Association between microRNA polymorphisms and the risk of inflammatory bowel disease

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Abstract. Common single nucleotide polymorphisms (SNPs) in precursor microRNAs may change their properties via altering the expression of miRNAs, resulting in diverse functional consequences. The present study evaluated the effects of four common SNPs in pro-miRNAs on the risk of inflammatory bowel disease (IBD) and IBD-associated colorectal cancer (IBD-CRC). In a hospital based case-control investigation in a Chinese population, 468 patients with IBD and 450 age- and gender-matched healthy subjects were enrolled in the present study. The SNPs were genotyped using a polymerase chain reaction (PCR)-restriction fragment length polymorphism technique. The expression levels of the miRNAs were detected by reverse transcription-PCR. For rs2910164, the risk of IBD was significantly increased in the GC and CC genotypes. The mean expression levels of mir-146a in the CC and GC genotypes were lower, compared with that of the GG genotype. For rs2292832, an increased risk of IBD was detected in the recessive model of the TT genotype, compared with the combination of the CT and CC genotypes. The [T] allele was found to be at increased significantly, with a 1.268-fold increased risk of IBD, compared with the [C] allele. The mean expression levele of mir-149 expression level in the TT genotype was lower, compared with that of the CC genotype. For rs11614913, the risk of IBD-CRC was significantly increased in the CC genotype, compared with the TT genotype. In the dominant model, the CC genotype had a high risk of IBD-CRC, compared with the combination of the CT and TT genotypes. These findings suggested that mir-146a rs2910164 and mir-149 rs2292832 may be associated with the increased risk of IBD via alterations in the expression levels of miRNAs. Therefore, mir-196a rs11614913 may contribute to the progression of IBD-CRC.

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Introduction

MicroRNAs (miRNAs) are small (~22 nucleotide), endogenous, non-coding RNAs, which downregulate expression via complimentary binding to the 3'-untranslated region of target messenger (m)RNAs, thereby repressing translation or decreasing mRNA stability (1). In previous years, >1,000 miRNAs have been identified in the human genome, which regulate 30% human genes (2,3). Increasing evidence has indicated that miRNAs are important in the development of several human diseases, predominantly by targeting genes, which are key regulators of cell proliferation, differentiation and survival, DNA repair and the immune response (4).

Crohn's disease (CD) and ulcerative colitis (UC) are the two predominant types of idiopathic inflammatory bowel disease (IBD). IBD is a gastrointestinal chronic inflammatory disorder, which has been empirically defined by clinical, pathological, endoscopic and radiological features (5). The worldwide prevalence rate is as high as 39.6/100,000 individuals, the incidence rate for CD varies between 0.1 and 16/100,000 individuals and for UC between 0.5 and 24.5. One of the most serious complications faced by patients with IBD is the potential development of colorectal cancer (CRC). Although IBD-associated CRC (IBD-CRC) accounts for only 1-2% of all cases of CRC, IBD with colon involvement is among the top three high-risk conditions for CRC (6). IBD is considered to arise in genetically susceptible individuals as a consequence of a dysregulated immune response, and involves complex pathophysiological mechanisms (7). Previous evidence indicates that genetic factors are important in the pathogenesis of IBD (8,9), thus genetic risk factors predisposing individuals to IBD remain to be fully elucidated.

The differential expression of miRNA is described in multiple autoimmune-associated disorders, including rheumatoid arthritis, lupus, psoriasis and asthma (10-12). It has been reported that there are changes in the expression levels of miRNA in epithelial cells of patients with active UC and CD, compared with healthy controls, as well as in the progression from normal colonic tissue to dysplastic tissue

in patients with IBD (13). It is well demonstrated that single nucleotide polymorphisms (SNPs) or mutations in miRNAs sequence may alter the expression of miRNAs (14). In addition, several studies have been performed to investigate the association between SNPs in miRNAs and susceptibility to CRC (15,16). Previous studies have shown that four common polymorphisms (rs2910164, rs11614913, rs3746444 and rs2292832) in pre-miRNAs (mir-146a, mir-196a, mir-499 and mir-149, respectively) are associated with an increased risk for several diseases, including CRC (16-18). The present study involved performing a case-control investigation to elucidate the association of these polymorphisms with the risk of the occurrence of IBD, and its progression to IBD-CRC. The results of this study may indicate markers for the occurrence of IBD and the risk of progression to IBD-CRC; this would help physicians identify and treat patients earlier.

Materials and methods

Patient cohorts and study design. Between January 2010 and December 2012, 468 patients with IBD and 450 healthy, unrelated, age- and gender-matched individuals (as a control group) from The First Hospital Affiliated of Henan Science and Technology University (Henan, China) were enrolled in the present study. The diagnosis of IBD was made on the basis of clinical, radiological, endoscopic and histological criteria (19). CD and UC were classified based on the Montreal classification (20). Individuals with other digestive system diseases, including gastric disease and hepatic disease, or chronic diseases, including hypertension and heart disease, were excluded from the investigation. The clinical data documented for the present study were as follows: Type of IBD (CD or UC), age, gender, age at diagnosis, disease localization, clinical symptoms and smoking status. The present study was approved by the Ethical Committee of The Chinese PLA General Hospital (Beijing, China) on 19 November, 2009 (approval no. 2009-039) and all patients provided written informed consent.

DNA extraction. Whole blood samples (~2 ml) from the patients and controls were collected and stored in Vacutainer tubes (BD Biosciences, Fanklin Lakes, NJ, USA), which contained the anticoagulant EDTA. Genomic DNA was extracted from the peripheral whole blood using a Qiagen Blood kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's protocol, and stored at -20˚C until use.

