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Association of the lncRNA HOTAIR Gene rs4759314 with Susceptibility to Recurrent Spontaneous Abortion in a Chinese Han Population

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This study was to examine the association between the long noncoding RNA (lncRNA) HOTAIR polymorphism (rs4759314, rs4759314) and the risk of recurrent spontaneous abortion (RSA) in Chinese Han women. 378 women with RSA and 361 pregnant women with normal pregnancies were selected as study subjects. The expression level of lncRNA HOTAIR was detected by fluorescence quantitative PCR. PCR restriction fragment length polymorphism (PCR-RFLP) method was used to detect genotyping. Logistic regression analysis was used to assess the independent factors of HOTAIR rs4759314 affecting recurrent miscarriage. The lncRNA HOTAIR gene expression level was significantly reduced in RSA patients. Rs1899663 polymorphism and rs4759314 GG genotype do not significantly increase the risk of developing RSA ($P > 0.050$). Rs4759314 AG genotype and rs4759314G allele significantly increased the risk of developing RSA ($P < 0.050$). Logistic analysis showed a significant correlation between rs4759314 and the occurrence of RSA (OR = 0.367, 95% CI = 0.232-0.580, $P < 0.050$). The lncRNA HOTAIR rs4759314 locus may serve as a biomarker for RSA susceptibility in the Chinese Han population.

Keywords: long noncoding RNA HOTAIR; recurrent spontaneous abortion; rs4759314; single nucleotide polymorphisms
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Introduction

Spontaneous abortion represents the most prevalent complication of early pregnancy, with an incidence of approximately 15%-25% of all pregnancies (Practice Committee of the American Society for Reproductive Medicine 2012). Recurrent spontaneous abortion (RSA) is defined as two or more consecutive spontaneous abortions with the same sexual partner, including consecutive biochemical pregnancies, with a prevalence of 1% to 5% in women of reproductive age (Barbaro et al. 2023). The risk of RSA increases with the number of miscarriages and with age (Van Der Ven 2015). A second pregnancy after RSA increases the risk of pregnancy complications and even infertility (Cozzolino et al. 2019). The disease of RSA is very complex and the known etiologic factors include chromosomal abnormalities, abnormal uterine morphology and endocrine abnormalities, infectious diseases, autoimmune diseases and environmental factors. However, there are still about 50% to 60% of RAS patients without any detectable etiology (Liu et al. 2021).

Long noncoding RNA (lncRNA) is RNA that is greater than 200 nucleotides in length and lacks protein-coding capacity (Zhang and Wang 2022). lncRNA has been shown to play important roles in various cellular functions, including influencing pluripotency and differentiation of embryonic stem cells, regulating apoptosis and the cell cycle, as well as in the origins, growth, evolution, selection, and other aspects of human life, evolution, selection and other important functions (Kopp and Mendell 2018). Huang et al. (2018) found that the number of differentially expressed lncRNAs in the chorionic tissues of the RSA patient group was significantly higher than that of the control group. HOTAIR is a member of the homeobox superfamily. This lncRNA is reverse transcribed from the HOXC locus and contains 2,158 nucleotides on chromosome 12q13.13

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(Zhang et al. 2016). HOTAIR has long been considered to be associated with the development of cancer. Recently, it has been demonstrated that the HOTAIR gene is fully expressed in placental tissues. It has been demonstrated that the HOTAIR gene is downregulated in women with RSA compared to healthy women (Che et al. 2019).

HOTAIR single nucleotide polymorphisms (SNPs) are able to regulate gene expression. Single nucleotide variants of HOTAIR have been found to be associated with recurrent failure of implantation and primary ovarian insufficiency (Jung et al. 2016; Cho et al. 2021). An Iranian study showed that HOTAIR may be involved in the pathologic process of RSA and could be considered as a potential therapeutic target for placental diseases. Variations in the HOTAIR gene may be a useful biomarker in determining susceptibility to RSA (Salimi et al. 2021). A Korean study showed that wild-type HOTAIR may have a potential protective effect against RSA (Park et al. 2022).

