

Original Article

Relationship between *H19* genetic polymorphisms and the risk of unexplained recurrent miscarriage in a Chinese population: a population-based study

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Abstract: Objective: This study was aimed to explore relationship between *H19* genetic polymorphisms (rs2067051, rs2251375, rs217727 and rs4929984) and the risk of unexplained recurrent miscarriage (RM) in a Chinese population. Methods: From June 2011 to April 2014, 312 female patients diagnosed with unexplained RM (RM group) and 357 normal pregnant women without history of spontaneous abortion and abnormal childbearing (control group) were selected. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to detect genetic polymorphisms of rs2067051, rs2251375, rs217727 and rs4929984 in the *H19* gene. Results: The frequency of heterozygote (CT), mutant type (TT) and T allele of rs217727 in the RM group were higher than that in the control group ($P < 0.05$). The subjects with TT genotype had 3.234 times higher risk of unexplained RM than those with wild type (CC) (OR = 3.234, 95% CI = 1.706~6.130), and the subjects with T allele exhibited 1.531 times higher risk of unexplained RM than those with C allele (OR = 1.531, 95% CI = 1.008~2.324). However, rs2067051, rs2251375 and rs4929984 genetic polymorphisms were not associated with the risk of unexplained RM ($P > 0.05$). Logistic regression analysis indicated that GACC haplotype was the protective factor for unexplained RM ($P < 0.05$), while uterine dysplasia, rs217727 polymorphism, GATC and GCTC haplotypes were independent risk factors for unexplained RM ($P < 0.05$). Conclusion: Our data indicated that rs217727 genetic polymorphism in the *H19* gene was an independent risk factor for unexplained RM in a Chinese population.

Keywords: *H19*, recurrent miscarriage, rs2067051, rs2251375, rs217727, rs4929984, genetic polymorphism

Introduction

Recurrent miscarriage (RM) is defined as the loss of three or more consecutive pregnancies, which may occur in approximately 1~3% of couples attempting to bear children [1, 2]. It has been reported that females aged over 35 years have an increased risk of RM, which may bring emotionally and physically injuries for couples [3, 4]. Risk factors for RM are believed to be involved with age, uterine conditions, family history of miscarriage, lifestyle factors, autoimmune diseases, endocrine dysfunctions, acquired and inherited thrombophilia, as well as genetic disorders [5-9]. Late pregnancy complications including intrauterine growth restriction, preterm labor and preeclampsia are also implicated in higher risk of RM [10-12]. However, approximately 50% RM patients fail to be diagnosed, namely unexplained RM, and for many couples with unexplained RM, it may

result in an unrelated finding or unnecessary treatment [13]. Unexplained RM is a challenging condition for both couples and clinicians, and hence, to investigate the nature of unexplained RM and risk factors are essential for treatment of unexplained RM.

H19 is an imprinted gene transcribing a long non-coding RNA, located on chromosome 11p-15.5 in an imprinted region near the *IGF2* gene, and *H19* gene is only expressed in maternally-inherited chromosome [14, 15]. *H19* gene contains 5 exons and 4 introns, with a length of 2.5 kb, which mainly exists in the cytoplasm and functions by regulating RNA or ribose [16]. Surprisingly, *H19* is widely expressed during human embryonic period, mainly in endoderm and ectoderm tissues, and it is involved in the normal development of embryo and differentiation of placental cells [17-19]. *H19* may play an important role in the processes of embryogen-

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Table 1. Genetic polymorphisms of rs2067051, rs2251375, rs217727 and rs4929984 in the *H19* gene

dbSNP	Function	Allele	Allele frequency (CHB)
rs2067051	Missense	G/A	G: 0.7561 A: 0.2439
rs2251375	Missense	C/A	C: 0.5556, A: 0.4444
rs217727	Missense	C/T	C: 0.6463, T: 0.3537
rs4929984	Missense	C/A	C: 0.6951, A: 0.3049

SNP: single nucleotide polymorphism; CHB: Chinese Han Beijing.

