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ORIGINAL RESEARCH

The lncRNA *ANRIL* Gene rs2151280 GG Genotype is Associated with Increased Susceptibility to Recurrent Miscarriage in a Southern Chinese Population

Di Che[1,*]
Zhenzhen Fang[2,*]
Hanran Mai[1,*]
Yufen Xu[1]
LanYan Fu[1]
Huazhong Zhou[1]
Linyuan Zhang[1]
Lei Pi[1]
Xiaoqiong Gu[1]

[1]Department of Clinical Biological Resource Bank, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, People's Republic of China; [2]Program of Molecular Medicine, Guangzhou Women and Children's Hospital, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, People's Republic of China

*These authors contributed equally to this work

Background: Genetic factors may play an important role in susceptibility to recurrent miscarriage. Some cardiovascular disease-related candidate genes have been shown to be associated with recurrent miscarriage. Long noncoding RNA *ANRIL* has been confirmed to be associated with susceptibility to various diseases, such as cardiovascular disease. However, it remains unclear whether the *ANRIL* gene polymorphism is related to recurrent miscarriage susceptibility.

Methods: Three *ANRIL* gene polymorphisms (rs2151280, rs1063192 and rs564398) were genotyped in 819 controls and 610 recurrent miscarriage patients through TaqMan real-time polymerase chain reaction. The odds ratios and 95% confidence intervals (CIs) were used to assess the strength of each association.

Results: Our results showed that the *ANRIL* rs2151280 GG genotype was associated with increased susceptibility to recurrent miscarriage (GG vs AA: adjusted OR=1.527, 95% CI=1.051–2.218, p=0.0262; GG vs AG/AA adjusted OR=1.460, 95% CI=1.021–2.089, p=0.0381). By combining the analysis of the risk genotypes in the three SNPs, we found that individuals with 2–3 risk genotypes had a significantly increased risk of recurrent miscarriage compared with those with a 0–1 risk genotype (adjusted OR=1.728, 95% CI=1.112–2.683, p=0.0149). This risk was more significant in subgroups of women less than 35–40 years of age and women with 2–3 miscarriages.

Conclusion: These results suggested that a specific SNP in the *ANRIL* gene may be associated with increased susceptibility to recurrent miscarriage in a southern Chinese population.

Keywords: recurrent miscarriage, lncRNA *ANRIL*, genetic susceptibility, polymorphism

Introduction

Recurrent miscarriage (RM) is defined as at least two consecutive miscarriages that occurred with the same husband before the 20th week of gestation.[1] Currently, the causes of recurrent miscarriage remain unknown. Accumulating evidence has suggested that immunological dysfunction, endocrine disorders, thrombophilia, abnormal placental function, parental chromosomal abnormalities and unhealthy life patterns are risk factors for recurrent miscarriage.[2–6] In recent years, research has found that genetic polymorphisms play an important role in the pathogenesis of miscarriage, and an increasing number of genetic polymorphisms are thought to be related to the occurrence of miscarriage.[6–9] Previous research has shown that there is an association between recurrent miscarriage and an increased risk of

Correspondence: Xiaoqiong Gu
Department of Clinical Biological Resource Bank, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, 9 Jinsui Road, Guangzhou, 510623, Guangdong, People's Republic of China
Tel/Fax +86-20-38076561
Email guxiaoqiong@gwcmc.org

cardiovascular disease in women later in life.[10] In addition, some cardiovascular disease-related genes have been found to be risk factors for miscarriage,[11–14] such as *plasminogen activator inhibitor-1 (PAI-1), angiotensin I-converting enzyme (ACE)*, *methylenetetrahydrofolate reductase (MTHFR)* and *apolipoprotein E (Apo E)*.[15–19]

Long noncoding RNA *ANRIL* is a novel large antisense noncoding RNA that was first identified within the 403-kb germline deletion with a history of melanoma and neural system tumors in a French family.[20] Recent evidence has suggested that numerous polymorphisms located in the *ANRIL* locus are associated with increased susceptibility to multiple diseases, such as cancers and cardiovascular disease.[21] For instance, *ANRIL* gene polymorphisms contribute to the risk of ischemic stroke and increased risk of coronary artery disease,[22,23] and *ANRIL* gene polymorphisms are significantly associated with breast cancer, glioma, endometriosis, melanoma and diabetes.[24–27] In addition, endometriosis, cardiovascular disease, diabetes and breast cancer are also high-risk factors for miscarriage. However, few studies have investigated whether *ANRIL* gene polymorphisms are associated with recurrent miscarriage. Therefore, we investigated whether *ANRIL* polymorphisms are related to recurrent miscarriage susceptibility. In the present case–control study, we investigated the association between *ANRIL* polymorphisms (rs2151280, rs1063192 and rs564398) and recurrent miscarriage susceptibility with 610 cases and 819 controls in a southern Chinese population.