Genotyping. The genotypes were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The primers (Beijing Genomics Institute, Beijing, China) and restriction endonucleases (New England Biolabs, Ipswich, MA, USA) used are summarized in Table I. The PCR reactions were performed using an AmpliTaq Gold PCR kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 μl, containing 1X PCR buffer, 0.2 mM dNTPs, 1 mM $MgCl_2$, 50 pmol of each primer, 20 ng genomic DNA and 1 unit of Ampli-Taq Glod DNA polymerase (Applied Biosystems, Foster City, CA, USA). The PCR parameters were as follows: 95˚C for 5 min, followed by 35 cycles of 95˚C for 30 sec, 30 sec at 58˚C for rs2910164, 30 sec at 60˚C for rs11614913 and 30 sec at 67˚C for rs3746444, 30 sec at 62˚C for rs2292832 and 30 sec at 72˚C, with a final elongation step at 72˚C for 10 min. Following PCR amplification, the products were digested overnight with specific restriction endonuclease at 37˚C. The digested products were electrophoresed on 3% agarose gels (Agarose bead Technologies, Madrid, Spain). Alpha Gel Imaging Systems (Alpha Inotech, Corporation, Santa Clara, CA, USA) was used to detect the electrophoresis results. The products of the genotyping assays are presented in Table I.

Quality control. For quality control purposes, 10% of the samples were randomly selected and sequence analysis was performed, with 100% concordance to the genotype, by PCR-RFLP.

Analysis of mir-RNA expression levels. Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The quality and quantity of the RNAs were assessed by a 260/280 optical density ratio. Reverse transcription reactions were performed using an AffinityScript QPCR cDNA synthesis kit (Agilent Technologies, La Jolla, CA, USA), according to the manufacturer's instructions. The mir-RNAs were detected using TaqMan MicroRNA assays (Applied Biosystems). After the genotyping of miRNA polymorphisms, ~5 ml peripheral blood was obtained from patients with IBD. Quantitative (q)PCR was performed in duplicates on an ABI 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). The qPCR reactions were performed in a total volume of 20 μl, containing 1 μl Taqman Small RNA Assay (X20), 1.33 μl reverse transcription reaction product, 10 μl TaqMan Universal PCR Master Mix and 7.67 μl nuclease-free water. The PCR parameters were as follows: 50˚C For 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Cycles of quantification (Cq) values were acquired, and the relative expression levels of the mature miRNAs were calculated using the Cq_{miRNA}/Cq_{U6} ratio (21).

Statistical analysis. Statistical analysis was performed using the SPSS 17.0 statistical software package (SPSS for Windows; SPSS, Inc, Chicago, IL, USA). A two-tailed χ^2 test was used to determine the differences in genotype distribution between patients and controls. The association between miRNA polymorphisms and IBD were assessed by calculating odds ratio (OR) and 95% confidence interval (95% CI) by logistic regression analysis. A non-parametric Mann Whitney U test was used to compare expression levels of miRNAs. The Hardy-Weinberg equilibrium test was used to evaluate whether there was stratification within the patients enrolled in the study. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of the study population. A total of 468 patients with IBD and 450 healthy individuals were recruited from The First Hospital Affiliated of Henan Science and Technology University for investigation in the present study.

The 468 patients with IBD included 227 patients with CD (48.5%) and 241 patients with UC (51.5%). The mean age of the IBD cohort was 42.2±9.8 years, and the male:female ratio was 1.11:1 (246:222 patients). The mean age of the control group was 41.9±8.6 years, and the male:female ratio was 1.11:1 (237:213 individuals). No significant differences were observed between the patients and controls in mean age (P=0.76) or gender distribution (P=0.35), suggesting adequate matching based on these two variables. The characteristics of the patients with IBD and controls are shown in Table II.

Genotypes. The genotype and allele distributions for the miRNA SNPs in 468 patients with IBD and 450 controls subjects are summarized in Table III. The distributions of genotypes among the two groups were in agreement with the Hardy-Weinberg equilibrium (rs2910164: patients: χ^2=0.367, P=0.545; controls: χ^2=3.573, P=0.059; rs11614913: patients: χ^2=0.521, P=0.479; controls: χ^2=1.046, P=0.306; rs3746444: patients: χ^2=2.827, P=0.093; controls: χ^2=2.542, P=0.111; rs2292832: patients: χ^2=0.308, P=0.579; controls: χ^2=0.448, P=0.504), providing no evidence of population stratification within the dataset. There were statistically significant differences between the frequencies of rs2910164 and rs2292832 genotypes between the IBD group and the healthy controls (χ^2=11.306, *df*=2.0, P=0.004 and χ^2=7.957, *df*=2.0, P=0.032, respectively). In order to assess whether the risk of IBD was associated with the genotype of the miRNA SNPs, logistic regression analysis was performed.

Genotypic distribution in patients and controls

Patients with IBD. As shown in Table III, statistically significant differences were found in mir-146a rs2910164 and mir-149 rs2292832 between the control and IBD groups. For rs2910164 in mir-146a, logistic regression analysis revealed that the risk of IBD was significantly increased in the GC genotype (OR=1.743, 95% CI=1.202-2.525, P=0.003) and CC genotype (OR=1.875, 95% CI=1.276-2.756, P=0.001), compared with the GG genotype. In addition, a similar trend of increased risk of IBD was detected in the recessive model, in which the GC and CC genotypes were combined (OR=1.799, 95% CI=1.269-2.551, P=0.001). The [C] allele was found to be associated with a significant 1.322-fold increased risk of IBD (OR=1.322, 95% CI=1.096-1.593, P=0.003), compared with the [G] allele, indicating that individuals carrying the G allele may have significantly increased IBD susceptibility. For rs2292832 in mir-149, an increased risk of IBD was detected in the recessive model in the TT genotype (OR=1.305, 95% CI=1.006-1.693, P=0.045), compared with the combination of the CT and CC genotypes. The [T] allele was found to be at a significant 1.268-fold increased risk of IBD (OR=1.268, 95% CI=1.035-1.555, P=0.022), compared with the [C] allele. These data showed that individuals carrying the T allele may have significantly increased IBD susceptibility. However, no associations were found between mir-196a rs11614913 or mir-499 rs3746444 and the risk of IBD in the allelic or genotypic analyses.

