Correlation Between Single Nucleotide Polymorphisms in Cxcr4 Microrna Binding Site and the Susceptibility to Knee Osteoarthritis in Han Chinese Population

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Abstract

Background:

This study aimed to investigate the relationship between single nucleotide polymorphisms (SNPs) at the microRNA target sequence in *CXCR4* and the susceptibility to knee osteoarthritis (KOA).

Methods:

A total of 305 patients with KOA and 305 healthy controls were recruited into this study. The genotypes of *CXCR4* rs1804029 and rs17848060 loci were analyzed.

Results:

The susceptibility to KOA of *CXCR4* rs1804029 G allele carriers was 1.33 times that of T allele carriers. The KOA susceptibility in individuals carrying T allele at *CXCR4* rs17848060 locus was 1.38 times that of individuals carrying A allele (95% CI: 1.17-1.57, $p < 0.001$). The G allele at *CXCR4* rs1804029 locus was the target of hsa-miR-146a-3p, while the A allele at *CXCR4* rs17848060 locus could be targeted by hsa-miR-20a-3p. The plasma level of hsa-miR-146a-3p was lower in rs1804029 G allele carriers than T allele carriers, whereas plasma level of hsa-miR-20a-3p was higher in rs17848060 T allele carriers than A allele carriers.

Conclusion:

The SNPs at rs1804029 and rs17848060 loci in *CXCR4* were significantly associated with the susceptibility to KOA in Han Chinese population.

1. Introduction

Osteoarthritis (OA) is a type of joint disease caused by the unbalanced processes of articular chondrocytes, stroma, and subchondral bone synthesis and breakdown induced by interaction of biological and mechanical factors (Honsawek et al. 2010). As a common clinically degenerative disease, OA is mainly manifested by degenerative changes in the articular cartilage bone, joint pain, difficulty in movement, and osteophyte formation, and is more common in middle-aged and elderly people (Loeser et al. 2012; Varady and Grodzinsky 2016). In the recent years, OA has become the fourth cause of disability in the elderly worldwide (Loeser 2013). By 2017, more than 100 million people worldwide were suffering from osteoarthritis, and the incidence of osteoarthritis is increasing year by year (Dobson et al. 2018).

However, the pathogenesis of OA has not been fully elucidated yet. During the progress of OA, infiltration of inflammatory factors in the joints and degradation of the articular cartilage occur in the early stages (Hochberg et al. 1995; Kapoor et al. 2011; Szebenyi et al. 2006). Chemokines are a class of soluble, chemically-inducible cytokines that can induce the chemotaxis of specific types of nearby immune cells (Gustavsson 2020; Miao et al. 2020; Shi et al. 2020). Chemokines act on target cells through

transmembrane G protein-coupled receptors and affect the morphology, proliferation, and differentiation of the target cells (Feil and Augustin 1998; Yu et al. 2006).

CXCR4 is located on chromosome 2q212, which encodes a seven-transmembrane G protein-coupled receptor that mediates signaling (D'Apuzzo et al. 1997). CXCR4 transduces signals by increasing intracellular calcium ion concentration and potentiates the chemotactic activity of lymphocytes (D'Apuzzo et al. 1997; Hu et al. 2017; Hu et al. 2017; Zheng et al. 2007). SDF-1/CXCR4 signaling pathway plays an important role in mediating inflammatory responses (Olive et al. 2008; Wang et al. 2018). A previous study showed that the expression of SDF-1 increases in the synovium of patients with rheumatoid arthritis (RA), and there are a large number of T cells expressing CXCR4 in the synovium, indicating that the SDF-1/CXCR4 signaling pathway is involved in the pathogenesis of RA (Buckley et al. 2000). Research from Nanki *et al*. revealed that memory T cells highly express CXCR4, and the endogenous SDF-1 concentration in the synovium of patients with RA is much higher than that in normal individuals. SDF-1 can promote the migration of memory T cells and inhibit T cell apoptosis, indicating that SDF-1 and its receptor CXCR4 may play important roles in T cell accumulation in the synovium of RA patients. Additionally, abolishing the SDF-1/CXCR4 signaling pathway, which has become a potential clinical strategy for OA treatment (Wang et al. 2017), can downregulate the expression of matrix degrading enzymes and reduce cartilage degeneration. These results demonstrate that SDF-1 and CXCR4 are involved in the occurrence and development of OA. Epidemiological research reported that *CXCR4* polymorphisms are correlated with cancer risk (Wu et al. 2016). In addition, the polymorphisms in *CXCR4* are associated with pre-eclampsia (PE) susceptibility and with the serum levels of SDF-1 in PE patients (Karakus et al. 2017).

MicroRNAs (miRNAs) are small non-coding RNAs with lengths between 18 and 22 nucleotides. miRNAs can directly bind to the 3'-untranslated region (3'-UTR) of the target gene to repress target mRNA translation. Multiple studies have proved that miRNAs are involved in a variety of human diseases through diverse mechanisms (Dasgupta et al. 2018; Paulmurugan). Nossent *et al*. proposed that single nucleotide polymorphisms (SNPs) in the miRNA binding sites in 3'-UTR of renin-angiotensin-aldosterone system (RAAS)-related genes influence arterial blood pressure and the risk of myocardial infarction (Nossent et al. 2011). Another report from Wei et al. showed that miRNA target site polymorphisms in the VHL-HIF1alpha pathway can predict the risk of renal cell carcinoma (Wei et al. 2014). These studies of the correlation between SNPs in miRNA binding sites and risk of diseases imply that SNPs might affect the susceptibility or development of human diseases by regulating the interaction between miRNAs and target genes.

In this study, we used the MirSNP (http://bioinfo.bjmu.edu.cn/mirsnp/search/) to select rs1804029 and rs17848060, two SNP sites located on the miRNA binding site of *CXCR4*, and investigated the correlation between the two SNPs and the susceptibility to knee osteoarthritis (KOA) in Chinese Han population.

2. Methods And Materials

2.1 Subjects

A total number of 305 KOA patients and 305 healthy controls in the First Affiliated Hospital of Kunming Medical University from May 2018 to October 2019 were enrolled in this study. The patients were diagnosed with KOA based on the symptom criteria of the American College of Rheumatology and at least one side of the knee (Kellgren-Lawrence grading ≥ 2) (Kellgren and Lawrence 1957). The Kellgren-Lawrence grading of control group was less than 2. Patients were excluded from the study if they had (1) arthritis other than KOA; (2) metabolic bone disease; (3) RA; (4) tumor; (5) diabetes or hypertension. This study was approved by the First Affiliated Hospital of Kunming Medical University medical ethics committee, and all the subjects had signed informed consent forms.