Materials and Methods

Study Subjects

According to the Declaration of Helsinki, the study was supported by the Ethics Committee of the Guangzhou Women and Children's Medical Center, and all participants signed an informed consent form (2018022202). Between June 2017 and June 2019, this study recruited 610 patients who had recurrent miscarriage, which was defined as two or more consecutive pregnancy losses before 20 weeks of gestation. The exclusion criteria were as follows: history of abnormal uterine anatomy; metabolic disorders; autoimmune conditions; arterial or venous thrombosis; liver or kidney dysfunction; and uterine abnormalities. The controls included 819 healthy controls who had at least two normal pregnancies and did not have a history of miscarriage or premature birth, and the healthy controls were age-matched to the case. Blood samples (2 mL) from each participant were collected for genetic analysis.

DNA Extraction and SNP Genotyping

Total genomic DNA from 200-μL venous blood samples (collected from participants) was isolated using the TIANamp Blood DNA Kit (Tiangen, Beijing, China) following the manufacturer's instructions. Three SNP genotyping probes for the *ANRIL* gene (rs2151280, rs1063192 and rs564398) were purchased from ABI (Applied Biosystems, CA, USA), and all SNP genotyping was performed in a 384-well plate according to the TaqMan real-time polymerase chain reaction protocol on an ABI Q6 instrument (QuantStudio™ 6 Flex Real-Time PCR System, Thermo Fisher Scientific, CA, USA). We have previously reported the details for this method.[28,29]

Statistical Analysis

Hardy-Weinberg equilibrium (HWE) was tested by the goodness-of-fit χ^2 test for the control group. The allele and genotype frequencies (between recurrent miscarriage patients and healthy controls) were evaluated by the two-sided chi-square test. The 95% confidence interval (CI) and odds ratio (OR) were used to assess the association between *ANRIL* gene polymorphisms and susceptibility to recurrent miscarriage. ORs and 95% CIs were calculated by unconditional logistic regression analyses. Furthermore, the adjusted OR and corresponding 95% CI were calculated to adjust for age through unconditional multiple logistic regression. A stratified analysis was performed for the age and number of miscarriages. All statistical tests consisted of bilateral analyses. SAS software (version 9.4; SAS Institute, Cary, NC, USA) was used to perform all statistical analyses. P-values less than 0.05 were considered statistically significant.

Results

Population Characteristics

In total, we enrolled 610 patients with recurrent miscarriage (20 to 46 years old) and 819 healthy controls (20 to 49 years old) (Table 1). There were no significant differences between recurrent miscarriage patients and age-related controls (32.43±5.41 vs 32.81±5.08 years, p=0.1796). In addition, approximately 59.18% of patients with recurrent miscarriage experienced two or three miscarriages, and more than 40.82% of patients experienced four or more miscarriages in this study.

Table 1 Frequency Distribution of Selected Characteristics in Recurrent Miscarriage and Controls

Variables	Cases (n =610)		Controls (n = 819)		*P*[a]
	No.	%	No.	%	
Age range, year	20–46		20–49		0.1796
Mean ± SD	32.43±5.41		32.81±5.08		
<35 35–40 >40	403 150 57	66.07 24.59 9.34	532 217 70	64.96 26.5 8.55	
No. of miscarriages/%					
2–3 ≥4	361 249	59.18 40.82			

Note: [a]Bilateral χ^2 test for distributions between recurrent miscarriage patients and controls.

Association Between *ANRIL* Gene Polymorphisms and Recurrent Miscarriage Susceptibility

We performed a goodness-of-fit χ^2 test to test whether the genotype frequency of the distributed SNPs deviated from the expected pattern (Table 2). The genotype distribution of all SNPs was consistent with the HWE in the control. All p-values were higher than 0.05 (p=0.319 for rs2151280, p=0.119 for rs564398 and p=0.85 for rs1063192), indicating that all analyzed SNPs were consistent with HWE in the control group.