Table 2. The primer sequences of rs2067051, rs2251375, rs217727 and rs4929984 in the *H19* gene

SNP	Sequence
rs2067051	5'-AATAATAATGACACGAGGTCCAGATGC-3' 5'-GATACTATGAATTTGGGTTTC-3'
rs2251375	5'-CCA TTT GGT AGT GAG GCA GGT AT-3' 5'-CAC CAT CCA TGT TTG CTT CTG GT-3'
rs217727	5'-CAT GAC TTA GTA GAC AGA TGA-3' 5'-GGT CCA AAC AGG GAA GAA ATA-3'
rs4929984	5'-CCTGAGAGGAGATTGAACTAC-3' 5'-CTTAACTATCAGAGCCTCCA-3'

SNP: single nucleotide polymorphism.

esis, embryoimplantation and growth of embryo, which may be closely related to embryo development [20, 21]. A previous study demonstrated that mutations in *H19* gene may be associated with preeclampsia and trophoblast abnormalities [22]. Also, abnormal expression of *H19* has been found in placental site trophoblastic tumor and gestational choriocarcinoma [23-25], which may largely affect the development of embryo. On the other hand, RM may be correlated with abnormalities of maternally-inherited imprinted genes [26, 27]. In this regard, we hypothesize that *H19* genetic polymorphisms may also function in the risk of unexplained RM. To better understand relationship between *H19* genetic polymorphisms with the risk of unexplained RM, a case-control study is performed to analyze *H19* genetic polymorphisms, including rs2067051, rs2251375, rs217727 and rs4929984, in patients with unexplained RM.

Materials and methods

Ethical statement

All patients were appropriately informed about the current study and given their informed con-

sents forms. The processes of this study were all conducted with the approval of the Ethics Committee of the Affiliated Hospital of Qingdao University.

Study subjects

This study is a retrospective study. Between June 2011 and April 2014, 312 unexplained RM patients diagnosed in genetic center and reproductive department of the Affiliated Hospital of Qingdao University were selected for the RM group. All patients received uterine curettage. Of these 312 patients, 57 cases suffered from 2 times of missed abortion and 255 cases suffered from ≥ 3 times of missed abortion. These patients had menolipsis time ranging from 4 to 21 weeks, with a mean gestational age of 12.37 ± 2.48 weeks. They presented clinical manifestations including a little vaginal bleeding, intrauterine pregnancy confirmed by B-ultrasound, embryo without significant beating heart tube embryo. Inclusion criteria: (1) patients with ≥ 2 times of consecutive abortion as the definition of the American Society for reproductive medicine (ASRM) [28, 29]; (2) no chromosome abnormality or hereditary disease history in couples; (2) no infection with mycoplasma, toxoplasmosis gondii, herpes simplex virus, cytomegalovirus or other virus infections. Exclusion criteria: (1) patients with genital malformations or cervical incompetence; (2) patients with abnormalities of endocrine function including thyroid dysfunction. Also, 357 normal pregnant females who had no history of spontaneous abortion or abnormal reproductive were selected for control group.

Screening for single nucleotide polymorphisms (SNPs)

SNPs in the *H19* gene in a Chinese population was gained from Hapmap databases and data were imported into Haploview 4.2 software. SNPs election was based on the following criterion: $r^2 > 0.8$ and MAF = 0.05. The adjacent SNPs with linkage disequilibrium inconfidence interval (0.70~0.98) were assigned into a same haplotype. Finally, four SNPs, including rs2067051, rs2251375, rs217727 and rs4929984, were identified in the *H19* gene. Mutations of SNPs in the *H19* gene were shown in **Table 1**.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Fasting peripheral venous blood (5 ml) were drawn from each subject in the morning and

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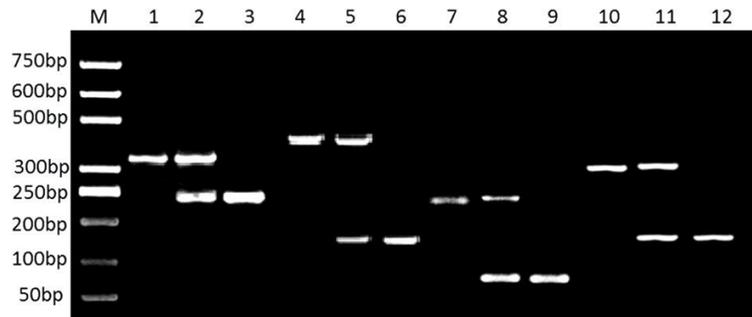


