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Single nucleotide polymorphisms in the promoter region of mir-133a-1 and in pre-mir-152 rs1707 may contribute to the risk of asthma in a Chinese Han population

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Abstract. – OBJECTIVE: microRNAs (miRNAs) play important roles in the pathogenesis of asthma. Single-nucleotide polymorphisms (SNPs) in the promotor regions of miRNAs or pre-miRNAs are involved in the alteration of miRNA expression levels or their maturation process and can contribute to asthma pathogenesis.

PATIENTS AND METHODS: A total of 591 asthma cases and 621 controls were recruited for this study to evaluate the genetic effects of the following five single nucleotide polymorphisms (SNPs) on the development of asthma: rs8089787 and rs9948906 in the promoter region of mir-133a-1, pre-mir-499 rs37464444, pre-mir-152 rs1707, pre-mir-155 rs5186. The genotypes were determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

RESULTS: Logistic regression analysis revealed that the CT and CT+TT genotypes in the mir-133a-1 rs8089787 (CT vs. CC, OR = 0.413, 95% CI: 0.315-0.541; CT+TT vs. CC, OR = 0.443, 95% CI: 0.342-0.574, respectively) were significantly associated with a decreased risk for asthma in sample of the Chinese Han population, compared with CC genotype. Similarly, the CT and CT+TT genotypes in the mir-133a-1 rs9948906 (CT vs. CC, OR = 0.398, 95% CI: 0.300-0.528; CT+TT vs. CC, OR = 0.403, 95% CI: 0.306-0.532, respectively) were associated with a decreased risk of asthma. However, the C alleles of both mir-133a-1 rs8089787 (C vs. T, OR = 1.867, 95% CI: 1.486-2.345) and rs9948906 (C vs. T, OR = 2.177, 95% CI: 1.690-2.804) were significantly associated with an increased risk for asthma. The CT genotype frequencies of pre-mir-152 rs1707 (CT vs. TT, OR = 4.730, 95% CI: 2.425-9.223) were significantly associated with an increased risk for asthma in a Chinese Han population ($p < 0.001$). The C allele frequencies of pre-mir-152 rs1707 (C vs. T, OR = 6.671, 95% CI: 3.146-14.147) was also significantly associated with an increased risk of asthma in a Chinese Han population ($p < 0.001$). However, the genotype and allele frequencies of rs5186, located in pre-miR-155, did not significantly differ between the cases and the controls; as well as those of rs3746444 in pre-miR-499.

CONCLUSIONS: Our study provided evidence that polymorphisms of rs8089787 and rs9948906 in the promotor region of mir-133a-1 and pre-mir-152 rs1707 may contribute to the risk of asthma in a Chinese Han population.

Key Words: asthma; single nucleotide polymorphism; promoter region; pre-miRNAs

Introduction

Allergic asthma is a complex and chronic inflammatory disorder characterized by wheeze, chest tightness, shortness of breath and reversible airway obstruction[1]. The occurrence of asthma continues to rise. It is a common and serious health problem worldwide. The mechanism for allergy and the subsequent development of allergic diseases in only certain people is yet not clearly defined[2,3]. Allergic asthma is influenced by genetic and environmental factors[4-6], genetic factors may especially play important roles in the pathogenesis of asthma[7-10]. Although the precise mechanism of asthma remains unclear, evidence has shown that activated Th2 lymphocytes and the elaboration of certain cytokines such as interleukin (IL)-13, and IL-5 are responsible for the cascade of eosinophil activation and IgE production necessary for allergic inflammation[11,12].

The microRNAs (miRNAs) are small, noncoding or non-messenger, single-stranded RNA molecules[13,14]. miRNAs can regulate post-transcrip-

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tion of the targets (mRNAs) by partially pairing with their 3'-untranslated regions (3'-UTR)[15]. Studies have shown that miRNAs played important roles in asthma[1,16,17], such as miR-133a[18,19] and miR-499[20], miR-152[21], miR-155[22]. The miRNAs regulate multiple signal transduction pathways that are related to the pathogenesis of asthma[1,23-26]. For example, the miR-152 binding site in the 3'-UTR of the asthma-susceptibility marker HLA-G has been identified[21]. Interleukin (IL)-13 induces airway hyperresponsiveness (AHR), which is a feature of allergic bronchial asthma[27]; RhoA (a monomeric GTP-binding protein) is up-regulated to augment the contractility of bronchial smooth muscle (BSM) in asthmatic mice[19,28,29]. Moreover, both IL-13 and RhoA are negatively regulated by miR-133a in BSM of asthmatic animals[18]. The results of these studies provided us with new opportunities for uncovering the etiopathogenesis of asthma in depth.