The single locus analysis suggested that two *ANRIL* gene SNPs modified recurrent miscarriage susceptibility. The rs2151280 GG genotype of the *ANRIL* gene showed increased recurrent miscarriage susceptibility (GG vs AA: adjusted OR=1.527, 95% CI=1.051–2.218, p=0.0262; GG vs AG/AA adjusted OR=1.460, 95% CI=1.021–2.089, p=0.0381). There was a negative association of the rs1063192 variant allele of the *ANRIL* gene with recurrent miscarriage susceptibility. By combining the analyses of the risk genotypes in the three SNPs, we found that individuals with 2–3 risk genotypes had a significantly increased risk of recurrent miscarriage compared with those with 0–1 risk genotypes (adjusted OR=1.728, 95% CI=1.112–2.683, p=0.0149).

Stratified Analysis of Selected Polymorphisms and Recurrent Miscarriage Susceptibility

We further explored the association between three selected SNPs of the *ANRIL* gene and the risk of recurrent miscarriage susceptibility by stratifying age and the number of miscarriages (Table 3). By combining all risk genotypes, we found that carrying 2–3 risk genotypes had a higher risk in women less than 35–40 years of age (OR=2.903, 95% CI=1.198–7.033, p=0.0183) and in women with 2–3 miscarriages (adjusted OR=1.900, 95% CI=1.155–3.126, p=0.0115) compared to women carrying 0–1 risk genotypes. However, this important finding needs to be further validated in studies with large sample sizes.

Discussion

Genetic factors play important roles in the pathogenesis of miscarriage. Polymorphism in many genes is thought to be related to miscarriage. In the present case-control study, we investigated the associations between *ANRIL* gene polymorphisms (rs2151280, rs564398 and rs1063192) and recurrent miscarriage susceptibility in a southern Chinese population. Among the three SNPs, we found that the rs2151280 GG allele was associated with an increased risk of recurrent miscarriage. However, the rs1063192 and rs564398 alleles were not related to recurrent miscarriage susceptibility.

LncRNA gene polymorphisms are associated with susceptibility to abortion. Studies have found that polymorphisms in some genes that regulate cell invasion and migration are related to susceptibility to abortion.[30–32] Our previous research found that polymorphism in the lncRNA *CCAT2* rs6983267 gene, which functions in regulating cell invasion and migration, is related to susceptibility to abortion (248 patients and 392 controls).[33] Then, we tried to verify the results in a larger-sample population by assessing other functionally similar genes. Similarly, we found that lncRNA *HULC* polymorphisms that specifically regulate cell invasion and migration are related to susceptibility to miscarriage, and the result suggests that the rs7770772 GC/CC alleles, rs1041279 GG allele and rs17144343 GA/AA allele of the *HULC* gene are associated with decreased susceptibility to recurrent miscarriage.[34] We further explored the relationship between other functional

Table 2 Genotype and Allele Frequencies of *ANRIL* in RM Patients and Controls

Genotype/Allele	RM (N =610)	Controls (N=819)	*P*-value[a]	OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value[b]
ANRIL/rs2151280 A >G (HWE = 0.319)							
AA	278(45.57)	402(49.08)	0.0933	1	/	1	/
AG	264(43.28)	352(42.98)	/	1.085(0.869–1.353)	0.4717	1.098(0.880–1.371)	0.4070
GG	68(11.15)	65(7.94)	/	**1.513(1.042–2.196)**	**0.0295**	**1.527(1.051–2.218)**	**0.0262**
Dominant	332(54.43)	417(50.92)	0.1886	1.151(0.933–1.421)	0.1888	1.165(0.944–1.438)	0.1548
Recessive	542(88.85)	754(92.06)	**0.0398**	**1.455(1.018–2.081)**	**0.0396**	**1.460(1.021–2.089)**	**0.0381**
ANRIL/rs564398 T >C (HWE = 0.119)							
TT	479(78.52)	642(78.39)	0.8892	1	/	1	/
TC	117(19.18)	161(19.66)	/	0.974(0.747–1.271)	0.8460	0.973(0.746–1.270)	0.8404
CC	14(2.30)	16(1.95)	/	1.173(0.567–2.426)	0.6675	1.217(0.586–2.525)	0.5982
Dominant	131(21.48)	177(21.61)	0.9506	0.992(0.769–1.280)	0.9506	0.994(0.770–1.284)	0.9659
Recessive	596(97.70)	803(98.05)	0.6571	1.179(0.571–2.434)	0.6564	1.224(0.591–2.534)	0.5870
ANRIL/rs1063192 A > G (HWE = 0.85)							
AA	427(70.00)	553(67.52)	0.4739	1	/	1	/
AG	168(27.54)	239(29.18)	/	0.910(0.720–1.151)	0.4320	0.909(0.719–1.149)	0.4241
GG	15(2.46)	27(3.30)	/	0.719(0.378–1.370)	0.3161	0.729(0.383–1.389)	0.3366
Dominant	183(30.00)	266(32.48)	0.3175	0.891(0.710–1.118)	0.3182	0.891(0.710–1.117)	0.3170
Recessive	595(97.54)	792(96.70)	0.3496	0.740(0.390–1.403)	0.3554	0.750(0.395–1.423)	0.3780
Combined risk-effect of genotypes *							
0–1	560(91.80)	780(95.24)	0.0083	**1**	**/**	**1**	**/**
2–3	**50(8.20)**	**39(4.76)**		**1.712(1.103–2.657)**	**0.0165**	**1.728(1.112–2.683)**	**0.0149**