Patients with CD and controls. As shown in Table III, a statistically significant difference in mir-146a rs2910164 was found between the controls and patients with CD. Logistic regression analysis revealed that the risk of CD was significantly increased in the GC genotype (OR=1.793, 95% CI=1.120-2.869, P=0.015) and CC genotype (OR=1.820, 95% CI=1.117-2.965, P=0.016), compared with the GG genotype. In addition, a similar trend of increased risk of CD was detected in the recessive model, in which GC and CC genotypes were combined (OR=1.804, 95% CI=1.156-2.816, P=0.009). The [C] allele was found to be at a significant 1.288-fold increased risk of CD (OR=1.288, 95% CI=1.023-1.623, P=0.032), compared with the [G] allele, indicating that individuals carrying the G allele may have significantly increased CD susceptibility.

Patients with UC and controls. As shown in Table III, there were statistical significances in mir-146a rs2910164 and mir-149 rs2292832 between the controls and patients with UC. For rs2910164 in mir-146a, logistic regression analysis revealed that the risk of UC was significantly increased in the GC genotype (OR=1.696, 95% CI=1.069-2.689, P=0.025) and the CC genotype (OR=1.927, 95% CI=1.119-3.097, P=0.007), compared with the GG genotype. In addition, a similar trend of increased risk of UC was detected in the recessive model, in which GC and CC genotypes were combined (OR=795, 95% CI=1.162-2.772, P=0.008). The [C] allele was found to be at a significant 1.354-fold increased risk of UC (OR=1.354, 95% CI=1.079-1.699, P=0.009), compared with the [G] allele, indicating that individuals carrying the G allele may have significantly increased UC susceptibility. For rs2292832 in mir-149, logistic regression analysis revealed that the risk of UC was significantly increased in the TT genotype (OR=1.891, 95% CI=1.049-3.407, P=0.034), compared with the CC genotype. Increased risk of IBD was detected in the recessive model in the TT genotype (OR=1.485, 95% CI=1.081-2.039, P=0.015), compared with combination of the CT and CC genotypes. The [T] allele was found to be at a significant 1.565-fold increased risk of UC (OR=1.565, 95%CI=1.207-2.029, P=0.001), compared with the [C] allele, indicating that individuals carrying the T allele may have significantly increased UC susceptibility.

Association between the clinical data and mir-146a rs2910164 and mir-149 rs2292832 polymorphisms. To establish whether the investigated SNPs were associated with specific disease phenotype, the present study analyzed the association of genotypes with gender, location of CD, behavior of CD, extent of UC, severity of UC and smoking habits. As shown in Table IV, no statistically significant differences were found between the clinical characteristics and the mir-146a rs2910164 and mir-149 rs2292832 polymorphisms (P>0.05).

Association between miRNA polymorphism and miRNA expression levels. To further investigate the functional relevance of the miRNA polymorphisms, the present study compared between the genotypes and the expression levels of mir-146a and mir-149 (10 patients for each genotype). As shown in Fig. 1, the mean expression level of mir-146a in the CC and GC genotypes were lower, compared with that of the GG genotype (P=0.000 and P=0.003, respectively). The mean expression level of mir-149 in the TT genotype was lower, compared with that in the CC genotype (P=0.010).

Table I. Primary information from genotyping assays of microRNA single nucleotide polymorphisms.

Gene	Primer sequence (5'-3')	PCR product	Restriction endonuclease	Enzyme product
rs2910164	F-5'-CATGGGTTGTGTCAGTGTCAGAGCT-3' R-5'-TGCCTTCTGTCTCCAGTCTTCCAA-3'	147 bp	*Sac*I	G allele: 147 bp, C allele: 122+25 bp
rs11614913	F-5'-CCCCTTCCCTTCTCCTCCAGATA-3' R-5'-CGAAAACCGACTGATGTAACTCCG-3'	149 bp	*Msp*I	C allele: 125+24 bp, T allele: 149 bp
rs3746444	F-5'-CAAAGTCTTCACTTCCCTGCCA-3' R-5'-GATGTTTAACTCCTCTCCACGTGATC-3'	146	*Bcl*I	A allele: 120+26 bp, G allele: 126 bp
rs2292832	F-5'-TGTCTTCACTCCCGTGCTTGTCC-3' R-5'-TGAGGCCCGAAACACCCGTA-3'	254 bp	*Pvu*II	C allele: 254 bp, T allele: 194+60 bp

F, forward; R, reverse; PCR, polymerase chain reaction.

Table II. Characteristics of the patient and control populations.

