Novel missense mutation in WNT6 in 100 couples with unexplained recurrent miscarriage

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STUDY QUESTION: Do mutations and/or polymorphisms in coding sequences in Wingless-Type MMTV Integration Site Family, Member 6 (WNT6) play a role in unexplained recurrent miscarriage (unexplained RM) in Chinese couples?

SUMMARY ANSWER: We found four mutations in the coding sequences of WNT6 which appear to exist in a small proportion of Chinese women with unexplained RM.

WHAT IS KNOWN ALREADY: WNT6 has been proved to be essential for stromal cell proliferation during decidualization in mice, but in humans WNT6 has not been studied in recurrent miscarriage populations.

STUDY DESIGN, SIZE, DURATION: For this study, 100 couples with unexplained RM (at least three or more unexplained spontaneous miscarriages), and 100 ethnically matched fertile couples (at least one live birth and no history of pregnancy pathologies) were recruited. All the participants were chosen over a 7-year period from the National Research Center for Assisted Reproductive Technology and Reproductive Genetics at Shandong University, Jinan, China.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients were recruited following extensive clinical studies. Genomic DNA was isolated from peripheral blood. Mutation analysis in the coding regions of WNT6 was performed by PCR amplification and DNA sequences testing in all participants. Functional effects of missense variants were predicted using Polyphen-2 and sorting intolerant from tolerant (SIFT).

MAIN RESULTS AND THE ROLE OF CHANCE: Four rare novel mutations, including one missense mutation, were found in intron 1, exon 3 and the 3’ untranslated region of WNT6 in four women with unexplained RM. Gene software predictions showed that the missense mutation in exon 3 could alter the function of WNT6. No mutations or polymorphisms were detected in the male partners of the unexplained RM patients or in the fertile controls. To further validate the findings, we continued to screen this missense mutation site in another 100 peripheral blood samples of normal fertile females, and there was still no positive result.

LIMITATIONS, REASONS FOR CAUTION: There is no direct evidence to validate whether these novel mutations discovered in the present research are related to unexplained RM. Further studies are warranted to investigate the role of WNT6 in unexplained RM, including larger studies in an independent group.

WIDER IMPLICATIONS OF THE FINDINGS: These results provide evidence to suggest the importance of WNT6 in reproductive failure and may support the hypothesis that WNT6 is essential for stromal cell proliferation during decidualization.

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Key words: genetic association / miscarriage / point mutation / missense / WNT6 gene
Introduction

Recurrent miscarriage (RM) is characterized by three or more consecutive spontaneous miscarriages with or without previous live births in early pregnancy. There is no consensus on the number of pregnancy losses needed for RM, but there is a growing movement to offer extensive diagnostic procedures to patients suffering at least two spontaneous abortions (Jauniaux et al., 2006; Alijotas-Reig and Garrido-Gimenez, 2013). Although many different factors related to RM, including chromosomal, anatomical and endocrine aberrations, and infection, have been investigated, >50% of RM cases are still without identifiable factors and labeled as unexplained (unexplained RM).

Epidemiological and genetic studies suggest a multifactorial background for the cause of RM. However, very few large cohort studies utilizing molecular genetics have been undertaken on the parental aspect in humans where the ethnic background is not well characterized. Recent experimental evidence has led to the concept that the decidualized endometrium acts as a biosensor of embryo quality which, if disrupted, may lead to implantation of embryos destined to miscarry (Larsen et al., 2013). Up to now, studies of Wnt signaling have increasingly focused on dynamically influencing endometrial function in human endometrial stromal cells (Tulac et al., 2006; Liu et al., 2010). Inactivation of nuclear Wnt-β-catenin signaling limits blastocyst competency for implantation and decidualization (Xie et al., 2008). It has also been demonstrated in a recent study that Wnt6 plays an important role in stromal cell proliferation during decidualization in mice (Wang et al., 2013).

This study, based on the evidence mentioned above, aims to find out whether Wnt6 plays some kind of role in the context of unexplained RM in the Chinese Han population. The aim is to find a candidate gene to further elucidate the pathogenesis of unexplained RM.