Notes: *The risk genotypes used for the calculation were as follows: rs2151280 GG + rs564398 CC + rs1063192 AA. [a]χ^2 test for genotype distributions between recurrent miscarriage patients and controls. [b]Adjusted for age. Statistically significant values are shown in bold (P<0.05).
Abbreviations: OR, odds ratio; HWE, Hardy–Weinberg equation; RM, recurrent miscarriage.

lncRNA genes and susceptibility to abortion. Studies have found that polymorphisms in some genes that play an important role in cardiovascular diseases are related to susceptibility to miscarriage.[11–14] Our previous research also found that lncRNA *MALAT1*, which plays an important role in cardiovascular diseases, is related to susceptibility to miscarriage (248 patients and 392 controls).[35] Similarly, we used a relatively large sample size to verify this result. Therefore, in this study, with other functionally similar genes, we explored the relationship between polymorphism in the *ANRIL* gene, which plays an important role in cardiovascular disease, and susceptibility to miscarriage. We found that the rs2151280 GG allele was associated with an increased risk of recurrent miscarriage. Studies have shown that both *ANRIL* and *HULC* are involved in regulating cell motility functions.[36–39] Additionally, studies have found that the expression levels of *ANRIL* and *HULC* are abnormal in patients with osteosarcoma, multiple sclerosis and breast cancer.[40–42] However, research has shown that *ANRIL* plays a more important role in cardiovascular disease. *ANRIL* gene polymorphisms contribute to the risk of ischemic stroke and increased risk of coronary artery disease.[22,23] In this research, we focus on highlighting some genes related to cardiovascular disease as risk factors for miscarriage and provide new perspectives for the study of genetic susceptibility to recurrent miscarriage.

ANRIL is located on the chromosome 9p21 locus and belongs to the long noncoding RNA group. *ANRIL* is considered to be a molecular scaffold for chromatin-modifying complexes, and it can control gene expression by modifying histone tails.[43] Recently, accumulative evidence has suggested that *ANRIL* gene polymorphisms are associated with a variety of diseases, especially cardiovascular diseases, hypertension, endometriosis, breast cancer and diabetes.[24,25,44–46] Moreover, cardiovascular diseases, hypertension, endometriosis, breast cancer and diabetes are also high-risk factors for miscarriage.[12,47–49] Thus, the *ANRIL* gene polymorphism may be related to the onset of miscarriage. To the best of our

Table 3 Stratification Analysis for Associations Between *ANRIL* Polymorphism and Recurrent Miscarriage Risk in a South Chinese Population

Variable	Combined Effect of Risk Genotype (Cases/Controls)*		P	OR (95% CI)	P	Adjust OR	P[a]
	0–1	2–3					
Age							
<35	374/507	29/25	0.1070	1.573 (0.906–2.729)	0.1076	/	/
35–40	135/209	15/8	**0.0151**	**2.903 (1.198–7.033)**	**0.0183**	/	/
>40	51/64	6/6	0.7086	1.255 (0.382–4.124)	0.7084	/	/
No. of miscarriage/%							
2–3	331/780	30/39	**0.0200**	**1.813 (1.107–2.968)**	**0.0180**	**1.900 (1.155–3.126)**	**0.0115**
4≥	229/780	20/39	0.0576	1.747 (0.999–3.054)	0.0504	1.727 (0.986–3.025)	0.0561

Notes: *The combination of risk genotypes used for the calculation was as follows: rs2151280 AA + rs564398 CC + rs1063192 AA. [a]Adjusted for age. Statistically significant values are shown in bold (P<0.05).