These studies suggest that HOTAIR gene polymorphisms may be associated with RSA. However, the relationship between HOTAIR gene polymorphisms and RSA susceptibility in the Chinese Han population has not been reported. The association between LncRNA HOTAIR gene polymorphisms and RSA susceptibility in the Chinese Han population, as well as the specific LncRNA HOTAIR genotypes that increase the risk of developing RSA, remain unknown. Therefore, this study was conducted with 378 hospitalized cases and 361 controls, aiming to investigate the relationship between HOTAIR gene polymorphisms (rs4759314, rs1899663) and RSA in China, with a view to obtaining a new biomarker of RSA susceptibility in the Chinese Han Chinese population, and to provide a preliminary theoretical basis for the prediction and diagnosis of RSA.

Materials and Methods

Ethical statement

All subjects signed an informed consent form before enrollment. The study was approved by the Longyan First Affiliated Hospital of Fujian Medical University and strictly followed the principles of the Declaration of Helsinki.

Study subjects

A total of 378 women with RSA attending Longyan First Affiliated Hospital of Fujian Medical University from 2020 to 2023 were selected as the experimental group for the study, and another 361 normal pregnant women with age-matched pregnancies who underwent health checkups in the hospital were selected as the control group. The study subjects were all Chinese Han Chinese from Fujian, Jiangxi or Guangzhou provinces. The term Chinese Han population was used as a uniform abbreviation. The diagnostic criteria for RSA were two or more spontaneous miscarriages by the same husband, and normal pregnancies with regular menstruation in the control group.

Exclude patients with a history of alcohol consumption; exclude patients with a history of smoking; patients with any history of pelvic surgery, cancer or genetic syndromes were excluded. Healthy controls with unexplained infertility in family were excluded. Exclude study subjects with metabolic disorders, autoimmune diseases, hypertension, endocrine disorders, arteriovenous thrombosis, uterine abnormalities, hepatic or renal dysfunction, or chromosomal abnormalities in the embryo.

Diastolic blood pressure (DBP) and systolic blood pressure (SBP) were measured with an Ormon Hem-7136 monitor. High-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), and triglycerides (TG) were measured using a Roche automated biochemistry analyzer. The patients' fasting blood sugar (FBG) were examined by the hexokinase method using a Beckman automatic biochemistry analyzer AU5811 provided by Beckman Coulter Systems, Germany.

Clinical samples collection

Fasting venous blood was taken 5 mL, centrifuged at 4°C, 3,000 *g* for 15 min, and the supernatant was placed in a ribonuclease-free EP tube and stored at −80°C.

Genotyping

The HOTAIR rs4759314 and rs1899663 gene polymorphisms were identified through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). DNA quality was examined using agarose gel electrophoresis and UV gel imaging system, and qualified samples with DNA sample concentration between the range of 20-20 ng/μL and OD260/280 values between the range of 1.7-2.0 were selected for subsequent genotyping experiments. The total volume of the PCR reaction was 20 μl, comprising 10 μl of DNA polymerase 2× master mix, 1 μl of DNA template, 1 μl of each of the upstream and downstream primers, and 7 μl of distilled water. The PCR cycling conditions were as follows: an initial denaturation at 95°C for 5 minutes. A total of 35 cycles were conducted: the temperature was maintained at 95°C for 40 seconds, followed by an annealing step at 55°C for 40 seconds and an extension step at 72°C for 40 seconds. The final extension step was allowed to run for 15 minutes. The final extension at 72°C for 15 minutes was placed at 4°C for storage. Polymorphisms of rs4759314 and rs1899663 were identified through the use of AluI and MboI restriction endonucleases, respectively. The resulting digestion products were then separated by electrophoresis on a 2% agarose gel for DNA staining. A random selection of 10% of the samples was subjected to PCR analysis, with the results being found to be consistent.

