtml) and Sorting Intolerant From Tolerant (SIFT; http://sift.bii.a-star.edu.sg/www/SIFT_BLink_submit.html) to predict the effect of any amino acid substitution. The alignment of amino acid sequence in various species was accomplished using Clustal Omega Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/). The chi-squared and Fisher's exact tests were performed using the Statistical Package for Social Sciences version 13.0 (SPSS, USA). *P*-values <0.05 were considered statistically significant. TargetScanHuman6.2 (www.targetscan.org) was used to find microRNA binding sites.

Results

Two novel heterozygous mutations (c.28G>T in exon 1 and c.*158C>T in exon 4) were detected in patient 7, while these mutations were not found in the 42 controls (**Fig. 1A–C**). The former was a missense mutation from G to T at nucleotide position 28 in exon 1, resulting in an alanine/serine substitution at codon 10 (p.A10S). Alanine 10 is a WNT9B residue that is conserved across humans, mice and rats (**Fig. 1D**). Although alanine is replaced by leucine and isoleucine in chicken and zebrafish, respectively, these amino acids belong to the same class as alanine, which is hydrophobic. The latter was an exchange from C to T at the 158th nucleotide from the translation termination codon, which was not in the coding sequence but in the 3′-untranslated region (UTR). TargetScanHuman6.2 predicted that it was within the binding site for miR-3926 and miR-548s.

In addition, this study identified six known single-nucleotide polymorphisms (SNP): rs4968281 in exon 2, rs34072914 in exon 3, rs12602170 in intron 1, rs79877551, rs11654422 and rs11654424 in intron 2, which were present both in the MRKH and control groups. Rs4968281 (c.317T>C) was a missense mutation, leading to a substitution of methionine to threonine (p.M106T). This residue is not conserved at all across different species (**Fig. 1D**). Rs34072914 was a synonymous mutation from G to T (c.399G>T). The incidence rates of SNP were not significantly different between the two groups (Table 2). PolyPhen-2 and SIFT analyses of p.A10S and p.M106T predicted them to be benign.

Discussion

MRKH syndrome is a potentially harmful disease, although it is not life-threatening. Based on the incidence of 1 in 4000–5000 female newborns, there are over 130,000 MRKH patients in China. Such patients lose the ability to engage in sexual intercourse and are infertile. The former condition can be restored in part by vaginoplasty, while the latter is permanently lost. Surrogate IVF can assist this patient population in having children of their own in several countries (e.g. the USA, Belgium and Holland). However, this procedure is forbidden in China and in many other countries for

Table 1 Primers and PCR conditions for *WNT9B* gene amplification.

Primer	*Primer sequence (5′—3′)*	*Product length* (bp)	*Annealing temperature* (°C)
Exon 1F	TGGGGAGCCTCCCAATCTC	380	Variable[a]
Exon 1R	GAACTCCGACTGCGAAGTGG		
Exon 2F	GACCGGAATCTGGAAAACAGG	618	62
Exon 2R	TCTCATTTGGGACCGTGCT		
Exon 3F	GCGGCAGGCAACCTCTAA	460	66
Exon 3R	AGTTCACGGCTCTTCATGTATT		
Exon 4F	GTTCAGTCGCGTAAGTTGTCT	1243	58.3
Exon 4R	TGAACGGATGGCTTCTACC		

[a]Touchdown PCR: variable annealing temperatures in initial 30 cycles: 68°C for the first cycle, decreasing by 0.5°C per cycle, 59°C in the final 10 cycles.