Figure 1. Enzyme digestion identification of *H19* genetic polymorphisms (rs2067051, rs2251375, rs217727 and rs4929984) Note: M, DNA marker; 1-3, the genotypes of rs2067051 after enzyme digestion were GG (339 bp), GA (248/339 bp), AA (248 bp); 4-6, the genotypes of rs2251375 after enzyme digestion were CC (416 bp), CA (152/416 bp), AA (152 bp); 7-9, the genotypes of rs217727 after enzyme digestion were CC (236 bp), CT (62/236 bp), TT (62 bp); 10-12, the genotypes of rs217727 after enzyme digestion were CC (296 bp), CA (112/296 bp), AA (112 bp).

Table 3. Comparison on baseline characteristics of subjects between the RM and control groups

Characteristic	RM group (n = 312)	Control group (n = 357)	P
Age	28.66 ± 5.01	28.07 ± 3.41	0.073
BMI	22.20 ± 3.64	22.11 ± 3.78	0.755
Smoking history (cases, %)	22 (7.05)	31 (8.68)	0.436
Menstrual cycle	28.43 ± 4.21	29.03 ± 3.99	0.059
Single gestation sac (cases, %)	281 (90.06)	329 (92.16)	0.341
Uterine dysplasia (cases, %)	44 (14.10)	21 (5.88)	0.001
Coagulation disorder (cases, %)	37 (11.86)	25 (7.00)	0.031

RM: recurrent miscarriage; BMI: body mass index.

put into ethylene diamine tetraacetic acid (EDTA)-contained tubes. Genomic DNA was extracted using phenol-chloroform method and stored at -20°C. PCR-RFLP was used to determine the genetic polymorphisms, including rs-2067051, rs2251375, rs217727 and rs4929984, in the *H19* gene. PCR primers of these SNPs were designed by Primer Premier Software (version 5.0; Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Shanghai Sangon Biological Engineering Technology Co., Ltd. (Shanghai, China). The primer sequences were listed in **Table 2**. The PCR reaction system (15 µL) contained 1.5 µL 10 × PCR buffer, 0.3 µL dNTPs (10 mmol/L), 0.25 µL upstream primer (10 pmol/µL), 0.25 µL downstream primer (10 pmol/µL), 0.25 µL Taq polymerase (5 µ/µL, TaKaRa), 1 µL template and ddH₂O supplemented to a 15 µL volume. Reaction conditions of PCR were as follows: 94°C

initial denaturation for 5 min, 94°C denaturation for 30 s, 60°C annealing for 30 s, and 72°C extension for 30 s. After 35-cycles of amplification, additional extensions were performed at 72°C for 10 min. During each cycle of PCR reaction, a negative control was set by replacing the template with sterile ddH₂O. PCR products (3 µl) were mixed with 6 × Loading Buffer, separated using 3% agarose gel electrophoresis in 100 V for 15 min and stained by Ethidium bromide (EB). The results were analyzed by gel imaging system. PCR products (6 µl), supplemented with 1.5 µL 10 × enzyme digestion buffer and 7.5 µL sterile ddH₂O, were subjected to restriction enzyme digestion in a 37°C water bath for 16 hours. The restriction enzyme digestion stopped and the product was separated by 3% agarose gel electrophoresis. After EB staining, the results were analyzed by gel imaging system. During each cycle of restriction enzyme digestion, a negative control was set to ensure accuracy of enzyme digestion. Specific restriction enzymes of HincII, XspI, EcoI and AseI (Takara Biotechnology Ltd., Dalian, China) were used to identify specific SNPs of PCR products, and gel electrophoresis was used to detect PCR fragment. An automated DNA sequencer (ABI370; Applied Biosystems, ABI Company, Oyster Bay, NY) was used to determine genotypes of SNPs in the *H19* gene (**Figure 1**).