Real time PCR

Detection of relative expression of lncRNA HOTAIR in serum. Total RNA was extracted from the serum of all study subjects and reverse transcribed into cDNA. RNA concentration and quality using the Nano-Drop2000 instru-

ment. cDNA was used as a template and detected using a fluorescence quantitative PCR instrument. Detection was performed using Applied Biosystems 7500 Real-Time PCR system (Thermo, USA). The reaction conditions were predenaturation at 95℃ for 5 min, cycling conditions were denaturation at 95℃ for 10 s, annealing at 60℃ for 30 s, and extension at 72℃ for 34 s, with a total of 40 cycles. LncRNA HOTAIR forward primer: 5′-GTGGTGCTGACAAAGCTTGGAA-3′, reverse primer: 5′-TCACTGGGTGCCATCGTAAGAA-3′; GAPDH forward primer: 5′-GGAGCGAGATCCCTCCAAAAT-3′, reverse primer: 5′-GGCTGTTGTCATACTTCTCATGG-3′. The primers were synthesized by Beijing RuiBiotech Biotechnology Co. And the relative expression level of lncRNA HOTAIR was calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as an internal reference.

Data analysis

SPSS 23.0 software was used to analyze the experimental data of this study. The data were expressed as mean ± standard deviation (x ± s), and an independent samples t-test was employed to compare the data between the two groups. Count data were expressed using examples, and the $\chi 2$ test was used to assess the role of gene frequencies of the lncRNA HOTAIR gene polymorphism in the development of RSA. A logistic regression analysis was conducted to assess the potential of lncRNA HOTAIR rs4759314 to act as an independent factor in the occurrence of RSA. $P < 0.05$ was considered a statistically significant difference.

Results

Comparison of baseline data of enrolled subjects

Clinical baseline indicators of the subjects were analyzed during the study. The mean age of the 378 patients with RSA included was 27.15 ± 2.02 years and the gestational week was 7.08 ± 1.01 weeks. During the same period, 361 women with normal pregnancies, with a mean age of 27.06 ± 1.92 years, who had a medical examination at our hospital were selected as healthy controls. The gestational week of the healthy control group was also counted and was 6.97 ± 1.03 weeks. There was no significant difference in age, gestational week, BMI, HDL, LDL, TG, SBP, DBP and FBG between the control group and the RSA patient group ($P > 0.050$). The mean number of miscarriages in the RSA patients was 3.94 ± 0.83, which was significantly higher than that of the healthy control group ($P < 0.050$). The expression level of lncRNA HOTAIR in the RSA patient group was significantly lower than that of the healthy control group ($P < 0.050$, Table 1).

Serum lncRNA HOTAIR expression levels in in RSA patients and healthy controls

In this study, the expression level of serum lncRNA HOTAIR was examined by fluorescence quantitative PCR in all groups of enrollees. Reduced levels of lncRNA HOTAIR expression were observed in patients with RSA compared with healthy pregnant women (Fig. 1A), and the difference was significant ($P < 0.0001$).

Genotypes and allele frequencies of lncRNA HOTAIR rs4759314 and rs1899663 in RSA patients

Table 2 presents the genotypes and gene frequencies of the lncRNA HOTAIR polymorphisms in the RSA patient group and the healthy control group. The Hardy-Weinberg equilibrium (HWE) test was conducted on the frequency distributions of the two loci between the groups, and the values of P^{HWE} were found to be greater than 0.05, indicating that the samples were representative, and the group survey information was reliable for subsequent analysis. The typing results demonstrated the presence of three genotypes at the rs1899663 locus of the lncRNA HOTAIR gene: GG (66.21%), GT (31.86%) and TT (1.94%). The frequencies of alleles G and T were 82.13% and 17.87%, respectively.

Table 1. General information of the enroll participants.