Materials and Methods

The study recruited 100 Chinese Han couples with unexplained RM, who sought treatment for unexplained recurrent miscarriage from the Center for Reproductive Medicine, Provincial Hospital Affiliated to Shandong University, over the period from 2006 to 2013. Recruiting criteria were: couples with a history of three or more unexplained miscarriage within the first trimester of gestation fathered by the same partner. Excluding factors were: abnormal chromosomes in either partner, reproductive duct abnormalities or abnormal female reproductive ducts were also ruled out from the control group by using karyotyping, hysterosalpingography and/or hysteroscopy and/or laparoscopy. Informed consent for molecular studies was obtained from all individuals. Our study was restricted to the Chinese Han ethnic group to minimize any misinterpretation related to ethnic stratification. The study was approved by the Shandong University Hospital Institutional Review Board.

Methods of candidate gene mutation screening have been described in detail previously (Dang et al., 2012). In general, DNA was extracted from peripheral blood samples (Qiaamp DNA Blood Mini Kit; Qiagen, Germany), according to standard procedures. Subsequently, all four exons and exon-intron boundaries of Wnt6 were amplified by polymerase chain reaction (PCR) with specific primers which were designed from the human sequence (Table I). The PCR products were first analyzed by agarose gel electrophoresis (AGE), and then the PCR products were sequenced using the same forward and reverse primers with Applied Biosystems BigDye terminator version 3.1 sequencing kit and run on an automatic genetic sequencer as per the manufacturer’s instructions (ABI 3730 XL; Applied Biosystems, USA). Any novel variant was confirmed by three or four independent PCR amplifications, followed by sequencing in forward and reverse directions.

Results

Four novel variants were identified in Wnt6 in samples from four women with unexplained RM; these included one missense mutation in exon 3, one synonymous mutation in exon 3 one mutation in intron 1, and one mutation in the 3′ untranslated region (Fig. 1 and Table I).

There were no variations, including single-nucleotide polymorphisms, found in any of the control women nor in the male partners of unexplained RM patients.

Gene software predictions showed that the missense mutation in exon 3 could alter the function of Wnt6.

unexplained RM
**Discussion**

This is the largest case–control study in Chinese Han couples with a history of unexplained RM so far, and four novel mutations in WNT6 were identified. One of them is a missense variant which resides in the WNT1 domain; one is located in the 3′ UTR which has an unknown role in unexplained RM; and the other two variations are considered meaningless for DNA transcription. All four mutations were present in maternal samples and no mutations or polymorphisms were detected in male partners of unexplained RM patients or in fertile controls (Fig. 1A and B).

WNT6 is a member of the WNT family which has been identified as having at least nineteen major genes encoding secreted glycoproteins that are involved in intercellular signaling during the process of embryogenesis (Parr et al., 1993; Cadigan and Nusse, 1997; MacDonald et al., 2009). WNT6, located in Chromosome 2, consists of 4 exons and one
main functional domain (WNT1), which encodes the first pan-epidermal Wnt signaling molecule and promotes epithelial remodeling, myogenesis, and epithelial-mesenchymal transformation. It also activates endodermal genes in the endomesoderm gene regulatory network (Schubert et al., 2002; Croce et al., 2011). The novel missense variant we identified causes a Leu148Arg substitution in the WNT1 domain, which is the most important functional region in WNT6. This substitution is most likely to affect protein function given its occurrence at a position that is highly conserved in all mammals (Fig. 1C). Predicting the possible effects of the mutation by using gene function software suggests that the mutation would be an important amino acid substitution in the range of probably damaging in the PolyPhen software with a score of 0.971 and damaging in the SIFT software online with a score of 0.01 (Supplementary Table SII). The other mutation was located in the 3’UTR of WNT6, which may be associated with target traits via binding microRNA to affect the expression of mRNA. Unfortunately, no related reports have been published. The synonymous mutation and intron point mutation, called silent SNPs, may alter the primary and secondary structure and affect the stability of mRNA, consequently, changing translation efficiency (Comeron, 2004). More research of these silent SNPs should be carried out to test their potential effects in WNT6.