Statistical method

SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, in which measurement data was expressed as mean ± standard deviation. Also *t* test was used in comparison between groups, and these two groups should be characterized by normal distribution. Categorical data was expressed as ratio or percent and examined using Chi square

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Table 4. Comparison on genotype and allele frequency of rs2067051, rs2251375, rs217727 and rs4929984 in the H19 gene between the RM and control groups

Genotype/Allele	RM group (n = 312) [n (%)]	Control group (n = 357) [n (%)]	P	OR	95% CI	P ^a	OR ^a	95% CI ^a
rs2067051								
GG	195 (62.82)	205 (57.42)		1 (Ref.)	1 (Ref.)		1 (Ref.)	1 (Ref.)
GA	96 (30.45)	122 (33.89)	0.262	0.827	0.594~1.153	0.384	0.826	0.538~1.269
AA	21 (6.73)	30 (8.68)	0.308	0.736	0.407~1.329	0.468	0.754	0.351~1.617
G	486 (78.04)	532 (74.37)		1 (Ref.)	1 (Ref.)		1 (Ref.)	1 (Ref.)
A	138 (21.96)	182 (25.63)	0.149	0.83	0.644~1.069	0.063	0.662	0.429~1.023
rs2251375								
CC	122 (39.10)	132 (36.97)		1 (Ref.)	1 (Ref.)		1 (Ref.)	1 (Ref.)
CA	134 (42.95)	156 (43.70)	0.671	0.929	0.663~1.303	0.441	0.847	0.555~1.292
AA	56 (17.95)	69 (19.33)	0.553	0.878	0.571~1.350	0.964	0.987	0.547~1.997
C	378 (60.58)	420 (58.82)		1 (Ref.)	1 (Ref.)		1 (Ref.)	1 (Ref.)
A	246 (39.42)	294 (41.18)	0.514	0.93	0.747~1.157	0.972	0.99	0.547~1.792
rs217727								
CC	125 (40.06)	214 (59.94)		1 (Ref.)	1 (Ref.)		1 (Ref.)	1 (Ref.)
CT	134 (42.95)	117 (32.78)	< 0.001	1.961	1.407~2.733	0.01	1.67	1.129~2.470
TT	53 (16.99)	26 (7.28)	< 0.001	3.49	2.077 to 5.862	< 0.001	3.234	1.706~6.130
C	384 (61.54)	545 (76.33)		1 (Ref.)	1 (Ref.)		1 (Ref.)	1 (Ref.)
T	240 (38.46)	169 (23.67)	< 0.001	2.016	1.591-2.553	0.046	1.531	1.008~2.324
rs4929984								
CC	158 (50.64)	182 (49.30)		1 (Ref.)	1 (Ref.)		1 (Ref.)	1 (Ref.)
CA	119 (38.14)	135 (37.82)	0.927	1.015	0.733~1.407	0.414	1.189	0.785~1.081
AA	35 (9.80)	40 (12.89)	0.975	0.992	0.601~1.638	0.562	0.808	0.393~1.662
C	435 (69.71)	499 (68.21)		1 (Ref.)	1 (Ref.)		1 (Ref.)	1 (Ref.)
A	189 (30.29)	215 (31.79)	0.944	0.9917	0.785~1.253	0.174	0.746	0.489~1.138

RM: recurrent miscarriage; OR: odd ratio; 95% CI: 95% confidence interval; Ref.: reference; a, a corrected value in the logistic regression analysis; the correction factors include age, body mass index, smoking history, menstrual cycle and single gestation sac.

test. And Hardy-Weinberg equilibrium (HWE) was performed for odds ratio (OR) with 95% confidence region (CI) of genotype and allele. Unconditional logistic regression analysis was carried out for estimation of independent risk factors of unexplained RM. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.

Results

The comparison on baseline characteristics of subjects between the RM and control groups

The patients in the RM group had mean age of 28.66 ± 5.01 years, aged ranging from 18 to 45 years, and the normal pregnant females in the control group had mean age of 28.07 ± 3.41 years, aged ranging from 18 to 38 years. There were no significant difference in age, body mass index (BMI), smoking history, menstrual cycle and numbers of gestational sac between two groups ($P > 0.05$). While significant

differences were observed regarding the incidence of coagulation disorder and uterine dysplasia between the RM group and the control group ($P < 0.05$), as shown in Table 3.