Characteristic	CD (n=227)	UC (n=241)	Control (n=450)
Age (year)	43.8±4.7	41.7±10.1	41.9±8.6
Gender			
Male	118 (52.0)	128 (53.1)	237 (52.7)
Female	109 (48.0)	113 (46.9)	213 (47.3)
Age at diagnosis (year)	27.6±9.4	29.1±2.7	
≤16	72 (31.7)	-	-
17-40	121 (53.3)	-	-
>40	34 (15.0)	-	-
Disease location CD, n (%)			-
Ileum	84 (37.0)	-	-
Colon	61 (26.9)	-	-
Ileocolon	82 (36.1)	-	-
Disease behavior CD, n (%)			-
Inflammatory	151 (66.5)	-	-
Stricturing	56 (24.7)	-	-
Penetrating	20 (8.8)	-	-
Disease extent UC, n (%)			-
Ulcerative proctitis	-	31 (12.9)	-
Left-sided UC	-	109 (45.2)	-
Extensive UC	-	101 (41.9)	-
Disease severity UC, n (%)			-
Clinical remission	-	43 (17.8)	-
Mild UC	-	41 (17.0)	-
Moderate UC	-	118 (49.0)	-
Severe UC	-	39 (16.2)	-
Smoking, n (%)	51 (22.5)	57 (23.7)	126 (28.0)

UC, ulcerative colitis; CD, Crohn's disease.

Association between mir-RNAs polymorphisms and the risk of IBD-CRC. Among the patients with IBD enrolled in the present study, 42 patients, including 12 patients with CD and 30 patients with UC, developed IBD-CRC. The male:female ratio was 1.33 [24:18 patients]. The mean age of diagnosis with IBD-CRC was 48.2±8.7 years, the mean duration between age at diagnosed of IBD and age at diagnosis with IBD-CRC was 22.7±10.1 years. In order to identify the association

Table III. Association between single nucleotide polymorphisms in miRNAs and the risk of inflammatory bowel disease.

Genotype	Control (n=450) n (%)	Inflammatory bowel disease (n=468)			Crohn's disease (n=227)			Ulcerative coltits (n=241)		
		n (%)	OR (95 CI)	P-value	n (%)	OR (95 CI)	P-value	n (%)	OR (95 CI)	P-value
mir-146a rs2910164										
Genotype										
GG	97 (21.6)	62 (13.2)	1.00		30 (13.2)	1.00		32 (13.3)	1.00	
GC	202 (44.9)	225 (48.1)	1.743 (1.202-2.525)	0.003[a]	112 (49.3)	1.793 (1.120-2.869)	0.015[a]	113 (46.9)	1.696 (1.069-2.689)	0.025[a]
CC	151 (33.5)	181 (38.7)	1.875 (1.276-2.756)	0.001[a]	85 (37.5)	1.820 (1.117-2.965)	0.016[a]	96 (39.8)	1.927 (1.119-3.097)	0.007[a]
Additive			1.310 (1.089-1.576)	0.004[a]		1.274 (1.016-1.599)	0.036[a]		1.332 (1.067-1.664)	0.011[a]
Dominant										
GC+GG	299 (66.5)	287 (61.3)	1.00		142 (62.5)	1.00		145 (60.2)	1.00	
CC	151 (33.5)	181 (38.7)	1.249 (0.953-1.636)	0.107	85 (37.5)	1.185 (0.850-1.653)	0.316	96 (39.8)	1.311 (0.948-1.812)	0.101
Recessive										
GG	97 (21.6)	62 (13.2)	1.00		30 (13.2)	1.00		32 (13.3)	1.00	
CC+GC	353 (78.4)	406 (86.8)	1.799 (1.269-2.551)	0.001[a]	197 (86.8)	1.804 (1.156-2.816)	0.009[a]	209 (86.7)	1.795 (1.162-2.772)	0.008[a]
Allele										
G	396 (44.0)	349 (37.3)	1.00		172 (37.9)	1.00		117 (36.7)	1.00	
C	504 (56.0)	587 (62.7)	1.322 (1.096-1.593)	0.003[a]	282 (62.1)	1.288 (1.023-1.623)	0.032[a]	305 (63.3)	1.354 (1.079-1.699)	0.009[a]
mir-196a rs11614913										
Genotype										
TT	135 (30.0)	137 (29.3)	1.00		71 (31.3)	1.00		66 (27.4)	1.00	
CT	213 (47.3)	214 (45.7)	0.990 (0.730-1.342)	0.948	106 (46.7)	0.946 (0.654-1.370)	0.770	108 (44.8)	1.037 (0.713-1.508)	0.849
CC	102 (22.7)	117 (25.0)	1.130 (0.791-1.614)	0.500	50 (22.0)	0.932 (0.598-1.453)	0.756	67 (27.8)	1.344 (0.877-2.058)	0.174
Additive			1.059 (0.887-1.265)	0.525		0.964 (0.773-1.202)	0.745		1.156 (0.933-1.4330	0.184
Dominant										
CT+TT	348 (77.3)	351 (75.0)	1.00		177 (78.0)	1.00		174 (72.2)	1.00	
CC	102 (22.7)	117 (25.0)	1.137 (0.839-1.541)	0.407	50 (22.0)	0.964 (0.657-1.415)	0.851	67 (27.8)	1.314 (0.918-1.879)	0.135
Recessive										
TT	135 (30.0)	137 (29.3)	1.00		71 (31.3)	1.00		66 (27.4)	1.00	
CC+CT	315 (70.0)	331(70.7)	0.942 (0.667-1.330)	0.733	156 (68.7)	0.942 (0.667-1.330)	0.733	175 (72.4)	1.136 (0.803-1.609)	0.471
Allele										
T	483 (53.7)	488 (52.1)	1.00		248 (54.6)	1.00		240 (49.8)	1.00	
C	417 (46.3)	448 (47.9)	1.063 (0.885-1.277)	0.512	206 (45.4)	0.960 (0.756-1.204)	0.725	242 (50.2)	1.166 (0.934-1.454)	0.175

Table III. Continued.