2.2 *CXCR4* SNP analysis

Genomic DNA was extracted from the peripheral blood of the subjects using the QIAamp DSP DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA fragments containing rs1804029 and rs17848060 of *CXCR4* was amplified using PCR. The primer sequences were as follows: rs1804029-forward (F): 5'-CCA CCT CGC TTT CCT TTG GA-3', rs1804029-reverse (R): 5'-TAA AAC CTC TGC CCA GCA CG-3'; rs17848060-F: 5'-TGC TGA AAT CAA CCC ACT CCT-3', rs17848060-R: 5'-TCC CGT GGA ACG TTT TTC CT-3'. PCR was conducted as follows: 95℃ 5 min; 94℃ 30 s, 60℃ 30 s, 72℃ 30 s, 30 cycles; 72℃ 5 min. The PCR products were sequenced via Sanger sequencing (Shanghai Biotech Biotechnology Shanghai, China). Based on the sequencing results, the genotypes of rs1804029 and rs17848060 of *CXCR4* were analyzed, and 10% of the samples were randomly selected for repeated verification.

2.3 Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from the plasma of the subjects using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). PrimeScript RT Master Mix (TaKaRa, Dalian, China) was used to perform reverse transcription PCR to synthesize cDNA. Primer sequences for quantification of miRNAs were as follows: hsa-miR-146a-3p F: 5'-TTG CAC CAT CTC TGA AAA GCC-3'; hsa-miR-146a-3p R: 5'-TGT CTC CAG TCT TCC AAG CTC-3'; hsa-miR-20a-3p F: 5'-TGT GAC AGC TTC TGT AGC AC-3'; hsa-miR-20a-3p R: 5'-GGA CAG TTT GAT TGG GCG AC-3'. U6 was used as an internal control, and the primer sequences were: U6-F: 5'-CTC GCT TCG GCA GCA CA-3'; U6-R: 5'-TGG TGT CGT GGA GTC G-3'. The reaction conditions were: 95℃ 5 min, 1 cycle; 95℃ 10 s, 45 cycles; 68.7℃ 30 s, 45 cycles; 72℃ 30 s, 45 cycles. The program for fluorescence collection was: 95℃ 15 s; 60℃ 60 s; 95℃ 15 s; 60℃ 15 s. The expression level of miRNAs was calculated using the $2^{-\Delta\Delta CT}$ method.

2.4 Dual-luciferase reporter assay

Reporter plasmids harboring SNP sites at rs1804029 and rs17848060 loci in *CXCR4* were synthesized from Shanghai Integrated Biotech Solutions (Shanghai, China) and were named as pGL3-CXCR4-rs1804029 T, pGL3-CXCR4-rs1804029 G, pGL3-CXCR4-rs17848060 T, and pGL3-CXCR4-rs17848060 A. To investigate the effect of SNP on miRNA and target sequence interaction, pGL3-CXCR4-rs1804029 T or pGL3-CXCR4-rs1804029 G was co-transfected with hsa-miR-146a-3p mimic or hsa-miR-146a-3p inhibitor,

and pGL3-CXCR4-rs17848060 T or pGL3-CXCR4-rs17848060 A was co-transfected with hsa-miR-20a-3p mimic or hsa-miR-20a-3p inhibitor, respectively, into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and cells were harvested at 24 h post transfection for dual-luciferase activity detection according to commercial manual instructions (Promega, Madison, WI, USA).

2.5 Statistical analysis

All statistical analyses in this study were conducted using Graphpad prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA). Hardy-Weinberg equilibrium was evaluated using the χ^2 test. Binary logistic regression was used to adjust age, gender, body mass index (BMI), smoking history, drinking history, knee injury history, and KOA family history. The odds ratio (OR) and the 95% confidence interval (CI) were applied to evaluate the correlation between the genotypes and allele frequencies of the rs1804029 and rs17848060 loci in *CXCR4* and their susceptibility to KOA. Multifactor dimensionality reduction (MDR) 3.0.2 software was employed to analyze the influence of the interaction between rs1804029 and rs17848060 loci in *CXCR4* and age, gender, BMI, smoking history, drinking history, knee injury history, KOA family history, and other baseline data on the susceptibility to KOA. False positive report probability (FPRP) was calculated, and the threshold of FPRP was set to 0.2. A significant result of FPRP < 0.2 was considered to be noteworthy, when the prior probability is 0.1 (He et al. 2013). All the tests were two-tailed, and P value less than 0.05 indicated a statistically significant difference.

3. Results

3.1 Comparison of subject baseline characteristics

The baseline characteristics of the 305 KOA patients and 305 healthy controls enrolled in this study are summarized in Table 1. The comparison results showed there was no significant difference in age, gender, BMI, smoking history, and drinking history among patients with KOA ($p > 0.05$) and control group. However, the proportion of KOA patients with a history of knee trauma and a family history of KOA was significantly higher than that of the control group ($p < 0.05$). Among the 305 KOA patients, 152 were Kellgren-Lawrence grading grade 2, 110 were grade 3, and 43 were grade 4.

Table 1
Comparison of Subject Baseline Characteristics between KOA patients and control group

	KOA (n = 305)	Control (n = 305)	P value
Age(years)	58.64 ± 9.78	60.06 ± 10.96	0.092
60	203(66.56%)	210(68.85%)	
≥ 60	102(33.44%)	95(31.15%)	
Gender			0.746
Male	159(52.13%)	163(53.44%)	
Female	146(47.87%)	142(46.56%)	
BMI(kg/m^2)	25.04 ± 2.55	25.40 ± 2.66	0.088
24	103(33.77%)	84(27.54%)	
≥ 24	202(66.23%)	221(72.46%)	
Smoking history			0.451
Ever	120(39.34%)	125(40.98%)	
Never	185(60.66%)	170(55.74%)	
Drinking history			0.877
Ever	127(41.64%)	121(39.67%)	
Never	178(58.36%)	174(57.05%)	
Knee injury history			0.011
Ever	34(11.15%)	16(5.25%)	
Never	271(88.85%)	279(91.48%)	
KOA family history			< 0.001
Ever	56(18.36%)	10(3.28%)	
Never	249(81.64%)	295(96.72%)	
Kellgren-Lawrence grading			
2	152(49.84%)		
3	110(36.07%)		
4	43(14.10%)		
KOA,Knee osteoarthritis BMI, Body mass index			