To date, the regulation of miRNA expression and maturation is still poorly defined. However, the processing of mature miRNAs may be regulated by single-nucleotide polymorphisms (SNPs) [30]. Many studies have demonstrated that SNPs in miRNAs are related to lung diseases, such as lung cancer and asthma. For example, Yuan et al[31] used a meta-analysis to demonstrate that mir-192a rs1164913 is significantly associated with the increased risk of lung cancer in Asians; mir-146a rs2910164 and mir-149 rs2292832 were associated with a decreased risk of asthma in a Chinese population[32], but mir-146a rs2910164 may play a role in Mexicans' susceptibility to asthma[33].

Although miRNAs mentioned above play important roles in asthma, it has not been reported that whether the SNPs in these miRNAs are associated with asthma risk. Therefore, we hypothesized that the SNPs in the promoter region of miR-133a-1, in pre-miR-499, pre-miR-152, and pre-miR-155 might contribute to the risk of asthma. This case-control study investigates the association between risk of asthma in a Chinese Han population and the following five SNPs: rs8089787 and rs9948906 in the promoter region of mir-133a-1, pre-mir-499 rs37464444, pre-mir-152 rs1707, and pre-mir-155 rs5186.

Patients and Methods

Patients

This case-control study included 591 asthma patients and 621 healthy controls, and was performed with the approval of the medical Ethics Committee of Yijishan Hospital of Wannan Medical College. All of the subjects were periodically enrolled between September 2008 and February 2012 at the Yijishan Hospital of Wannan Medical College in China. The diagnostic criteria of asthma were adopted from the criteria of Chinese Society of Allergology (2008) as follows: (1) continual episodes of wheezing and dyspnea for at least 1 year, with shortness of breath, cough, or chest tightness; (2) clinically diagnosed wheezing; (3) lung function measurement showing a significant reversibility to bronchodilator [≥12% in 1-s forced expiratory volume (FEV_1) and peak expiratory flow (PEF) after delivering bronchodilator] [34]. Healthy individuals, with no history of asthma, were set as controls; they were recruited from the Healthy Testing Center in the same hospital. Written informed consents were obtained from each participant before samples were taken.

DNA Extraction

Genomic DNA was extracted from the 200 μL EDTA-added peripheral blood samples using DNA isolation kits (Sangon Biotech, Shanghai, China) strictly according to the manufacturer's instructions.

Capture PCR and Shrimp Alkaline Phosphatase (SAP)

The PCR amplifications were performed in a total reaction volume of 4 μL containing 1 μL genomic DNA (10 ng/μL), 1 μL primer (10 μM), 1 μL dNTPs (2.5 mM each), 0.625 μL 10 × PCR buffer (containing 15 mM $MgCl_2$), 0.325 μL $MgCl_2$ (25 mM), 0.95 μL H_2O, 0.1 μL Hotstar-Taq DNA polymerase (5 U/μL) (Qiagen, Shanghai, China). Primer sequences were synthesized at Sangon Biotech (Shanghai) Co. Ltd. and are listed in Table I. For PCR analysis, denaturation was conducted at 94 ºC for 15 minutes, then 45 cycles were carried out as follows: 94 ºC for 20 s; 56 ºC for 30 s; and 72 ºC for 1 min, with a final elongation at 72 ºC for 3 min. Residual primers and nucleotides were disabled by incubation, with a total volume of 2 μL shrimp alkaline phosphatase (SAP) containing 0.17 μL 10 × SAP Buffer, 0.3 μL SAP enzyme (1 U/μL) (SEQUENOM, San Diego, CA) and 1.53 μL H_2O. The reactions were incubated at 37 ºC for 40 minutes, then deactivation of SAP (85 ºC, 5 min), and finally stored at 4 ºC. Thereafter, this reaction mixture was directly used for the following primer extension reaction (PEX).