Genotype	Control (n=450) n (%)	Inflammatory bowel disease (n=468) n (%)	OR (95 CI)	P-value	Crohn's disease ((n=227) n (%)	OR (95 CI)	P-value	Ulcerative colitis (n=241) n (%)	OR (95 CI)	P-value
mir-499 rs3746444										
Genotype										
AA	339 (75.3)	357 (76.3)	1.00		172 (75.8)	1.00		185 (76.8)	1.00	
AG	105 (23.3)	105 (22.4)	0.950 (0.697-1.293)	0.743	51 (22.5)	0.957 (0.654-1.402)	0.823	54 (22.4)	0.942 (0.648-1.370)	0.756
GG	6 (1.4)	6 (1.3)	0.950 (0.303-2.973)	0.929	4 (1.7)	1.314 (0.366-4.718)	0.675	2 (0.8)	0.611 (0.122-3.057)	0.548
Additive			0.955 (0.723-1.261)	0.745		1.000 (0.712-1.403)	0.998		0.912 (0.647-1.2850	0.599
Dominant										
AA	339 (75.3)	357 (76.3)	1.00		172 (75.8)	1.00		185 (76.8)	1.00	
AG+GG	111 (24.7)	111 (23.7)	0.950 (0.702-1.285)	0.737	55 (24.2)	0.977 (0.673-1.416)	0.901	56 (23.2)	0.924 (0.640-1.335)	0.676
Recessive										
AA+AG	444 (98.6)	462 (98.7)	1.00		223 (98.3)	1.00		239 (99.2)	1.00	
GG	6 (1.4)	6 (1.3)	0.961 (0.308-3.002)	0.945	4 (1.7)	1.327 (0.371-4.752)	0.663	2 (0.8)	0.619 (0.124-3.092)	0.559
Allele										
A	783 (87.0)	819 (87.5)	1.00		395 (87.0)	1.00		424 (88.0)	1.00	
G	117 (13.0)	117 (12.5)	0.956 (0.727-1.258)	0.748	59 (13.0)	1.000 (0.715-1.398)	0.998	58 (12.0)	0.915 (0.654-1.281)	0.607
mir-149 rs2292832										
Genotype										
CC	50 (11.1)	39 (8.3)	1.00		22 (9.7)	1.00		17 (7.1)	1.00	
CT	176 (39.1)	164 (35.0)	1.208 (0.755-1.933)	0.430	84 (37.0)	1.097 (0.624-1.930)	1.097	80 (33.2)	1.352 (0.734-2.490)	0.333
TT	224 (49.8)	265 (56.7)	1.517 (0.962-2.390)	0.073	121 (53.3)	1.228 (0.710-2.124)	0.463	144 (59.7)	1.891 (1.049-3.407)	0.034[a]
Additive			1.240 (1.019-1.509)	0.032[a]		1.112 (0.875-1.412)	0.385		1.385 (1.084-1.769)	0.009[a]
Dominant										
CC		39 (8.3)	1.00		22 (9.7)	1.00		17 (7.1)	1.00	
CT+TT		429 (91.7)	1.382 (0.890-2.147)	0.150	205 (90.3)	1.171 (0.690-1.987)	0.559	224 (92.9)	1.655 (0.932-2.939)	0.085
Recessive										
CT+CC		203 (43.3)	1.00		106 (46.7)	1.00		97 (40.3)	1.00	
TT		265 (56.7)	1.305 (1.006-1.693)	0.045[a]	121 (53.3)	1.142 (0.829-1.572)	0.417	144 (59.7)	1.485 (1.081-2.039)	0.015[a]
Allele										
C	276 (30.7)	242 (25.9)	1.00		128 (28.2)	1.00		114 (23.7)	1.00	
T	624 (69.3)	694 (74.1)	1.268 (1.035-1.555)	0.022[a]	326 (71.8)	1.127 (0.878-1.445)	0.348	368 (76.3)	1.565 (1.207-2.029)	0.001[a]

[a]P<0.05. mir, microRNA; OR, odds ratio; 95 CI, 95% confidence interval.

Table IV. Correlation between mir-146a rs2910164 and mir-149 rs2292832, and clinical characteristics in patients with CD and UC.