3.2 The correlation between SNPs on *CXCR4* and KOA susceptibility

We analyzed the genotype frequencies of *CXCR4* rs1804029 and rs17848060 loci in 305 healthy subjects, and found that their distributions followed the Hardy-Weinberg equilibrium ($p > 0.05$, Table 2). After adjusting for age, gender, BMI, smoking history, drinking history, knee injury history, and KOA family history, the results showed that the KOA susceptibility of individuals carrying TG genotype at rs1804029 locus of *CXCR4* increased 1.28 times than that of individuals carrying TT genotype (95% CI: 1.01–1.55, $p = 0.048$). However, compared with TT genotype carriers, GG genotype carriers had no significant change in the susceptibility to KOA (p = 0.15). The additive model and the recessive model were not related to KOA susceptibility, but the KOA susceptibility was significantly increased by 1.32 times (95% CI: 1.06–1.57, p = 0.016) in dominant model. The KOA susceptibility of G allele carriers was 1.33 times that of T allele carriers (95% CI: 1.09–1.54, $p = 0.006$).

Table 2
The correlation of genotypes of *CXCR4* gene SNP locus and the allele frequency with KOA susceptibility

	KOA(n = 305)	Control(n = 305)	HWE *p*-value	Crude OR(95%CI)	Adjusted OR(95%CI)	*p* value
rs1804029						
TT	255(83.61%)	276(90.49%)	0.15	1.00(reference)	1.00(reference)	
TG	43(14.10%)	27(8.85%)		1.72(1.04–2.87)	1.28(1.01–1.55)	0.048
GG	7(2.30%)	2(0.66%)		3.79(0.78–18.40)	1.62(0.83–2.01)	0.150
Additive model				1.08(0.86–1.37)	1.04(0.92–1.18)	0.544
Dominance model				1.87(1.15–3.04)	1.32(1.06–1.57)	0.016
Recessive model				3.56(0.73–17.27)	1.57(0.81–1.95)	0.179
T	553(90.66%)	579(94.92%)		1.00(reference)	1.00(reference)	
G	57(9.34%)	31(5.08%)		1.93(1.22–3.03)	1.33(1.09–1.54)	0.006
rs17848060						
AA	241(79.02%)	269(88.20%)	0.09	1.00(reference)	1.00(reference)	
AT	51(16.72%)	33(10.82%)		1.723(1.08–2.76)	1.29(1.03–1.54)	0.030
TT	13(4.26%)	3(0.98%)		4.84(1.36–17.18)	1.72(1.12–2.03)	0.015
Additive model				1.12(0.88–1.41)	1.06(0.94–1.20)	0.392
Dominance model				1.98(1.27–3.09)	1.35(1.11–1.59)	0.003
Recessive model				4.48(1.26–15.89)	1.65(1.08–1.95)	0.023
A	533(87.38%)	571(93.61%)		1.00(reference)	1.00(reference)	
T	77(12.62%)	39(6.39%)		2.12(1.41–3.17)	1.38(1.17–1.57)	< 0.001

KOA, Knee osteoarthritis；HWE, Hardy-Weinberg equilibrium；OR, Odds ratio. CI, Confidence interval；

As shown in Table 2, based on the AA genotype of the *CXCR4* rs17848060 locus, after adjusting for age, gender, BMI, smoking history, drinking history, knee injury history, and KOA family history, the frequencies of AT and TT genotypes in KOA patients were significantly higher than those in the control group ($p < 0.05$). The locus additive model was not significantly related to KOA susceptibility, but the dominant model and recessive model were significantly related to KOA susceptibility (OR = 1.35, 95% CI: 1.11–1.59, $p = 0.003$; OR = 1.65, 95% CI: 1.08–1.95, $p = 0.023$). The KOA susceptibility of T allele carriers was 1.38 times higher than that of individuals carrying A allele at *CXCR4* rs17848060 locus (95% CI: 1.17–1.57, $p < 0.001$).

3.3 Stratified analysis

To further explore the impact of subject baseline data on the SNPs in *CXCR4* and KOA susceptibility, a stratified analysis of subject baseline data was performed. In males, young people (≤60 years old), obese people (BMI ≥ 24 kg/m^2), smokers, people with drinking history, people with no knee injury history, people with no KOA family history, and individuals carrying G allele (TG + GG) at the rs1804029 locus were more susceptible to KOA than TT genotype carriers ($p < 0.05$). However, in females, people aged over 60 years, non-obese (BMI <24 kg/m^2) individuals, non-smokers, people with no drinking history, people with knee injury history, and people with KOA family history, there was no significant difference in the susceptibility risk of KOA between the rs1804029 locus G allele (TG + GG) carriers and the TT genotype carriers ($p < 0.05$, Table 3). These results indicate that a stratified process for factors including gender, age, BMI, smoking history, drinking history, knee injury history, and KOA family history might significantly affect the correlation between the SNP at *CXCR4* rs1804029 locus and the susceptibility to KOA.

Table 3
Stratified analysis for the association of genotype and allele frequency of *CXCR4* rs1804029 locus with the susceptibility to KOA

	KOA(*n* = 305)	Control(*n* = 305)	Adjusted OR(95%CI)	*p* value
Gender				
Male				
TT	128(80.50%)	148(90.80%)	1.00(reference)	
TG + GG	31(19.50%)	15(9.20%)	1.45(1.09–1.79)	0.013
Female				
TT	127(86.99%)	128(90.14%)	1.00(reference)	
TG + GG	19(13.01%)	14(9.86%)	1.37(0.66–2.85)	0.512
Age				
60				
TT	168(82.76%)	191(90.95%)	1.00(reference)	
TG + GG	35(17.24%)	19(9.05%)	1.39(1.05–1.70)	0.020
≥ 60				
TT	87(85.29%)	85(89.47%)	1.00(reference)	
TG + GG	15(14.71%)	10(10.53%)	1.47(0.62–3.44)	0.505
BMI(kg/m^2)				
24				
TT	90(87.38%)	77(91.67%)	1.00(reference)	
TG + GG	13(12.62%)	7(8.33%)	1.59(0.60–4.18)	0.480
≥ 24				
TT	165(81.68%)	199(90.05%)	1.00(reference)	
TG + GG	37(18.32%)	22(9.95%)	1.38(1.06–1.70)	0.019
Smoking history				
Ever				
TT	99(82.50%)	118(92.19%)	1.00(reference)	
TG + GG	21(17.50%)	10(7.81%)	1.49(1.03–1.89)	0.035

KOA, Knee osteoarthritis BMI, Body mass index OR, Odds ratio. CI, Confidence interval