Table I. Primers for MALDI-TOF-MS genotyping analysis.

microRNAs	SNP ID	Primer Sequences	
hsa-mir-133a-1	rs8089787	Forward	5'-ACGTTGGATGCCCCTCTCAAGACATTGAAA-3'
		Reverse	5'-ACGTTGGATGCTATTGTGTGGATAATACTTG-3'
		Competitor	5'-TCAACAGTAAAACCACATTAT-3'
hsa-mir-133a-1	rs9948906	Forward	5'-ACGTTGGATGACTGCCACTGCTTCTTTTAG-3'
		Reverse	5'-ACGTTGGATGGCCACTGATATTCCTACACC-3'
		Competitor	5'-CCACTGCTTCTTTTAGCATTATAG-3'
pre-mir-499	rs37464444	Forward	5'- ACGTTGGATGGGCTGTTAAGACTTGCAGTG-3'
		Reverse	5'- ACGTTGGATGGGAAGCAGCACAGACTTG-3'
		Competitor	5'- cccCTCTCCACGTGAAC-3'
pre-mir-152	rs1707	Forward	5'- ACGTTGGATGCCTTTGTGACTTCAAGAACC-3'
		Reverse	5'- ACGTTGGATGAGTTCAGCATGAGGAAGAGG-3'
		Competitor	5'- ccACTTCAAGAACCCTGACT-3'
pre-mir-155	rs5186	Forward	5'- ACGTTGGATGCCACATAATGCATTTTCTCC-3'
		Reverse	5'- ACGTTGGATGAGAACATTCCTCTGCAGCAC-3'
		Competitor	5'- CAATTCTGAAAAGGAGCTAA-3'

Primer Extension

The primer extension reactions were conducted in a total reaction volume of 2 μL containing 0.2 μL 10 × iPlex Buffer, 0.2 μL iPlex termination mix, 0.804 μL primer (10 μM), 0.401 μL iPlex enzyme (SEQUENOM), and 0.755 μL H_2O. Reaction conditions consisted of extension, denaturation at 94 ºC for 30 s, 5 cycles were performed at 52 ºC for 5 s, and 80 ºC for 5 s. Then 40 cycles were executed at 94 ºC for 5s, 52 ºC for 5s and 80 ºC for 5 s, with a final elongation at 72 ºC for 3 min.

Clean Resin

The iPlex reaction products were desalted by adding 6 mg clean resin (SEQUENOM) to 25 μL water. After incubation for 30 minutes, the reaction mixtures were centrifuged at 3500 × g for 5 min.

MALDI-TOF MS Analysis

The samples were dispensed onto a 384 SpectroCHIP® Array using a Nanodispenser and introduced into a MassArray Compact mass spectrometer (SEQUENOM). Automated spectra acquisition was performed using Spectroacquire (SEQUENOM). Genotype data were extracted by the MassArray Typer software version 3.4 (SEQUENOM).

Statistical Analysis

The genotypes and allele frequencies were counted directly. Hardy-Weinberg equilibrium (HWE) of each SNP was examined in controls and cases using the chi-squared (χ^2) test. Differences of variants in the genotypes or alleles between the cases and controls were also evaluated using the χ^2 test. The associations between each SNP polymorphism and risk of asthma were estimated using logistic regression analysis. Statistical significance was defined as $p < 0.05$. Bonferroni's correction was applied by multiplying the p-values by the number of SNPs. All analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the Study Population

Among the DNA samples of 591 patients and 621 controls, the genotyping for 5 controls in mir-133a-s rs8089787 failed even after repetition for three times. For the other SNPs, the genotyping was successful in all asthma cases and controls. Characteristics of cases and controls including age, sex, forced volume vital capacity (FVC), forced expiratory volume at 1-s intervals (FEV_1)/FVC %, forced expiratory flow (FEF) 25%-75%, and peak expiratory flow (PEF) in this study are summarized in Table II. As shown in Table II, there was no significant difference was detected on age and sex between the cases and the controls by the t-test and χ^2 test, respectively. There were statistic differences on FVC, FEV_1/FVC%, FEF 25%-75% and PEF between the cases and the controls by the *t*-tests ($p < 0.05$). The observed genotype frequencies for two polymorphisms of mir-133a-1 rs8089787 and rs9948906, pre-mir-499 rs3746444, pre-mir-152 rs1707, pre-mir-152 rs5186 in the controls and cases all fall within Hardy-Weinberg equilibrium (data not shown).

Table II. Demographics of the patients and controls

	Cases (n = 591)	Controls (n = 621)	*p*-value
Age, years (mean ± SD)	44.9 ± 15.3	44.7 ± 15.5	0.959[a]
Gender, n (male / female)	356 / 235	385 / 236	0.530[b]
FVC (mean ± SD)	3.10±1.15	4.10 ± 0.95	0.045[a]
FEV1/FVC (%) (mean ± SD)	64.80 ± 11.41	77.25 ± 13.01	0.031[a]
FEF25%-75% (mean ± SD)	1.30 ± 0.43	2.81 ± 1.22	0.001[a]
PEF (mean ± SD)	3.75 ± 1.68	6.77 ± 2.25	0.002[a]

FVC: forced vital capacity; FEV1: forced expiratory volume in 1 s; FEF: forced expiratory flow; PEF: peak expiratory flow. a *p*-value was calculated by t-test for the categorical data; b *p*-value was calculated by Chi-square test for the categorical data.