	mir-146a rs2910164						mir-149 rs2292832					
	CD (n=227)			UC (n=241)			CD (n=227)			UC (n=241)		
Characteristic	GG (n=30)	GC (n=112)	CC (n=85)	GG (n=32)	GC (n=113)	CC (n=96)	CC (n=22)	CT (n=84)	TT (n=121)	CC (n=17)	CT (n=80)	TT (n=144)
Gender, n (%)												
Male	15 (50.0)	61 (54.5)	42 (49.4)	17 (53.1)	59 (52.2)	52 (54.2)	14 (58.3)	48 (55.8)	56 (47.9)	6 (54.5)	40 (54.1)	82 (52.6)
Female	15 (50.0)	51 (45.5)	43 (50.6)	15 (46.9)	54 (47.8)	44 (45.8)	8 (41.7)	36 (44.2)	65 (52.1)	11 (45.5)	46 (45.9)	62 (47.4)
Location of CD, n (%)												
Ileum	12 (40.0)	38 (33.9)	34 (40.0)				9 (40.9)	33 (39.3)	42 (34.7)			
Colon	7 (23.3)	34 (30.4)	20 (23.5)				7 (31.8)	22 (26.2)	32 (26.4)			
Ileocolon	11 (36.7)	40 (35.7)	31 (36.5)				6 (27.3)	29 (34.5)	47 (38.9)			
Behavior of CD, n (%)												
Inflammatory	21 (70.0)	76 (67.9)	54 (63.5)				13 (59.1)	59 (70.2)	79 (65.3)			
Stricturing	7 (23.3)	27 (24.1)	22 (25.9)				8 (36.4)	22 (26.2)	26 (21.5)			
Penetrating	2 (6.7)	9 (8.0)	9 (10.6)				1 (4.5)	3 (3.6)	16 (13.2)			
Extent of UC, n (%)												
Ulcerative proctitis				4 (12.5)	16 (14.2)	11 (11.5)				3 (17.6)	12 (15.0)	17 (11.8)
Left-sided UC				16 (50.0)	52 (46.0)	41 (42.7)				8 (47.1)	37 (46.2)	64 (44.4)
Extensive UC				12 (37.5)	45 (39.8)	44 (45.8)				6 (35.3)	31 (38.8)	63 (43.8)
Severity of UC, n (%)												
Clinical remission				5 (15.6)	20 (17.7)	18 (18.8)				3 (17.6)	15 (18.8)	25 (17.4)
Mild UC				5 (15.6)	19 (16.8)	17 (17.7)				3 (17.6)	12 (15.0)	26 (18.2)
Moderate UC				16 (50.0)	55 (48.7)	47 (49.0)				9 (52.9)	41 (51.2)	68 (47.2)
Severe UC				6 (18.8)	19 (16.8)	14 (14.5)				2 (11.9)	12 (15.0)	25 (17.4)
Smoking, n (%)	6 (20.0)	26 (23.2)	19 (22.4)	7 (21.9)	25 (22.1)	25 (26.0)	5 (22.7)	21 (25.0)	25 (20.7)	2 (11.8)	19 (23.7)	36 (25.0)

mir, microRNA; CD, Cronh's disease; UC, ulcerative colitis.

Table V. Association between single nucleotide polymorphisms in microRNAs and the risk of IBD-associated CRC.

Genotype	Healthy control (n=100) n (%)	IBD control (n=100) n (%)	CRC (n=42) n (%)	OR (95 CI) (CRC, vs. healthy control)	P-value (CRC, vs. healthy control)	OR (95 CI) (CRC, vs. IBD control)	P-value (CRC, vs. IBD control)
mir-146a rs2910164							
Genotype							
GG	23 (23.0)	14 (14.0)	6 (14.2)	1.00		1.00	
GC	45 (45.0)	48 (48.0)	18 (42.9)	1.533 (0.536-4.389)	0.426	0.875 (0.292-2.626)	0.812
CC	32 (32.0)	38 (38.0)	18 (42.9)	2.156 (0.741-6.274)	0.159	1.105 (0.365-3.349)	0.860
Additive				1.539 (0.918-2.580)	0.102	1.103 (0.650-1.870)	0.717
Dominant							
GC+GG	48 (68.0)	62 (62.0)	24 (57.1)	1.00		1.00	
CC	32 (32.0)	38 (38.0)	18 (42.9)	1.594 (0.759-3.346)	0.218	1.224 (0.588-2.546)	0.589
Recessive							
GG	23 (23.0)	14 (14.0)	6 (14.2)	1.00		1.00	
CC+GC	77 (77.0)	86 (86.0)	36 (85.8)	1.792 (0.671-4.784)	0.244	0.977 (0.348-2.743)	0.964
Allele							
G	91 (45.4)	76 (38.0)	30 (35.7)	1.00		1.00	
C	109 (54.5)	124 (62.0)	54 (64.3)	1.555 (0.915-2.642)	0.103	1.103 (0.649-1.874)	0.716
mir-196a rs11614913							
Genotype							
TT	33 (33.0)	34 (34.0)	10 (23.8)	1.00		1.00	
CT	51 (51.0)	48 (48.0)	18 (42.9)	1.165 (0.479-2.8320	0.737	1.275 (0.542-3.102)	0.592
CC	16 (16.0)	18 (18.0)	14 (33.3)	2.887 (1.054-7.908)	0.039[a]	2.644 (0.980-7.134)	0.055
Additive				1.702 (1.012-2.861)	0.045[a]	1.630 (0.982-2.706)	0.059
Dominant							
CT+TT	84 (84.0)	82 (82.0)		1.00		1.00	
CC	16 (16.0)	18 (18.0)		2.625 (1.139-6.051)	0.024[a]	2.278 (1.004-5.170)	0.049[a]
Recessive							
TT	33 (33.0)	34 (34.0)		1.00		1.00	
CC+CT	67 (67.0)	66 (66.0)		1.576 (0.692-3.591)	0.279	1.648 (0.725-3.750)	0.233
Allele							
T	117 (58.5)	116 (58.0)	38 (45.2)	1.00		1.00	
C	83 (41.5)	84 (42.0)	46 (54.8)	1.706 (1.021-2.852)	0.041[a]	1.672 (1.001-2.793)	0.050

Table V. Continued.