	KOA(*n* = 305)	Control(*n* = 305)	Adjusted OR(95%CI)	*p* value
Never				
TT	156(84.32%)	158(89.27%)	1.00(reference)	
TG + GG	29(15.68%)	19(10.73%)	1.55(0.83–2.87)	0.218
Drinking history				
Ever				
TT	101(79.53%)	120(96.00%)	1.00(reference)	
TG + GG	26(20.47%)	5(4.00%)	1.84(1.38–2.12)	< 0.001
Never				
TT	154(86.52%)	156(86.67%)	1.00(reference)	
TG + GG	24(13.48%)	24(13.33%)	1.01(0.55–1.86)	0.967
Knee injury history				
Yes				
TT	32(94.12%)	16(94.12%)	1.00(reference)	
TG + GG	2(5.88%)	1(5.88%)	1.00(0.08–11.87)	1.000
No				
TT	223(82.29%)	260(90.28%)	1.00(reference)	
TG + GG	48(17.71%)	28(9.72%)	1.37(1.09–1.64)	0.009
KOA family history				
Yes				
TT	47(83.93%)	9(90.00%)	1.00(reference)	
TG + GG	9(16.07%)	1(10.00%)	1.72(0.19–15.33)	0.990
No				
TT	208(83.53%)	267(90.51%)	1.00(reference)	
TG + GG	41(16.47%)	28(9.49%)	1.36(1.05–1.67)	0.021
KOA, Knee osteoarthritis□BMI, Body mass index□OR, Odds ratio. CI, Confidence interval□				

In males, people less than 60 years, people with obesity, people with no smoking history, people with drinking history, people with no knee injury history, people with no KOA family history, compared with AA genotype carriers, individuals carrying T allele (AT + TT) at rs17848060 locus had higher KOA

susceptibility ($p < 0.05$). In females, people aged over 60 years, people with non-obese (BMI ≤24 kg/m^2), people with no smoking history, people with no drinking history, people with knee injury history, there was no significant difference in susceptibility to KOA between individuals carrying T allele (AT + TT) and individuals carrying AA genotypes at rs17848060 locus ($p > 0.05$, Table 4). These data demonstrate that stratified gender, age, BMI, smoking history, drinking history, knee injury history, and KOA family history has a significant effect on the correlation between the SNP at *CXCR4* rs17848060 locus and KOA susceptibility risk.

Table 4
Stratified analysis for the association of genotype and allele frequency of *CXCR4* rs17848060 locus with the susceptibility to KOA

	KOA(*n* = 305)	Control(*n* = 305)	Adjusted OR (95%CI)	*p* value
Gender				
Male				
AA	121(76.10%)	143(87.73%)	1.00(reference)	
AT + TT	38(23.90%)	20(12.27%)	1.43(1.09–1.76)	0.010
Female				
AA	120(82.19%)	126(88.73%)	1.00(reference)	
AT + TT	26(17.81%)	16(11.27%)	1.71(0.87–3.34)	0.160
Age				
60				
AA	159(78.33%)	186(88.57%)	1.00(reference)	
AT + TT	44(21.67%)	24(11.43%)	1.40(1.10–1.70)	0.007
≥ 60				
AA	82(80.39%)	83(87.37%)	1.00(reference)	
AT + TT	20(19.61%)	12(12.63%)	1.69(0.78–3.67)	0.257
BMI(kg/m^2)				
24				
AA	77(74.76%)	76(90.48%)	1.00(reference)	
AT + TT	26(25.24%)	8(9.52%)	3.21(1.37–7.53)	0.010
≥ 24				
AA	164(81.19%)	193(87.33%)	1.00(reference)	
AT + TT	38(18.81%)	28(12.67%)	1.25(0.95–1.57)	0.109
Smoking				
Ever				
AA	92(76.67%)	108(84.38%)	1.00(reference)	
AT + TT	28(23.33%)	20(15.63%)	1.64(0.87–3.11)	0.169

KOA, Knee osteoarthritis BMI, Body mass index OR, Odds ratio. CI, Confidence interval

	KOA(*n* = 305)	Control(*n* = 305)	Adjusted OR (95%CI)	*p* value
Never				
AA	149(80.54%)	161(90.96%)	1.00(reference)	
AT + TT	36(19.46%)	16(9.04%)	1.44(1.11–1.74)	0.007
Drinking				
Ever				
AA	99(77.95%)	112(89.60%)	1.00(reference)	
AT + TT	28(22.05%)	13(10.40%)	1.46(1.07–1.82)	0.020
Never				
AA	142(79.78%)	157(87.22%)	1.00(reference)	
AT + TT	36(20.22%)	23(12.78%)	1.73(0.98–3.06)	0.079
Knee injury history				
Yes				
AA	28(82.35%)	14(82.35%)	1.00(reference)	
AT + TT	6(17.65%)	3(17.65%)	1.00(0.22–4.61)	1.000
No				
AA	213(78.60%)	255(88.54%)	1.00(reference)	
AT + TT	58(21.40%)	33(11.46%)	1.40(1.13–1.66)	0.002
KOA family history				
Yes				
AA	41(73.21%)	9(90.00%)	1.00(reference)	
AT + TT	15(26.79%)	1(10.00%)	3.29(0.38–28.24)	0.459
No				
AA	200(80.32%)	260(88.14%)	1.00(reference)	
AT + TT	49(19.68%)	35(11.86%)	1.82(1.14–2.92)	0.017
KOA, Knee osteoarthritis□BMI, Body mass index□OR, Odds ratio. CI, Confidence interval□				

3.4 False positive report rate

False positive report rate of correlation between SNPs on *CXCR4* rs1804029 and rs17848060 loci and KOA susceptibility and stratified analysis of baseline data are summarized in Table 5. In the stratified

analysis, among people with a history of drinking, individuals with TG or GG genotypes at rs1804029 locus were more susceptible to KOA than those with TT genotype, when the prior probability was 0.1, the FPRP value was less than 0.2 (Table 5). However, similar results were not obtained when analyzing other genotype frequencies and conducting stratified analysis for other factors, which indicated that the sample size of stratified analysis of drinking history was small, and the results may be biased (He et al. 2013). Therefore, the related results still need to be validated in large sample sizes.