Parameters	Controls Group (n =361)	RSA Group (n = 378)	*P values*
Age (years)	27.06 ± 1.92	27.15 ± 2.02	0.153
Gestation (weeks)	6.97 ± 1.03	7.08 ± 1.01	0.773
BMI (kg/m^2)	22.84 ± 2.83	23.14 ± 2.89	0.776
Miscarriages	1.03 ± 0.74	3.94 ± 0.83	0.022
HDL (mmol/L)	1.16 ± 0.16	1.16 ± 0.15	0.421
LDL (mmol/L)	2.08 ± 0.43	2.11 ± 0.40	0.489
TG (mmol/L)	1.27 ± 0.27	1.27 ± 0.26	0.444
SBP (mmHg)	112.78 ± 9.57	112.44 ± 9.97	0.375
DBP (mmHg)	73.17 ± 4.29	73.31 ± 4.57	0.156
FBG (mmol/L)	4.51 ± 0.32	4.50 ± 0.30	0.479
HOTAIR	1.01 ± 0.24	0.50 ± 0.13	0.000

BMI, body mass index; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; TG, triglyceride; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose.

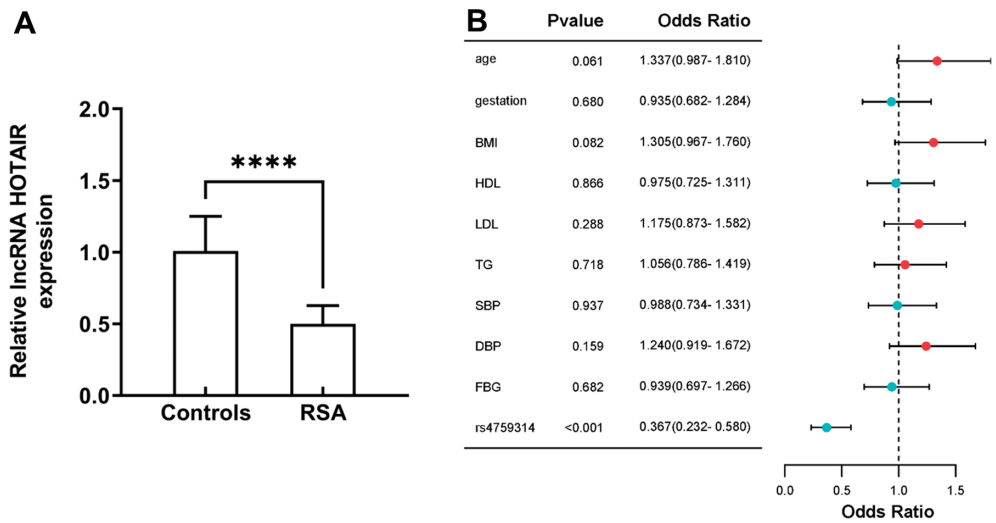

Fig. 1. Expression and significant of HOTAIR in the onset of RSA.
(A) Relative expression levels of serum lncRNA HOTAIR gene. (B) Logistic regression analysis to assess the association between rs4759314 and RSA.

Table 2. The genotype and allele frequencies of lncRNA HOTAIR polymorphisms.

Genotype/Allele	Controls Group (n = 361)	RSA Group (n = 378)	OR (95% CI)	*P*
Rs1899663 (n, %)				
G/G	239 (66.21)	220 (58.20)	1	-
G/T	115 (31.86)	129 (35.44)	1.219 (0.893-1.663)	0.213
T/T	7 (1.94)	15 (4.12)	2.328 (0.932-1.080)	0.063
G	593 (82.13)	569 (78.16)	1	-
T	129 (17.87)	159 (21.84)	1.285 (0.991-1.665)	0.058
P^{HWE}	0.10	0.47		
Rs4759314 (n, %)				
AA	331 (91.69)	302 (79.89)	1	-
AG	28 (7.76)	74 (19.58)	2.897 (1.825-4.598)	< 0.001
GG	2 (0.55)	2 (0.53)	1.096 (0.153-7.829)	0.927
A	690 (95.57)	678 (89.68)	1	-
G	32 (4.43)	78 (10.32)	24.743 (17.107-35.788)	< 0.001
P^{HWE}	0.11	0.26		

The rs4759314 locus demonstrated the presence of AA (91.69%), AG (7.76%), and GG (0.55%) genotypes, respectively. The allele frequencies were A (95.57%) and G (4.43%), respectively.