Genotype and Allele Frequencies of Mir-133a-1 rs8089787 and rs9948906 May Contribute to the Risk of Asthma

The genotype distributions and allele frequencies of mir-133a-1 rs8089787 and rs9948906, pre-mir-499 rs3746444, pre-mir-152 rs1707, pre-mir-152 rs5186 in all subjects are shown in Table III and Table IV, respectively.

The genotype frequencies of mir-133a-1 rs8089787 were 79.7% (CC), 17.8% (CT), 2.5% (TT) in the asthma patients and 63.5% (CC), 34.3% (CT), 2.3% (TT) in the controls. When the mir-133a-1 rs8089787 CC homozygote genotype was used as the reference group, the CT and CT+TT genotypes of mir-133a-1 rs8089787 were significantly associated with a decreased risk for asthma (CT vs. CC, OR = 0.413, 95% CI: 0.315-0.541; CT+TT vs. CC OR = 0.443, 95% CI: 0.342-0.574, respectively). However, the mir-133a-1 rs8089787 TT variant genotype was not associated with the risk of asthma (TT vs. CC, OR = 0.889, 95% CI: 0.424-1.865), compared with the mir-133a-1 rs8089787 CC wild-type homozygote. The genotype frequencies of mir-133a-1 rs9948906 were 83.9% (CC), 15.2% (CT), 0.8% (TT) in the asthma patients, and 67.8% (CC), 30.9% (CT), and 1.3% (TT) in the controls. When the mir-133a-1 rs9948906 CC genotypes were used as the reference group, the CT genotype and CT+TT genotype of mir-133a-1 rs9948906 were associated with a significantly decreased risk of asthma (CT vs. CC, OR = 0.398, 95% CI: 0.300-0.528; CT+TT vs. CC, OR = 0.403, 95% CI: 0.306-0.532, respectively). However, the mir-133a-1 rs9948906 TT variant genotype was not associated with the risk for asthma (TT vs. CC, OR = 0.530, 95 % CI: 0.172-1.634).

As shown in Table IV, there were significant differences in the allelic distribution of mir-133a-1 rs8089787 and rs9948906 polymorphisms among cases and controls. The C allele of rs8089787 was significantly associated with an increased risk of asthma (C vs. T, OR = 1.867, 95% CI: 1.486-2.345) ($p < 0.001$). Similarly, the C allele of rs9948906 was also associated with a significantly increased risk of asthma (C vs. T, OR = 2.177, 95% CI: 1.690-2.804) ($p < 0.001$).

Genotype and Allele Frequencies of Pre-mir-152 rs1707 May also Contribute to the Risk of Asthma

The genotype frequencies of pre-mir-152 rs1707 were 91.7% (TT), 8.3% (CT), 0% (CC) in the asthmatic patients and 98.7% (TT), 1.3% (CT), 0% (CC) in the controls. When the pre-mir-152 rs1707 TT homozygote genotype was used as the reference group, the CT genotype of pre-mir-152 rs1707 was significantly associated with an increased risk for asthma (CT vs. TT, OR = 4.730, 95% CI: 2.425-9.223) ($p < 0.001$), as shown in Table III.

There were significant differences in the allelic distribution of pre-mir-152 rs1707 polymorphisms among cases and controls. The C allele of pre-mir-152 rs1707 was significantly associated with an increased risk of asthma. (C vs. T, OR = 6.671, 95% CI: 3.146-14.147) ($p < 0.001$), as shown in Table IV.

No Significant Association Between two Polymorphisms in pre-mir-499 rs37464444, pre-mir-155 rs5186 and the Risk of Asthma

There are no significant differences in the genotype frequencies and allelic distribution of pre-mir-499 rs37464444 and pre-mir-155 rs5186 polymorphisms among cases and controls. The results showed that there was no association between the single nucleotide polymorphisms of pre-mir-499

Table III. Logistic regression analysis of associations between SNPs in hsa-mir-133a-1 (rs8089787 and rs9948906), pre-mir-499 rs37464444, pre-mir-152 rs1707, pre-mir-155 rs5186 and risk of asthma.