Table 5
FPRP value of the correlation between SNPs in *CXCR4* gene and KOA susceptibility

Genotype	OR (95%CI)*	Statistical power	Prior probability		
			0.1	0.01	0.001
rs1804029 TG vs TT	1.28(1.01–1.55)	0.912	0.260	0.795	0.975
rs1804029 Dominance model	1.32(1.06–1.57)	0.865	0.254	0.789	0.974
rs1804029 G vs T	1.33(1.09–1.54)	0.877	0.253	0.788	0.974
rs17848060 AT vs AA	1.29(1.03–1.54)	0.902	0.259	0.793	0.975
rs17848060 TT vs AA	1.72(1.12–2.03)	0.813	0.207	0.742	0.967
rs17848060 Dominance model	1.35(1.11–1.59)	0.932	0.250	0.786	0.974
rs17848060 Recessive model	1.65(1.08–1.95)	0.775	0.214	0.750	0.968
rs17848060 T vs A	1.38(1.17–1.57)	0.908	0.246	0.782	0.973
rs1804029 TG + GG vs TT					
Male	1.45(1.09–1.79)	0.856	0.237	0.773	0.972
Age≥60 years	1.39(1.05–1.70)	0.932	0.245	0.781	0.973
BMI ≥ 24 kg/m^2	1.38(1.06–1.70)	0.889	0.246	0.782	0.973
Smoking	1.49(1.03–1.89)	0.788	0.232	0.769	0.971
Drinking	1.84(1.38–2.12)	0.692	**0.197**	0.792	0.964
No Knee injury history	1.37(1.09–1.64)	0.902	0.247	0.783	0.973
No KOA family history	1.36(1.05–1.67)	0.855	0.249	0.784	0.973
rs17848060 AT + TT vs AA					
Male	1.43(1.09–1.76)	0.923	0.239	0.776	0.972
Age≥60 years	1.40(1.10–1.70)	0.759	0.243	0.780	0.973
BMI ≥ 24 kg/m^2	1.25(0.95–1.57)	0.912	0.265	0.798	0.981
Never Smoking	1.44(1.11–1.74)	0.933	0.238	0.775	0.927
Drinking	1.46(1.07–1.82)	0.942	0.236	0.772	0.933
No Knee injury history	1.40(1.13–1.66)	0.892	0.243	0.780	0.954
No KOA family history	1.82(1.14–2.92)	0.904	**0.198**	0.731	0.965

BMI, Body mass index；KOA, Knee osteoarthritis；OR, Ratio ratio. CI, Confidence interval. *$p < 0.05$；

3.5 MDR analysis of the association between SNPs at CXCR4 rs1804029 and rs17848060 loci and baseline information

In this study, we used MDR to analyze the relevance between the interaction of SNP at rs1804029 and rs17848060 loci with several factors (age, gender, BMI, smoking history, drinking history, knee injury history, KOA family history) of the subjects and the susceptibility of KOA. The effect of various factors on KOA susceptibility is shown in Fig. 1A. As presented in Fig. 1B, the strongest interaction appeared between drinking history and SNP at rs1804029 site. The best prediction model for the interaction of various factors on the prediction of KOA susceptibility risk was the interaction between drinking, knee injury history, knee osteoarthritis family history, and SNPs at *CXCR4* rs1804029 and rs17848060 sites (accuracy = 0.6262, cross-validation consistency = 9/10, $x^2 = 4.38$, $p = 0.04$, Table 6).

Table 6
MDR analysis for the interaction between SNPs at *CXCR4* rs1804029 and rs17848060 sites, and baseline data from enrolled subjects

Model	Testing Balanced accuracy(%)	Cross-validation Consistency	x^2	p
KOA	57.54	10/10	3.60	0.06
KOA,rs17848060	56.39	6/10	1.51	0.22
K,KOA,rs17848060	58.36	5/10	2.00	0.16
K,KOA,rs1804029,rs17848060	60.66	7/10	3.14	0.08
D,K,KOA,rs1804029,rs17848060	62.62	9/10	4.38	**0.04**
A,D,K,KOA,rs1804029,rs17848060	57.70	4/10	1.65	0.20
G,B,S,D,KOA,rs1804029,rs17848060	53.44	2/10	0.30	0.58
G,A,B,S,D,KOA,rs1804029,rs17848060	56.56	9/10	1.05	0.30
G,A,B,S,D,K,KOA,rs1804029,rs17848060	59.51	10/10	2.21	0.13

G,gender; A, Age; B, BMI; S, smoking; D, drinking; K, Knee injury history; KOA, Knee osteoarthritis family history.

3.6 Interaction between CXCR4 and hsa-miR-146 or hsa-miR-20a-3p

Bioinformatic assays predicted that hsa-miR-146 could target CXCR4 harboring G allele at rs1804029 site, and hsa-miR-20a-3p could bind to CXCR4 with A allele at rs17848060 loci (Fig. 2A and 2C). Reporter plasmids including pGL3-CXCR4-rs1804029T, pGL3-CXCR4-rs1804029G, pGL3-CXCR4-rs17848060A, and pGL3-CXCR4-rs17848060T were constructed for dual-luciferase assays. hsa-miR-146a-3p mimic significantly reduced fluorescence activity when co-transfected with the rs1804029 G allele, while hsa-miR-146a-3p inhibitor addition in cells transfected with pGL3-CXCR4-rs1804029G obviously increased

luciferase activity. However, transfection of hsa-miR-146a-3p mimic or inhibitor failed to affect the luciferase activity in cells delivered with pGL3-CXCR4-rs17848060T (Fig. 2B). Similarly, co-transfection of hsa-miR-20a-3p mimic and pGL3-CXCR4-rs17848060A greatly reduced the luciferase signal, while co-transfection of hsa-miR-20a-3p inhibitor and pGL3-CXCR4-rs17848060A improved the luciferase activity. Delivery of the hsa-miR-20a-3p mimic or inhibitor had no effect on the luciferase activity in cells transfected with pGL3-CXCR4-rs17848060T (Fig. 2D). Altogether, these results suggested that CXCR4 carrying G allele at rs1804029 site or carrying A genotype at rs17848060 site could be targeted by hsa-miR-146a-3p or hsa-miR-20a-3p, respectively.