The most frequent genotypes in the rs1899663 locus of the lncRNA HOTAIR gene in the healthy women's population (n = 361) and in the RSA patients (n = 378) were GG (66.21%) and GG (58.20%), followed by GT (31.86%) and GT (35.44%), respectively. The alleles with the highest frequencies were G (82.13%) and G (78.16%), respectively. In addition, we observed that both rs1899663 GT polymorphism (OR = 1.219 [0.893-1.663]) and rs1899663 TT polymorphism (OR = 2.328 [0.932-1.080]) increased the risk of RSA prevalence, but the difference was not significant ($P > 0.050$). For those carrying the T allele of rs1899663 (OR = 1.285 [0.991-1.665]), the risk of developing the disease was 1.29 times higher than for those carrying the G allele, but the difference was not significant ($P > 0.050$).

In the rs4759314 locus of the lncRNA HOTAIR gene in both study subgroups, the most frequent genotypes were AA (91.69%) and AA (79.89%), followed by AG (7.76%) and AG (19.58%). The alleles with the highest frequencies were A (95.57%) and A (89.68%). rs4759314 AG polymorphism (OR = 2.897 [1.825-4.598]) significantly increased the risk of prevalence of RSA, which was 2.90 times higher than that of the rs4759314 AA polymorphism ($P < 0.050$). The rs4759314 GG polymorphism (OR = 1.096 [0.153-7.829]) also slightly increased the risk of developing RSA, 2.90 times that of the rs4759314 AA polymorphism, but not significantly ($P > 0.050$). Women carrying the G allele of rs4759314 (OR = 24.74 [17.107-35.788]) were 24.74 times more at risk of developing RSA than women carrying the A

allele, a significant difference ($P < 0.050$).

Logistic assessment rs4759314 independent factors affecting recurrent abortion

Binary logistic regression analysis showed a significant association between rs4759314 and the occurrence of RSA (OR = 0.367, 95% CI = 0.232-0.580, $P < 0.050$, Fig. 1B).

Discussion

RSA seriously affects women's reproductive health. The etiology of RSA disease is very complex and still about 50% to 60% of patients with RAS are undetectable for any etiology (Liu et al. 2021). This may be related to functional or non-functional SNPs in specific genes (Daher et al. 2012). LncRNAs affect pluripotency and differentiation of embryonic stem cells. SNPs account for more than 1% of the genetic variation in the human genome and are common genetic variants. Similarly, SNPs in lncRNAs exhibit analogous functions to those observed in SNPs in protein-coding genes. They play a pivotal role in regulating gene expression and disease susceptibility. SNPs in lncRNAs affect the expression, function, and structure of lncRNAs by interfering with the binding of transcription factors. SNPs may also disrupt the association between miRNAs or proteins and lncRNAs. Consequently, SNPs can affect the susceptibility to diseases such as cancer (Abdi and Latifi-Navid 2022). HOTAIR is one of the lncRNAs involved in genome modification and was the first lncRNA found to have a trans-regulatory role. HOTAIR plays an important role in many diseases, such as breast, liver, pancreatic, cervical, and gastric cancers, etc. (Kim et al. 2015; Han et al. 2018; Wang et al. 2018a,b; Wu et al. 2018). HOTAIR affects tumor proliferation and metastasis (Elsayed et al. 2018). Gupta et al. (2010) showed that the expression of HOTAIR was significantly higher in gastric cancer tissues than in paracancerous tissues. There was a genotype-specific effect between the SNP of HOTAIR and its high expression (Guo et al. 2015). Rs920778 pure TT genotype with the T allele has been associated with an increased risk of several cancers (Zhang et al. 2014; Guo et al. 2016; Pan et al. 2016; Qiu et al. 2016; Li et al. 2017), but Hassanzarei et al. (2017) showed that that the rs920778 C allele variant was significantly associated with an increased risk of breast cancer. This may be due to the fact that the frequency of the HOTIAR SNP rs920778 allele varies in different populations (Iacoviello et al. 2016). Most of the studies of LncRNA HOTAIR polymorphisms have been conducted in Chinese populations, and a few have been conducted in Japanese, Korean, and Iranian populations. Most of these studies have been associated with cancer susceptibility. For example, the results of studies in Japanese populations showed that the rs920778 polymorphism did not affect the overall presence of cancer and had a weak effect on specific cancer types in Japanese populations (Iacoviello et al. 2016). LncRNA HOTAIR polymorphisms have been less studied in European populations, focusing on cancer: the HOTAIR rs18996630 GT genotype increased the risk of cancer in Ukrainian women by 2.7-fold (Volkohon et al. 2020).