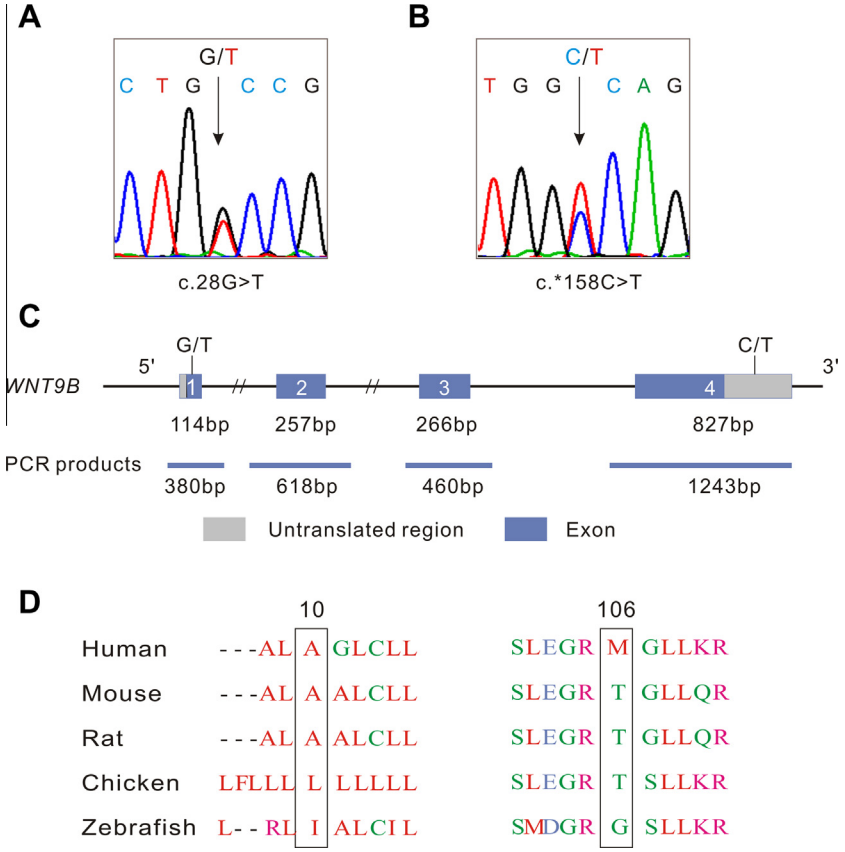

Fig. 1 (**A** and **B**) Chromatograms of two novel heterozygous mutations of the *WNT9B* gene in patient 7: a heterozygous substitution of G with T (c.28G>T, p.A10S) in exon 1 (**A**) and a heterozygous substitution of C with T (c.*158C>T) in exon 4. (**C**) Structure of the *WNT9B* gene and location of the two novel mutations. (**D**) Alignments of wild-type amino acid sequences at the location of the two missense mutations (p.A10S and p.M106T) in various species: alanine 10 is a WNT9B residue that is conserved across humans, mice and rats. Although alanine is replaced by leucine and isoleucine in chicken and zebrafish, respectively, these amino acids belong to the same class as alanine, which is hydrophobic. Methionine 106 is not conserved across different species.

both cultural and ethical reasons. Therefore, determining the aetiology of this syndrome may be the most feasible way to decrease the number of cases, especially in China.

The aetiology of MRKH syndrome remains unclear. Environmental disturbances were initially thought to be possible causes of the condition. However, retrospective analyses of maternal pregnancy histories failed to identify any association between the syndrome and drug use, illness or exposure to known teratogens (Morcel et al., 2007). Several familial occurrences (Griffin et al., 1976; Jones and Mermut, 1972; Kvint and Wilhelmsson, 1988; Tiker et al., 2000) and linked malformations in relatives (Wottgen et al., 2008) suggested that it might be a genetic defect. Because MRKH patients are infertile, linkage analysis cannot be carried out. Several association studies using a candidate-gene approach have been performed (Burel et al., 2006; Clément-Ziza et al.,

Table 2 *WNT9B* variants in Chinese females with MRKH syndrome and controls.

Location	*Variation*	*Genotype frequency* (n)		*Allele frequency* (%)	
		MRKH	*Control*	*MRKH*	*Control*
Exon 1	Novelp.A10S	GG: 41	GG: 42	G: 98.8	G:100
	c.28G>T	GT: 1	GT: 0	T: 1.2	T: 0
Exon 4	Novel	CC: 41	CC: 42	C: 98.8	C: 100
	c.*158C>T	CT: 1	CT: 0	T: 1.2	T: 0
Exon 2	rs4968281	TT: 11	TT: 12	T: 56.0	T: 52.4
	c.317T>C	TC: 25	TC: 20	C: 44.0	C: 47.6
	p.M106T	CC: 6	CC: 10		
Exon 3	rs34072914	GG: 38	GG: 37	G: 94.0	G: 94.0
	c.399G>T	GT: 3	GT: 5	T: 6.0	T: 6.0
	Synonymous	TT: 1	TT: 0		
Intron 1	rs12602170	CC: 18	CC: 21	C: 69.0	C: 70.2
	78−120C>G	CG: 22	CG: 17	G: 31.0	G: 29.8
		GG: 2	GG: 4		
Intron 2	rs79877551	TT: 15	TT: 17	T: 64.3	T: 63.1
	334+86T>C	TC: 24	TC: 19	C: 35.7	C: 36.9
		CC: 3	CC: 6		
Intron 2	rs11654422	GG: 8	GG: 10	G: 53.6	G: 52.4
	334+98G>A	GA: 29	GA: 24	A: 46.4	A: 47.6
		AA: 5	AA: 8		
Intron 2	rs11654424	GG: 3	GG: 7	G: 40.5	G: 41.7
	334+114G>A	GA: 28	GA: 21	A: 59.5	A: 58.3
		AA: 11	AA: 14		

There were no statistically significant differences between the MRKH and control groups.