Frequency distribution of H19 gene polymorphisms

The genotype frequency in the RM group and the control group were in accord with the Hardy-Weinberg (HWE) equilibrium with confirmation of chi-square test (RM group: rs2067051: $P = 0.059$; rs2251375: $P = 0.075$; rs217727: $P = 0.102$; rs4929984: $P = 0.087$; control group: rs2067051: $P = 0.058$; rs2251375: $P = 0.064$; rs217727: $P = 0.079$; rs4929984: $P = 0.055$). The results indicated that no significant difference was observed in genotype and allele frequency of rs2067051, rs2251375 and rs49299843 between the RM group and the control group ($P > 0.05$). The frequency of heterozygote (CT), mutant type (TT) and T allele of rs217727 in the RM group were higher than that in the

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Table 5. Haplotype analysis of rs2067051, rs2251375, rs217727 and rs4929984 in the H19 gene in the RM and control groups

Haplotype				RM group (n = 312)	Control group (n = 357)	P	OR	95% CI
rs2067051	rs2251375	rs217727	rs4929984					
A	A	C	C	13 (4.16)	19 (5.32)	0.176	0.693	0.407~1.181
A	C	C	A	8 (2.56)	13 (3.64)	0.329	0.734	0.393~1.369
A	C	C	C	19 (6.09)	26 (7.28)	0.387	0.827	0.537~1.273
A	C	T	C	13 (4.17)	12 (3.36)	0.509	1.209	0.688~2.122
G	A	C	A	23 (7.37)	28 (7.84)	0.715	0.926	0.615~1.396
G	A	C	C	39 (12.5)	60 (16.81)	0.023	0.701	0.515~0.954
G	A	T	A	11 (3.53)	9 (2.52)	0.278	1.418	0.752~2.672
G	A	T	C	27 (8.65)	14 (3.92)	0.001	2.211	1.383~3.534
G	C	C	A	25 (8.01)	32 (8.96)	0.299	0.815	0.553~1.200
G	A	C	A	64 (20.51)	86 (24.09)	0.111	0.809	0.623~1.051
G	A	C	C	18 (5.77)	16 (4.48)	0.282	1.307	0.802~2.130
G	A	T	A	37 (11.86)	20 (6.41)	< 0.001	2.302	1.540~3.441

RM: recurrent miscarriage; OR: odd ratio; 95% CI: 95% confidence interval.

Table 6. The logistic regression analysis for risk factors of unexplained RM

Risk factor	B	OR value	95% CI	P value
Uterine dysplasia	1.364	3.539	1.731-7.235	0.001
Coagulation disorder	0.718	2.050	0.998-4.210	0.051
rs217727	0.577	1.780	1.286-2.465	0.001
GATC	-0.857	0.424	0.190-0.950	0.037
GACC	-1.433	0.239	0.112-0.507	< 0.001
GCTC	2.501	12.197	4.609-32.281	< 0.001

RM: recurrent miscarriage; OR: odd ratio; 95% CI: 95% confidence interval.

control group ($P < 0.05$). The subjects with TT genotype had 3.234 times higher risk of unexplained RM than those with wild type (CC) (OR = 3.234, 95% CI = 1.706~6.130), and the subjects with T allele exhibited 1.531 times higher risk of unexplained RM than those with C allele (OR = 1.531, 95% CI = 1.008~2.324) (Table 4).

Haplotype analysis of rs2067051, rs2251375, rs217727 and rs4929984 in the H19 gene

The haplotypes of rs2067051, rs2251375, rs217727 and rs4929984 were presented in Table 5. Different haplotype frequency in the RM group and the control group was analyzed by the software Shesis. The haplotype analysis indicated that GATC and GCTC haplotype frequency in the RM group were higher than that in the control group ($P < 0.05$), but GACC haplotype frequency in the RM group was lower than that in the control group ($P < 0.05$). However,

no significant difference was observed concerning the frequency of other haplotypes (AACC, ACCA, ACCC, ACTC, GACA, GACC, GATA, GCCA, GCCC and GCTA) between the two groups ($P > 0.05$).

Logistic regression analysis for risk factors of unexplained RM

With unexplained RM as dependent variable and with age, BMI, smoking (yes/no), menstrual cycle (yes/no), single gestation sac (yes/no), uterine dysplasia (yes/no), coagulation disorder (yes/no), rs217727 genotype (CC, CT and TT), GATC haplotype (yes/no), GACC haplotype (yes/no), and GCTC haplotype (yes/no) as independent variables, logistic regression analysis showed that GACC haplotype was a protective factor of unexplained RM ($P < 0.05$), while uterine dysplasia, rs217727 polymorphism, GATC and GCTC haplotypes were independent risk factors of unexplained RM ($P < 0.05$) ($P < 0.05$). rs217727 polymorphism increased the risk of unexplained RM by 1.856 times (as shown in Table 6).