Abbreviations: OR, odds ratio; HWE, Hardy–Weinberg equation; RM, recurrent miscarriage.

knowledge, this study was the first to investigate the association between *ANRIL* gene polymorphisms (rs2151280, rs564398 and rs1063192) and susceptibility to recurrent miscarriage in a southern Chinese population. Pasmant et al found that an *ANRIL* gene polymorphism (rs2151280) is significantly associated with the number of plexiform neurofibromas in neurofibromatosis type 1 patients.[50] Poi et al found that the *ANRIL* rs2151280 polymorphism is associated with worse progression-free survival in patients with multiple myeloma.[51] The present study found that the rs2151280 polymorphism in the *ANRIL* gene T allele was associated with an increased risk of recurrent miscarriage, which may be related to the regulation of the inflammatory response by the *ANRIL* gene.[52] Wang et al found that the *ANRIL* rs564398 polymorphism is associated with an increased risk of HBV-related gestational diabetes mellitus in a Chinese population.[53] Other studies have confirmed that the *ANRIL* rs564398 variant is significantly associated with myocardial infarction after multivariate adjustments for coronary artery disease.[54] However, the present study found that the *ANRIL* rs564398 variant allele was not related to susceptibility to recurrent miscarriage. Because there are differences in susceptibility genes between different ethnic groups, the results of genetic susceptibility need to be verified in different populations. Deng et al found that the *ANRIL* rs2151280 variant genotype may increase susceptibility to glioma in a Chinese Han population.[55] Similarly, a study in an Italian population found that *ANRIL* rs2151280 polymorphism is associated with optic glioma development.[56] Research has shown that the rs1063192 polymorphism is related to genetic risk factors for normal-tension glaucoma.[57,58] Hu et al found that the *ANRIL* rs1063192 polymorphism is significantly associated with a decreased risk of glaucoma.[59] Moreover, Li et al confirmed that the rs1063192 variant is significantly associated with esophageal squamous cell carcinoma.[60] In contrast, Chen et al found that the rs3217986 polymorphism is not associated with the risk of intracranial aneurysm in a Chinese population.[61] Similarly, the present study demonstrated that there was a negative association of the *ANRIL* rs1063192 variant allele with recurrent miscarriage susceptibility. The above results suggest that *ANRIL* gene polymorphisms play different roles in various diseases. Our results need to be verified in populations of different genetic backgrounds.

Research has shown that women who have experienced recurrent miscarriage have a higher risk of miscarriages during the next pregnancy than those who have given birth successfully.[62] Many studies have shown that advanced age is a risk factor for miscarriage, and women over 40 years old are five times more likely to have miscarriages than women 31 to 35 years old.[63,64] High maternal age is an important risk factor for spontaneous miscarriage. The risk of spontaneous miscarriage in women aged 20–24 years is 8.9%, compared with 74.7% for women aged more than 40 years.[64] By combining all risk genotypes, the present study demonstrated that carrying 2–3 risk genotypes had a higher risk in women less than 35–40 years of age and in women with 2–3 miscarriages compared to women carrying 0–1 risk genotypes. These results require a larger sample study for verification, and the molecular mechanism of these functions deserves further exploration.

This case-control study is the first to assess the association between *ANRIL* gene polymorphism and susceptibility to recurrent miscarriage. Some limitations in our research should be addressed. First, only three SNPs (rs2151280, rs564398 and rs1063192) were investigated in our study, and more SNPs should be included in future studies. Second, our sample size was not large enough, resulting in limited statistical power. Future research will require a larger sample size to validate the association of the *ANRIL* gene polymorphism with recurrent miscarriage susceptibility in a southern Chinese population. Third, we only studied the relationship between the *ANRIL* gene polymorphism and susceptibility to recurrent miscarriage, and we did not detect *ANRIL* gene expression in patients.

In conclusion, this case-control study confirmed that the *ANRIL* gene rs2151280 GG genotype is associated with increased susceptibility to recurrent miscarriage. These results increase our understanding of the function of this gene in recurrent miscarriage and provide valuable insights into its role in disease pathogenesis. However, larger sample sizes and additional experiments should be performed to confirm the role of the *ANRIL* polymorphism in recurrent miscarriage susceptibility.

Data Sharing Statement

Please contact the author for data requests.

Ethical Approval

The study was supported by the Ethics Committee of the Guangzhou Women and Children's Medical Center, and all participants signed informed consent forms (2018022202).

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Author Contributions

All authors contributed significantly to this work. All authors made substantial contributions to the conception and design of the study as well as the acquisition, analysis and interpretation of data. All authors participated in drafting the article or revising it critically for important intellectual content, and all authors agreed to submit the manuscript to the current journal. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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