3.7 Quantification of hsa-miR-146a-3p and hsa-miR-20a-3p in plasma

In order to confirm the interaction between *CXCR4* and hsa-miR-146a-3p or hsa-miR-20a-3p, we used RT-qPCR to quantify the plasma levels of hsa-miR-146a-3p and hsa-miR-20a-3p. As shown in Fig. 3A and 3B, the abundance of hsa-miR-146a-3p and hsa-miR-20a-3p in the plasma of KOA patients was lower than those in the control group ($p < 0.001$). In both KOA patients and control groups, subjects carrying TG and GG genotypes at *CXCR4* rs1804029 locus had significantly lower plasma levels of hsa-miR-146a-3p than subjects with TT genotype at rs1804029 site ($p < 0.001$,Figure 3C, 3D). Meanwhile, subjects with AT and TT genotypes at *CXCR4* rs17848060 locus had higher plasma levels of hsa-miR-20a-3p than subjects with AA genotype at *CXCR4* rs17848060 locus ($p < 0.001$, Fig. 3E and 3F). These results showed that individuals carrying G allele at *CXCR4* rs1804029 site had lower expression level of hsa-miR146a-3p in the plasma, whereas individuals with T genotype at rs17848060 site had higher levels of hsa-miR-20a-3p in the plasma.

4. Discussion

In the current research, we studied the correlation between *CXCR4* polymorphism at rs1804029 and rs17848060 loci and the susceptibility to KOA in 305 cases of KOA patients and 305 healthy controls in Chinese Han population. The results revealed that individuals harboring G allele or T allele at *CXCR4* rs1804029 site were more susceptible to KOA. Moreover, subjects with G allele at rs1804029 site had lower level of hsa-miR-146a-3p in the plasma, while hsa-miR-20a-3p was highly expressed in subjects with T allele at rs17848060 locus. Dual-luciferase assays further proved that hsa-miR-146a-3p could bind to *CXCR4* carrying G at rs1804029 locus, and *CXCR4* with A genotype at rs17848060 site was one of the target genes of hsa-miR-20a-3p. Therefore, it was inferred that SNPs at *CXCR4* rs1804029 and rs17848060 loci were significantly associated with KOA susceptibility in the Chinese Han population.

CXCR4 is located on chromosome 2q21 and contains two exons and one intron (Federsppiel et al. 1993; Herzog et al. 1993; Wegner et al. 1998). Generally, the 3'-UTR of a gene has a regulatory region for gene expression. Gene mutations in the regulatory region can affect regulation of gene expression; therefore, SNPs in regulatory region are related to the occurrence and development of several diseases (An et al. 2006; Hashemi et al. 2018; Revathidevi et al. 2016; Seegers et al. 2002). The rs1804029 site is located at

the 3'-UTR targeted binding site of hsa-miR-146a-3p on *CXCR4*. The G to T allele mutation at this site can break the interaction between hsa-miR-146a-3p and the 3'-UTR of *CXCR4*. Similarly, the rs17848060 site is within the binding site of the hsa-miR-20a-3p and *CXCR4*. The A > T allele mutation can affect the binding between hsa-miR-20a-3p and 3'-UTR of *CXCR4*. After adjusting for age, gender, BMI, smoking history, drinking history, knee injury history, and KOA family history, the KOA susceptibility of heterozygosity (TG) at the *CXCR4* rs1804029 locus and dominant model was significantly increased.

Although there was no significant increase in KOA susceptibility of homozygosity (GG), additive model and recessive model, the overall analysis showed that the KOA susceptibility of carriers with G allele at *CXCR4* rs1804029 locus was statistically increased. The reason may be that the sample size of the G allele carrier of the *CXCR4* rs1804029 locus was small, especially the sample size of the subjects carrying GG genotype was small, which led to a large error in statistical analysis. The KOA susceptibility of the heterozygous, homozygous, dominant model, and recessive model of *CXCR4* rs17848060 locus had significantly increased. Although the KOA susceptibility of the additive model was not significantly increased, *CXCR4* with T allele at rs17848060 locus was a susceptible gene for KOA.

Stratified analysis is very important in analyzing the correlation between gene SNPs and development of disease (Wang et al. 2018; Wang et al. 2017; Wu et al. 2015). In the present study, stratified analysis was conducted for the baseline information of enrolled subjects. As expected, stratified analysis of factors including gender, age, BMI, smoking history, drinking history, knee injury history, and KOA family history had a great impact on the correlation between the SNPs at *CXCR4* rs17848060 locus and KOA susceptibility, indicating the necessity of stratified analysis for different groups of subjects. In addition, in the stratified analysis, compared with subjects with TT genotype at rs1804029 locus, the KOA susceptibility was significantly higher in the subjects who carried TG and GG genotypes and had drinking history. When the prior probability was 0.1, the FPRP was less than 0.2, suggesting that the sample size was small and the results of the analysis may be biased.

MDR was applied to analyze the interaction between SNPs at *CXCR4* rs1804029 and rs17848060 loci and the factors including age, gender, BMI, smoking history, drinking history, knee injury history, and KOA family history. The results showed that the interaction between drinking, knee injury history, and knee osteoarthritis family history and SNPs at rs1804029 and rs17848060 loci had the greatest predictive value for KOA susceptibility, indicating that the occurrence of KOA may be induced by multiple factors.

To probe the potential mechanism, dual-luciferase experiments and bioinformatic assays were conducted to investigate the interaction between SNPs at *CXCR4* rs1804029 and rs17848060 sites and predicted miRNAs; experimental data demonstrated the presence of *CXCR4* with G allele at rs1804029, and *CXCR4* with A allele at rs17848060 locus. Although the levels of miRNA in plasma were consistent with the results of dual-luciferase assay, we did not have data on the expression level of *CXCR4* protein in articular cartilage tissue, and further experiments were needed for further verification.

In summary, there are still some limitations in this study that need to be overcome; in particular, the limitation of the sample size affects the objectivity of the results, which need to be further verified in a

large sample size. In addition, potential mechanisms need to be deeply explored in both in vitro and in vivo models.

5. Conclusion

We found that the SNPs at *CXCR4* rs1804029 and rs17848060 loci were significantly associated with KOA susceptibility in Chinese Han population. It was speculated that the reason may be related to the differential binding efficiency of miRNAs on CXCR4 carrying different alleles at rs1804029 and rs17848060 loci, which probably affected the regulation of *CXCR4* expression.

Declarations

Ethics approval and consent to participate

This study was approved by the First Affiliated Hospital of Kunming Medical University medical ethics committee with the following reference number: 2018-L-21, and all the subjects had signed informed consent forms.

Consent for publication

Not applicable

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Guoliang Wang designed the research study and contributed essential reagents or tools; Yang Wang, Yanlin Li, Di Jia and Jiali Zheng analysed the data; Guoliang Wang and Yang Wang wrote the paper.