Genotype	Healthy control (n=100) n (%)	IBD control (n=100) n (%)	CRC (n=42) n (%)	OR (95 CI) (CRC, vs. healthy control)	P-value (CRC, vs. healthy control)	OR (95 CI) (CRC, vs. IBD control)	P-value (CRC, vs. IBD control)
mir-499 rs3746444							
Genotype							
AA	73 (73.0)	76 (76.0)	30 (71.4)	1.00		1.00	
AG	25 (25.0)	22 (22.0)	11 (26.2)	1.071 (0.468-2.447)	0.871	1.267 (0.483-3.318)	0.630
GG	2 (2.0)	2 (2.0)	1 (2.4)	1.217 (0.106-13.928)	0.875	1.267 (0.076-21.099)	0.869
Additive				1.080 (0.530-2.203)	0.832	1.222 (0.535-2.791)	0.634
Dominant							
AA	73 (73.0)	76 (76.0)	30 (71.4)	1.00		1.00	
AG+GG	27 (27.0)	24 (24.0)	12 (28.6)	1.081 (0.485-2.411)	0.848	1.081 (0.485-2.411)	0.848
Recessive							
AA+AG	98 (98.0)	98 (98.0)	41 (97.6)	1.00		1.00	
GG	2 (2.0)	2 (2.0)	1 (2.4)	1.195 (0.105-13.548)	0.886	1.195 (0.072-19.706)	0.901
Allele							
A	171 (85.5)	87 (87.0)	71 (84.5)	1.00		1.00	
G	29 (14.5)	13 (13.0)	13 (15.5)	1.080 (0.531-2.197)	0.833	1.225 (0.534-2.811)	0.631
mir-149 rs2292832							
Genotype							
CC	12 (12.0)	8 (8.0)	3 (7.1)	1.00		1.00	
CT	40 (40.0)	34 (34.0)	16 (38.1)	1.600 (0.398-6.434)	0.508	1.225 (0.293-5.371)	0.760
TT	48 (48.0)	58 (58.0)	23 (54.8)	1.917 (0.492-7.462)	0.348	1.057 (0.258-4.340)	0.938
Additive				1.305 (0.749-2.273)	0.348	0.934 (0.537-1.656)	0.839
Dominant							
CC	12 (12.0)	8 (8.0)	3 (7.1)	1.00		1.00	
CT+TT	88 (88.0)	92 (92.0)	39 (92.9)	1.773 (0.473-6.637)	0.395	1.130 (0.285-4.488)	0.862
Recessive							
CT+CC	52 (52.0)	42 (42.0)	19 (45.2)	1.00		1.00	
TT	48 (48.0)	58 (58.0)	23 (54.8)	1.311 (0.636-2.703)	0.463	0.877 (0.424-1.812)	0.722
Allele							
C	64 (32.0)	50 (25.0)	22 (26.2)	1.00		1.00	
T	136 (68.0)	150 (75.0)	62 (73.8)	1.326 (0.750-2.345)	0.332	0.939 (0.525-1.681)	0.833

[a]P<0.05. IBD, inflammatory bowel disease; CRC, colorectal cancer; OR, odds ratio; 95 CI, 95% confidence interval.

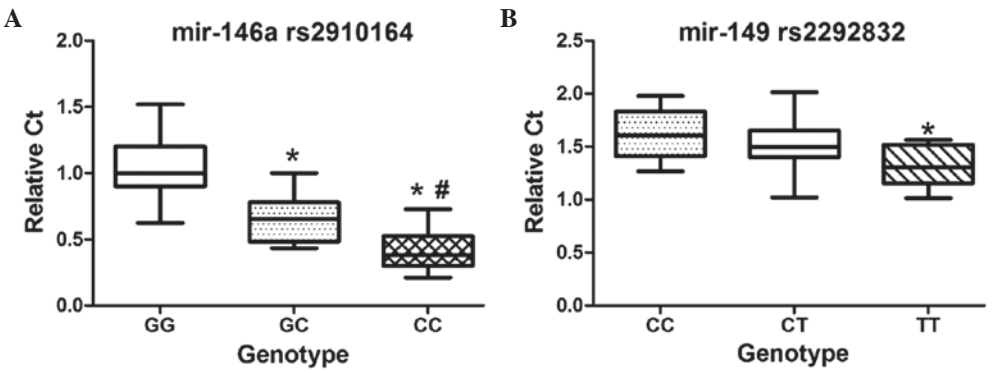

Figure 1. Association between miRNA polymorphisms and miRNA expression levels. (A) Expression level of mir-146a in the rs2910164 genotypes (*P<0.05, compared with GG; #P<0.05, compared with GC). (B) Expression level of mir-149 in the rs2292832 genotypes (*P<0.05, compared with CC). Data are expressed as the mean ± standard deviation. mir/miRNA, microRNA.

between mirRNA polymorphisms and the risk of IBD-CRC, the present study performed analyses among the patients with IBD-CRC, 100 gender- and age-matched healthy individuals, and 100 patients with IBD, whose gender ratio, age and follow-up duration were matched with those of the patients with IBD-CRC. As shown in Table V, for mir-196a rs11614913, the risk of IBD-CRC was significantly increased in the CC genotype (OR=2.887, 95% CI=1.054-7.908, P=0.039), compared with the TT genotype. In the dominant model, the CC genotype had a higher risk of IBD-CRC (OR=2.625, 95% CI=1.139-6.051, P=0.024), compared with the combination of the CT and TT genotypes. Therefore, the [C] allele may be at higher risk of IBD-CRC (OR=1.706, 95% CI=1.021-2.852, P=0.041).

Discussion

The present study involved two innovative aspects. First, it was the first investigation, to the best of our knowledge, of the effects of four common polymorphisms (rs2910164, rs11614913, rs3746444 and rs2292832) in pre-miRNAs mir-146a, mir-196a, mir-499 and mir-149, respectively, on the risk of occurrence of IBD. The results revealed statistically significant differences in mir-146a rs2910164 and mir-149 rs2292832 between the healthy control and IBD groups, and the SNPs in mir-146a and mir-149 decreased the expression levels of mature miRNA. Second, the association between these polymorphisms with the risk of IBD-CRC was examined, which revealed that mir-196a rs11614913 may be associated with the risk of IBD-CRC.