2005; Drummond et al., 2008a; Drummond et al., 2008b; Lalwani et al., 2008; Liatsikos et al., 2010; Oppelt et al., 2005; Ravel et al., 2009, 2012; Zenteno et al., 2004). However, these studies did not reveal plausible causative mutations. A recent study detected a frameshift mutation of *LHX1* in one out of 62 MRKH patients (Ledig et al., 2012), which is worthy of further identification. In recent years, recurrent copy number variants, such as microdeletions at 17q12 and 22q11.2, have been detected (Ledig et al., 2011; Nik-Zainal et al., 2011). However, direct targets of genes within the fragments have not yet been identified.

This study investigated the underlying cause of MRKH syndrome. Based on the importance of the murine *Wnt9b* gene in Müllerian duct development in mouse models, this gene has become a potential candidate gene for MRKH syndrome in humans. Only one study of the *WNT9B* gene in MRKH syndrome has been published to date. It included 11 Caucasian women and reported a known polymorphism, rs4968281 (Ravel et al., 2009). The current study, however, identified two novel heterozygous mutations in one out of 42 MRKH patients by sequencing of the *WNT9B* coding regions. Both variants were absent in controls and were likely to be causative.

The mutation c.28G>T (exon 1) was located in the hydrophobic core of the N-terminal signal peptide and led to the replacement of hydrophobic alanine by hydrophilic serine. The hydrophobic region of the signal peptide is essential for the transport of secretory proteins into the secretory pathway (von Heijne, 1990). Recent investigations have revealed the effects of signal peptide mutations of other genes on disease states (Beck et al., 2011; Crockett et al., 2011; Mencarelli et al., 2012). The substitution of amino acids in this study might have altered signal peptide hydrophobicity, resulting in a defect in WNT9B protein secretion. Therefore, this novel mutation might be a pathogenic variant of MRKH syndrome.

Another novel mutation, c.*158C>T in exon 4, was located in the 3′-UTR. The 3′-UTR is a regulatory region required for the appropriate expression of many genes, as it can specifically control the nuclear export, polyadenylation status, subcellular targeting and translation and degradation rates of mRNA (Conne et al., 2000). The relationship between mutations in the 3′-UTR and disease pathogenesis has been reported for several disease states (Conne et al., 2000; Sato et al., 2012; Takahashi and Ishiura, 1999). It has been suggested that the *WNT9B* gene and its 3′ conserved noncoding region should be explored for its role in human nonsyndromic cleft lip with cleft palate (Juriloff et al., 2006). One of the 3′-UTR regulatory mechanisms consists of microRNA binding to complementary sequence motifs in the 3′-UTR. TargetScanHuman6.2 suggested that c.*158C>T in *WNT9B* was within the binding site for miR-3926 and miR-548s, both of which have been subject to little or no research. Therefore, this mutation might prevent microRNA from binding the 3′-UTR and the post-transcriptional regulation of gene expression. This study

cannot exclude the possibility that this heterozygous mutation c.*158C>T in *WNT9B* is a causative variant of MRKH syndrome.

It was interesting that the two novel mutations were detected in the same patient (patient 7). Retrospective analysis of her clinical data failed to reveal any particular peculiarities. Because the aetiology of the condition appears to be multifactorial, it could be that the two mutations, if both were causative variants, have a synergistic effect on Müllerian duct development, thereby leading to MRKH syndrome.

Moreover, this study identified six known SNP in both MRKH patients and controls. The only missense mutation, c.317T>C (rs4968281), was once reported in four out of 11 Caucasian women with MRKH syndrome (Ravel et al., 2009). There were no significant differences in the mutation rate or allele frequency of these six SNP between the case and control groups. Thus, these SNP were not likely related to MRKH syndrome.

In summary, this work investigated mutations of the *WNT9B* gene in 42 Chinese females with MRKH syndrome for the first time. The two novel heterozygous mutations were plausibly pathogenic and shed light on the aetiology of MRKH syndrome. Functional studies and further research in a larger cohort are needed.

Acknowledgements

This work was supported by the Hubei Science and Technology Key Project (No. 2010CDA044) and the New Technology Foundation of Tongji Hospital (No. 2012-2-12). The authors are grateful to the patients and volunteers who participated in the study as well as all of their colleagues for their contributions to the physical examinations of patients and the collecting of samples.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 12 March 2013; refereed 14 June 2013; accepted 10 September 2013.