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Not applicable

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Figures

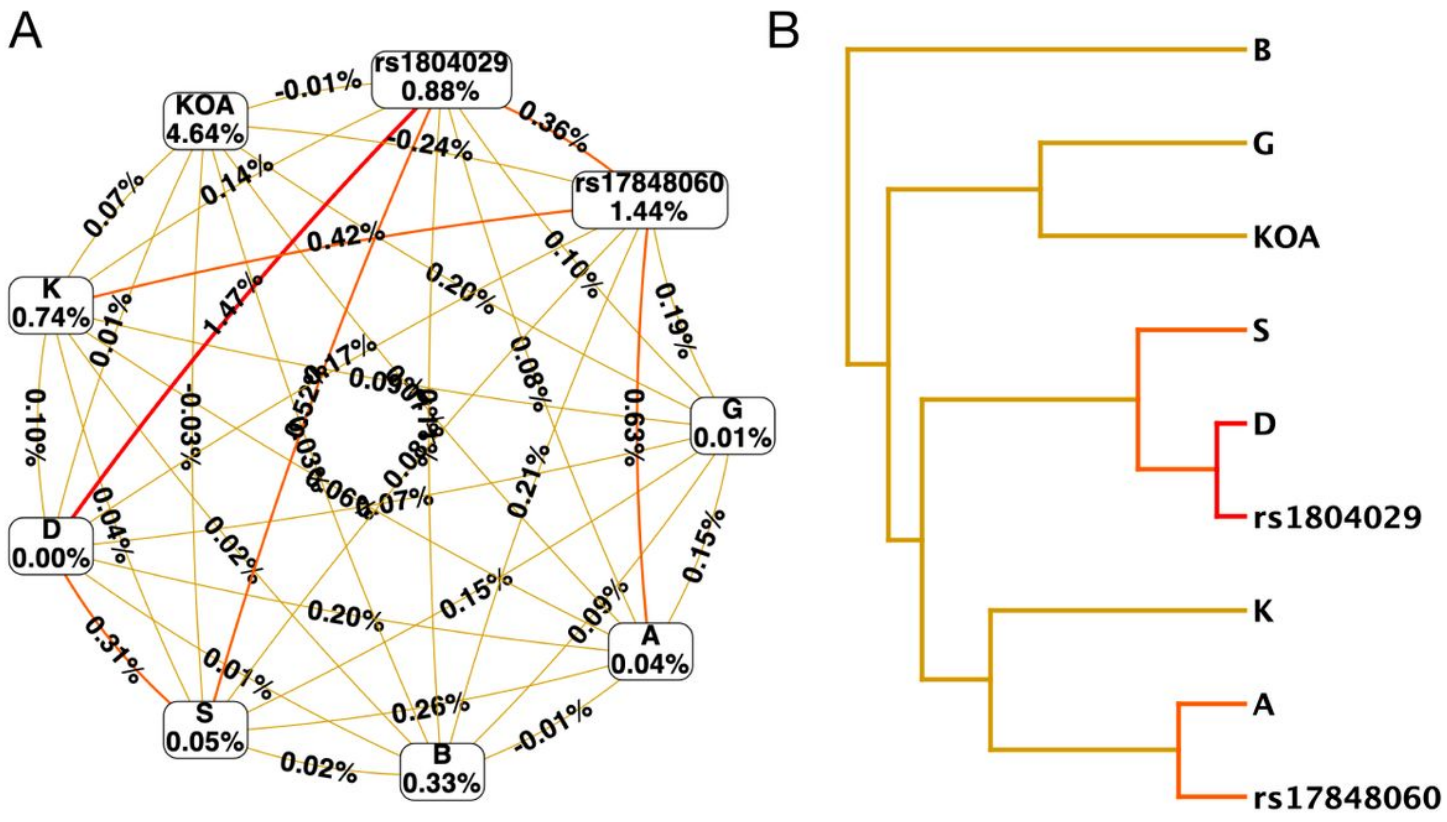

Figure 1

MDR analysis for the interaction between the SNPs on CXCR4 rs1804029, rs17848060 loci and the baseline data of the subject A.Cycle diagram. Data at the apex represented the influence of the factor on KOA susceptibility. Values on the line represented interactions between factors at two apexes. B. Dendrogram diagram. The red line and the closer distance indicated stronger interaction between factors. G, gender; A, Age; B, BMI; S, smoking; D, drinking; K, Knee injury history; KOA, Knee osteoarthritis family history.