Discussion

In the present study, we evaluated the association between H19 genetic polymorphisms and the risk of unexplained RM. Our study results implied that H19 rs217727 polymorphism may be correlated with an increased risk of unex-

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plained RM, while the other three SNPs (rs-2067051, rs2251375 and rs49299843) in the *H19* gene do not confer susceptibility to unexplained RM, suggesting *H19* gene, especially rs217727, may serve as a potential indicator of the increased risk of unexplained RM.

Initially, we found that the frequency of TT genotype, CT genotype and T allele of rs217727 was significantly higher in the patients with unexplained RM than that in the normal pregnant females. These results suggested that *H19* rs217727 polymorphism may be associated with an increased risk of unexplained RM. Currently, there is no effective treatment for females with unexplained RM, and each additional loss may worsen the prognosis for pregnancy and increase physical and psychological risks of unexplained RM for couples, especially for mothers [30, 31]. Previous studies have showed that the *H19* gene encodes a functional non-coding RNA suppressing growth and is abundantly expressed in human placenta, which is implicated in the pathogenesis of congenital growth disorders such as Beckwith-Wiedemann and Silver-Russell syndromes [32-34]. In some cases, the biallelic expression of *H19* gene may exist at early stage of females with normal pregnancy, while it may alter into mono-allelic expression around 10 weeks of gestation, which may regulate the maintenance of normal pregnancy [23, 35]. Further, the silence of *H19* gene imprinting in the placental tissues of the patients with preeclampsia may be correlated with severe hypertension, which may contribute to the development of preeclampsia and influence the fetal growth [23]. Koukoura *et al.* have demonstrated that hypomethylation combined with an increased expression of *H19* in placentas in pregnant females may be associated with the risk of fetal growth restriction [24], which may largely affect the fetal development. In this regard, we suspected that the *H19* polymorphisms may be also associated with the risk of unexplained RM.

We also found that patients with TT genotype of rs217727 had a higher risk of unexplained RM than those with CC genotype, and patients with T allele had an elevated risk of unexplained RM as compared to those with C allele. And also, the haplotype analysis clarified that ACTC and GCTC haplotypes were associated with the risk of unexplained RM. Additionally, logistic regres-

sion analysis showed that rs217727 polymorphism, ACTC and GCTC haplotypes were independent risk factors of unexplained RM. These results suggested that the subjects with TT genotype and T allele of rs217727 may be closely associated with the risk of unexplained RM, and the ACTC and GCTC haplotypes may act as indicators for the development of unexplained RM. *H19* rs217727 polymorphism has been reported to be strongly correlated with the methylation of multiple CpG sites within the *IGF2* gene and *H19* differentially methylated regions [36]. The presence of T alleles at *H19* rs217727 polymorphism in females may be positively associated with birth size, and the genotypes may be associated with fetal growth and risk for other pregnancy complications [37]. Recently, a previous study has demonstrated that the TT genotype of *H19* rs217727 polymorphism in pregnant females may be correlated with the birth weight of fetal [38]. Consequently, we propose that patients with rs217727 polymorphism, especially with TT genotype and T allele, were at high risk of unexplained RM. However, Ostojić *et al.* have suggested that genotype and allele frequency of *H19* polymorphism had no significant difference on RM patients and fertile females, implying that the *H19* polymorphism may be not associated with the risk of RM [39]. On the contrary, our study results declared *H19* polymorphism may be correlated with an increased risk of unexplained RM. We suspect geographic differences, various lifestyle or racial characteristics of different populations may largely affect the distribution of the *H19* gene polymorphism.

In conclusion, our study demonstrates that *H19* rs217727 polymorphism may be correlated with an increased risk of unexplained RM in a Chinese population, and the TT genotype and T allele of rs217727 polymorphism may be the risk factors for unexplained RM. Thus this study may provide a therapeutic target for early intervention of unexplained RM. Additionally, future investigations using larger patient sample-size are needed to support positive associations between *H19* polymorphisms and an increased risk of unexplained RM.

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Disclosure of conflict of interest

None.

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