It has been shown that RSA is associated with increased apoptosis of extrachorionic trophoblast cells (Minas et al. 2007). Whereas, HOTAIR is thought to be associated with trophoblast movement and embryo implantation (Huppertz 2019). Recently, it has been shown that HOTAIR SNPs are associated with primary ovarian insufficiency (POI) (Kim et al. 2020) and RSA (Park et al. 2022). Cho et al. (2021) found that HOTAIR rs4759314 AA genotype and rs795894 GC genotype reduced the risk of POI. By analyzing HOTAIR polymorphisms in a Korean population, Park et al. (2022) found that patients carrying the HOTAIR rs4759314 G allele and rs920778 C allele had a significantly higher risk of suffering from RSA, suggesting that wild-type HOTAIR may have a potentially protective effect against RSA. In the Iranian population, the rs920778 TC, rs1899663 GT and rs12826786 CT loci increased the risk of RSA (Salimi et al. 2021). In this experiment, we evaluated the association between two lncRNA HOTAIR gene polymorphisms (rs4759314, rs1899663) and the risk of RSA in Chinese Han women. The rs1899663 locus polymorphism increased the risk of RSA in our studied population, but not significantly. rs4759314 AG genotype significantly increased the risk of RSA. The G allele carrying rs4759314 had a much higher risk of RSA than the A allele. Our study suggests that the rs4759314 polymorphism may be associated with an increased risk of RSA, which is consistent with the findings in the Korean population (Park et al. 2022). However, the results of the study in the Iranian population showed that the rs4759314 locus polymorphism did not increase the risk of developing RSA (Salimi et al. 2021). The results of logistic regression analysis showed that age, BMI, LDL, TG and DBP were protective factors for RSA and these factors had an inhibitory effect on the occurrence of RSA. While gestational, HDL, SBP, FBG and rs4759314 had OR values less than 1, and these factors were risk factors for RSA. Among them, only rs4759314 was significantly associated with the occurrence of RSA. In conclusion, the rs4759314 locus polymorphism may be a marker of RSA susceptibility in the Chinese Han population.

In addition, in this study, the results of serum lncRNA HOTAIR relative expression assay showed that the relative expression of lncRNA HOTAIR in RSA patients was significantly lower than that in healthy women. This is consistent with the study of Li et al (2018): the rs4759314 G allele variant affects the transcriptional activity of the HOTAIR gene promoter and reduces the expression level of lncRNA HOTAIR.

There are some limitations of this study, such as the mechanism related to the influence of lncRNA HOTAIR polymorphisms on the pathogenesis of RSA is unknown, the relationship between the gene expression levels of dif-

ferent genotypes of HOTAIR is unclear, and the grouping of patients with RSA on the basis of age or the number of miscarriages was not discussed.

Conclusion

Overall, this trial investigated the association between two lncRNA HOTAIR gene polymorphisms (rs4759314, rs1899663) and RSA susceptibility in Chinese Han women. The results demonstrated that the relative expression of lncRNA HOTAIR was significantly decreased in RSA patients, and that the rs4759314 AG genotype and G allele may serve as biomarkers of RSA risk. This study provides a theoretical basis for the prediction of risk in RSA patients.

Conflict of Interest

The authors declare no conflict of interest.

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