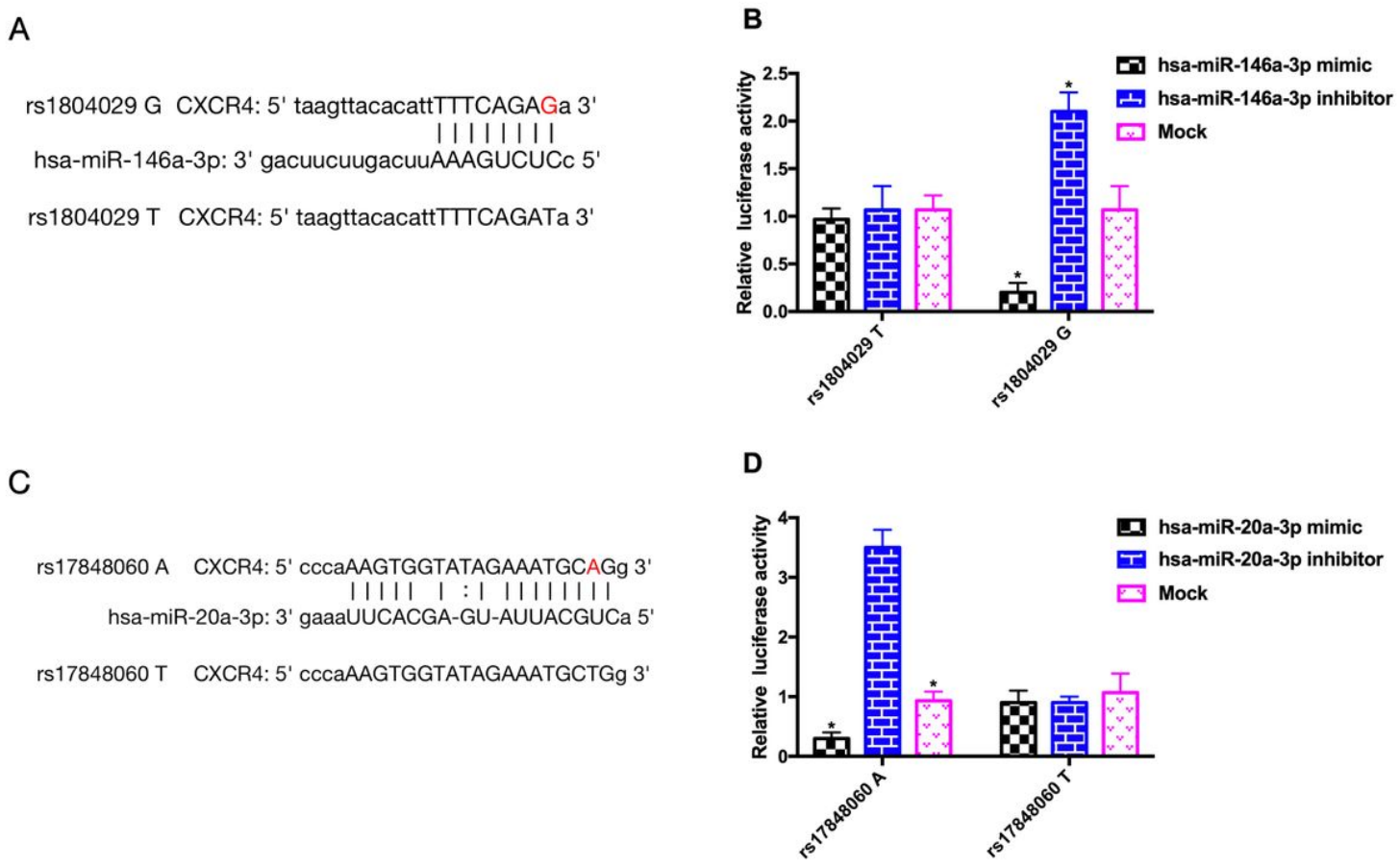

Figure 2

Prediction and verification of the binding sites between CXCR4 and microRNAs A. Prediction of hsa-miR-146a-3p target sequence on CXCR4; B.Dual-luciferase assay to investigate the binding between hsa-miR-146a-3p and CXCR4 carrying G at rs1804029 site; C. Prediction of hsa-miR-20a-3p target sequence on CXCR4; D. Dual-luciferase assay to investigate the binding between hsa-miR-20a-3p and CXCR4 carrying A at rs17848060 site. *, $p < 0.05$, compared to mock group.

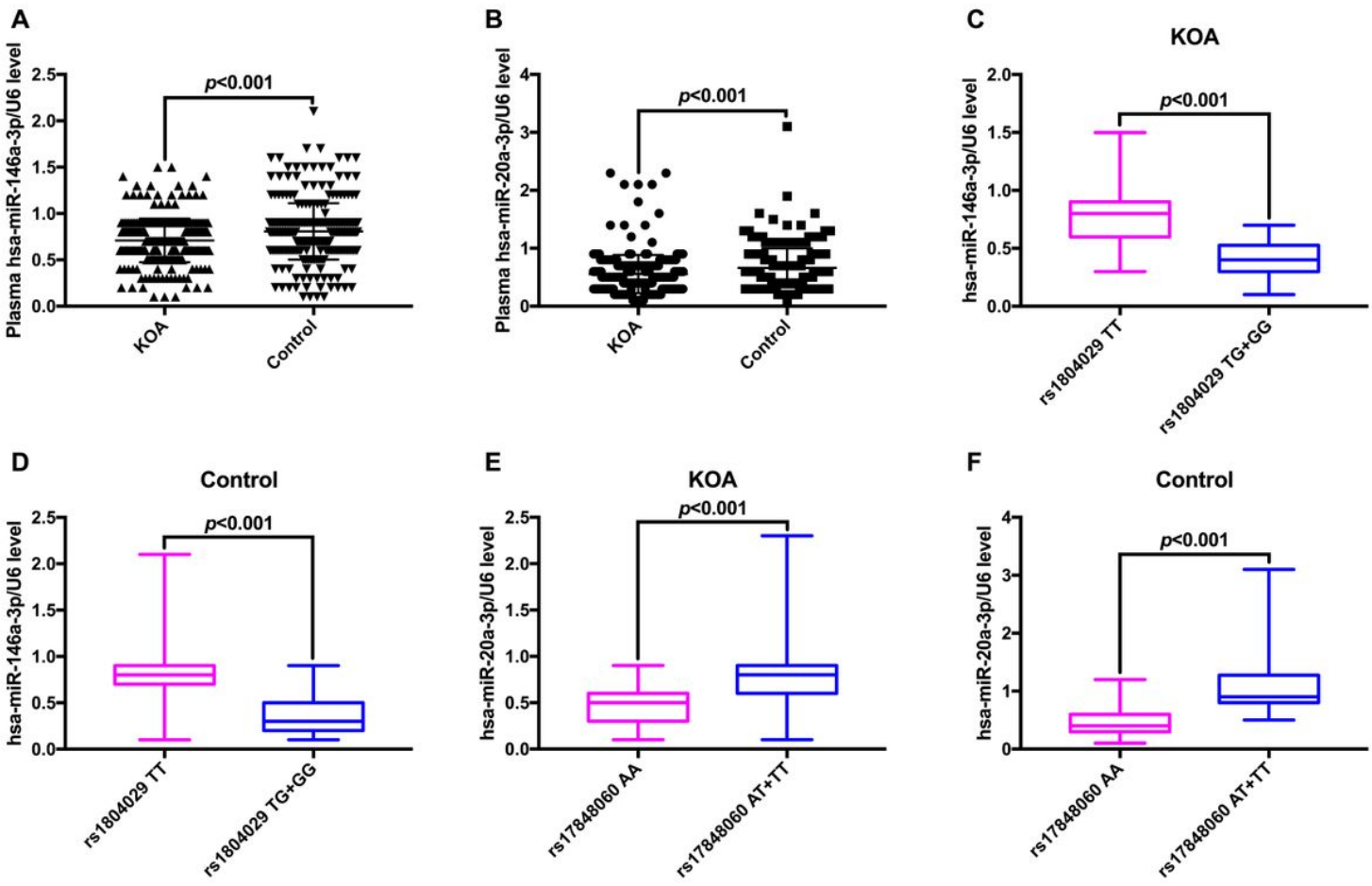

Figure 3

The level of has-miR-146a-3p and has-miR-20a-3p in plasma of subjects were quantified by RT-qPCR. A. The comparison of has-miR-146a-3p between KOA patients and healthy controls; B. The comparison of hsa-miR-20a-3p between KOA patients and healthy controls; C and D. The abundance of hsa-miR-146a-3p in KOA patients and control with diverse SNPs at CXCR4 rs1804029 site; E and F. The abundance of hsa-miR-20a-3p in KOA patients and control with diverse SNPs at CXCR4 rs17848060 site.