Abnormal endometrial development is a contributory factor to RM (Tuckerman et al., 2004). During the early weeks of gestation, uterine stromal cells change into decidualised cells into which the trophoblasts migrate and invade maternal spiral arteries in order to form an interface between maternal and fetal cells. These physiological changes allow the substantial exchange of blood supply to the growing fetus (Mandala and Osol, 2012). Abnormal endometrial receptivity has been thought to be one cause of unexplained RM, because retarded endometrial development in the peri-implantation period and luteal phase defect are associated with RM (Larsen et al., 2013; Weimar et al., 2013). This new insight into RM proposes that endometrial stromal cell proliferation and decidualization are requisite conditions for normal reproductive outcomes beyond embryo karyotypic disorders. In Wnt6 (Wnt6−/−) mutant female mice, the uteri showed a remarkably reduced decidual response compared with wild-type mice, and this Wnt6 deficiency also affected the expression of progesterone synthesis enzymes, 3β-hydroxysteroid dehydrogenase and cytochrome P450 cholesterol side-chain cleavage enzyme, in the mutant mice corpus luteum (Wang et al., 2013).

It is widely accepted that the canonical Wnt–β-catenin pathway is involved in the activation of gene regulatory networks during development and regeneration (Hobmayer et al., 2000; Mohamed et al., 2005; Lengfeld et al., 2009). This pathway is dynamically activated in the uterine compartments in close connection with embryo implantation and decidualization (Daikoku et al., 2004; Hayashi et al., 2009). Moreover, Wnt-β-catenin signaling is a functional player in mediating the functions of progesterone during the progression of human endometrial decidualization (Matsuoka et al., 2010; Macdonald et al., 2011). Therefore, the functions of WNT6 genes might be involved in the outcomes of pregnancy. Although the WNT family consists of 19 genes in mammals, few of these have been studied in the context of RM. In recent progress, research has focused on elucidating WNT6 gene roles in peri-implantation events and the relationship in vitro, but the relationship between Wnt signaling and reproductive mechanisms are still obscure. So far a limited number of the Wnt family knockout mice studies has revealed reproductive-related phenotypes (Chen et al., 2009). Therefore recent studies have found that many Wnt ligands and Wnt signaling-related genes are dynamically expressed in the uterine stroma during the process of uterine decidualization (Daikoku et al., 2004; Hayashi et al., 2007, 2009). This process is defined by mesenchymal-to-epithelial transformation of endometrial fibroblasts into secretory decidual cells (Gellersen et al., 2007). It is apparent that aberrant stromal cells are associated with reproductive disorders, such as endometriosis, adenomyosis or pregnancy loss (Mehasseb et al., 2010; Weimar et al., 2013).

Although RM is sporadic and multifactorial, genetic factors are still a crucial facet to shed light on this disease. Genetically, RM is generally caused by a fetal or maternal chromosomal abnormality. At the molecular level, it is complicated to localize a candidate gene amongst parental genes, because unexplained RM is still considered an idiopathic occurrence. Our study shows one novel mutation within WNT6 that might cause pregnancy loss. It indicates that the mutation likely accounts for unexplained RM in a small subset of couples with unexplained RM.

We have identified a novel missense mutation affecting the functional region of WNT6 in one maternal blood sample with unexplained RM, however, these are preliminary findings in a small group and these findings should be validated in an larger independent group. Additionally, we are no longer able to contact the patient we screened for the missense mutation due to the length of the study; therefore it is impossible to recognize the origin of mutation and its inheritance. On the other hand, although some abortuses are tested for karyotype, and they have been found to be of the normal karyotype, not all RM patients get karyotype tests due to the high costs involved. Further functional studies are necessary to determine the functional significance of the present mutation. This study should be considered as the prelude for further studies on a larger scale in other ethnic groups.
In summary, we discovered four novel variations in WNT6 in different maternal genomic DNA among 100 couples with unexplained RM. There were no mutations in the paternal genomic DNA. Among the four mutations, one is a missense mutation, which changes the encoding of amino acids and the biofunction of the protein, and may alter the function of WNT6. Our findings not only further support the physiological importance of WNT6 in the endometrial decidualization process but also represent an advancement in our understanding of the unexplained RM. This genetics study may indicate an explanation for certain patients with unexplained RM.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles
Y.M.Z., G.Y.L., Y.Y.F., Y.Q.C., and S.X.H. recruited the subjects and conducted the data analysis. G.Y.L., Y.Y.F., Y.Q.C., and S.X.H coordinated and performed the sequencing analysis. Y.M.Z. drafted the manuscript. J.L.M. contributed to the critical revision. Z.-J.C. and J.H.Y. designed the study, supervised the experiments and revised the manuscript. All authors critically reviewed the article and approved the final manuscript.

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Conflict of interest
None declared.

References