miRNAs are a class of endogenous, small, single-stranded, non-coding RNAs, which have emerged as key regulators of fundamental biological processes, including cell proliferation and differentiation, DNA repair and the immune response, via controlling the expression levels of >30% of human genes (22). miRNAs are initially transcribed as pri-miRNAs with several hundred nucleotides, which are further cleaved by nuclear Drosha into 60-70 nucleotide hairpin-structured pre-miRNAs. Pre-miRNAs are exported to the cytoplasm by Exportin-5 and are further processed into mature miRNAs. Mature miRNAs consist of ~22 nucleotides (1). To date, >1,000 miRNAs have been detected in humans, each of which may regulate multiple genes (23). Physiologically, miRNAs act as post-transcriptional regulators by complimentary binding to the 3' untranslated regions of target messenger RNA transcripts, leading to mRNA degradation or translational repression, and consequently to the downregulation of protein expression (2,20). miRNAs are considered to be key in the regulation of several biological processes, as well as in the induction of inflammatory and autoimmune diseases (24,25). Genetic mutations located with its mature sequence or within the 'seed' region may alter its normal function, leading to a pathological process. In previous years, several studies have indicated that polymorphisms in miRNA are associated with a number of diseases (16,18). Therefore, the present study hypothesized that there is an association between the four common polymorphisms in pri-miRNAs and the risk of IBD and potentially IBD-CRC.

In the present study, statistically significant differences were found in mir-146a rs2910164 and mir-149 rs2292832 between the control and IBD groups. For rs2910164 in mir-146a, the risk of IBD was significantly increased in the GC and CC genotypes, compared with the GG genotype. A similar trend of increased risk of IBD was detected in the recessive model, the [C] allele was found to be at a significant 1.322-fold increased risk of IBD, compared with the [G] allele, indicating that individuals carrying the G allele may have significantly increased IBD susceptibility. In addition, the patients with IBD with the CC or GC genotypes showed lower expression levels of mir-146a in the PBMCs, compared with those with the GG genotype. Previous studies have shown that miRNAs are important in the development of cells of the innate and adaptive immune system, and in regulating an immune response (26). Macrophages and dendritic cells recognize pathogens via pattern recognition receptors, among which Toll-like receptors (TLR) lead to downstream activation of signal transduction pathways and the regulation of inflammatory cytokines (27). The expression of mir-146a can be induced by exposure to lipopolysaccharide, peptidoglycan and flagellin trough TLR ligands (13). Of note, mir-146a is known to be a nuclear factor (NF)κB-dependent gene and reduces the expression of TNF-receptor-associated factor-6 and IL-1 receptor (ILR) associated kinase-1, which are two target genes of the TLR signaling cascade, thus prevent excess inflammation (28). Therefore, the [C] allele-associated reduced expression of mir-146a may affect the negative feedback signaling pathway and contribute to the enhancement of inflammation, which may be associated with the high risk of IBD. In the present study, a difference in the genotypic distribution of mir-149

rs2292832 was found between the control and IBD groups. The [T] allele was found to be at a significant 1.268-fold increased risk of IBD (OR=1.268, 95%CI=1.035-1.555, P=0.022), compared with the [C] allele. In addition, the IBD patients carrying the [T] allele had lower expression levels of mir-149. A previous study reported that mir-149 negatively regulates ILR-triggered inflammatory cytokine production, possibly through a mechanism directly targeting MyD88, involved in the TLR/NF-κB pathway (29), thus lower expression levels of mir-149 decrease the negative regulation of ILR-triggered inflammation.

The present study also analyzed the association between the miRNA SNPs and the risk of IBD-CRC. In comparing between the healthy controls and patients with IBD-CRC, the data showed that, in mir-196a rs11614913, the risk of IBD-CRC was significantly increased in the CC genotype (OR=2.887, 95% CI=1.054-7.908, P=0.039), compared with the TT genotype. In the dominant model, individuals with the CC genotype had a high risk of IBD-CRC (OR=2.625, 95% CI=1.139-6.051, P=0.024), compared with the combination of the CT and TT genotypes. The [C] allele may be associated with a high risk of IBD-CRC (OR=1.706, 95% CI=1.021-2.852, P=0.041). The analysis between the IBD-case controls and patients with IBD-CRC indicated that the CC genotype had a 2.278-fold increased risk of IBD-CRC (OR=2.278, 95% CI=1.004-5.170, P=0.049), compared with the combination of CT and TT genotypes. Several previous studies have suggested that the mir-149 rs2292832 polymorphism is associated with a significantly increased susceptibility of CRC in the TT genotype, compared with the TC and TC/CC genotypes (30). Xu *et al* (31) demonstrated that mir-149 is epigenetically silenced in CRC, and the downregulation of mir-149 is associated with hypermethylation of the neighboring CpG island. mRNA for Specificity protein 1, a potential oncogenic protein, was also identified as a target of mir-149. In addition, it has been reported that the target genes of mir-149, Akt 1 and E2F1, are involved in promoting cell growth and cell cycle progression (31-33). Thus, the present study hypothesized that there was an association between the mir-149 rs2292832 polymorphism and the risk of IBD-CRC.

In conclusion, the results of the present study suggested that mir-146a rs2910164 and mir-149 rs2292832 were associated with an increased risk of IBD in the Chinese population examined. In addition, an association was identifed between mir-196a rs11614913 and the risk of progression of IBD-CRC. As the number of patients with IBD-CRC was limited in the present study, a large sample size is required for further investigation. Based on the results in the current study, in clinical practice, testing for pre-miRNAs polymorphisms may help predict the occurrence of IBD and IBD-CRC, which would help physicians to make early measures for patients. For example, for patients with IBD and the CC genotype in mir-149 rs2292832, regular colonoscopy is necessary to detect early stages of pre-cancerous lesions.

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