SNP	Genotype	Cases		Controls		OR (95%CI)	*p*-value
		n	%	n	%		
hsa-mir-133a-1 rs8089787	CC	471	79.7	391	63.5	1.00	-
Asthma, n =591	CT	105	17.8	211	34.3	0.413 (0.315-0.541)	< 0.001
Controls, n =616	TT	15	2.5	14	2.3	0.889 (0.424-1.865)	0.757
	CT+TT	120	20.3	225	36.6	0.443 (0.342-0.574)	< 0.001
rs9948906	CC	496	83.9	421	67.8	1.00	-
Asthma, n = 591	CT	90	15.2	192	30.9	0.398 (0.300-0.528)	< 0.001
Controls, n = 621	TT	5	0.8	8	1.3	0.530 (0.172-1.634)	0.269
	CT+TT	95	16.0	200	32.2	0.403 (0.306-0.532)	< 0.001
pre-mir-499 rs37464444	TT	432	73.1	490	78.9	1.00	-
Asthma, n = 591	CT	154	26.1	127	20.5	1.375 (1.052-1.798)	0.020
Controls, n = 621	CC	5	0.8	4	0.6	1.418 (0.378-5.314)	0.604
	CT+CC	159	26.9	131	21.1	1.377 (1.056-1.794)	0.018
pre-mir-152 rs1707	TT	542	91.7	613	98.7	1.00	-
Asthma, n = 591	CT	49	8.3	8	1.3	4.730 (2.425-9.223)	< 0.001
Controls, n = 621	CC	0	0	0	0	-	-
pre-mir-155 rs5186	AA	544	92.0	563	90.7	1.00	-
Asthma, n = 591	AC	47	8.0	58	9.3	0.839 (0.561-1.254)	0.391
Controls, n = 621	CC	0	0	0	0	-	-

Table IV. The allele frequencies of hsa-mir-133a-1 (rs8089787 and rs9948906), pre-mir-499 rs37464444, pre-mir-152 rs1707, pre-mir-155 rs5186 in asthma patients and control subjects.

SNP	Allele	Asthma n (%)	Controls n (%)	OR (95% CI)	*p*-value
hsa-mir-133a-1					
rs8089787 C/T					
Asthma, n = 591	T	135 (11.4)	239 (19.4)	1.00	
Controls, n = 616	C	1047 (88.6)	993 (80.6)	1.867 (1.486-2.345)	< 0.001
rs9948906 C/T					
Asthma, n = 591	T	100 (8.5)	208 (16.7)	1.00	-
Controls, n = 621	C	1082 (91.5)	1034 (83.3)	2.177 (1.690-2.804)	< 0.001
pre-mir-499 rs37464444					
Asthma, n = 591	T	1018 (86.1)	1107 (89.1)	1.00	-
Controls, n = 621	C	164 (13.9)	135 (10.9)	1.321 (1.036-1.685)	0.025
pre-mir-152 rs1707					
Asthma, n = 591	T	1133 (95.9)	1234 (99.4)	1.00	-
Controls, n = 621	C	49 (4.1)	8 (0.6)	6.671 (3.146-14.147)	< 0.001
pre-mir-155 rs5186					
Asthma, n = 591	A	1135 (96.0)	1184 (95.3)	1.00	-
Controls, n = 621	C	47 (4.0)	58 (4.7)	0.845 (0.571-1.253)	0.402

rs37464444, pre-mir-155 rs5186 and the risk of asthma.

Discussion

In this hospital-based case-control study of asthma, we investigated the associations between rs8089787 and rs9948906 polymorphisms in the promotor region of mir-133a-1, as well as the mir-499 rs37464444, mir-152 rs1707, mir-155 rs5186 polymorphisms, and the risk of asthma in a sample of the Chinese Han population. We found that mir-133a-1 rs8089787 CT genotype and CT+TT genotype were associated with a decreased risk of asthma in this population; mir-133a-1 rs9948906 CT genotype and CT+TT genotype were also associated with a decreased the risk of asthma. Nevertheless, the C alleles of both mir-133a-1 rs8089787 and rs9948906 were significantly associated with an increased risk of asthma. Additionally, this study is the first to report a significant association between the CT genotype, the C allele of mir-152 rs1707 and an increased risk of asthma. In our experiment, however, no association between pre-mir-499 rs37464444, pre-mir-155 rs5186 and the risk of asthma was detected in a Chinese Han population.

These miRNAs in our study including miR-133a-1[18,19], miR-499[35-37], miR-152[21] and miR-155[22,38], may play important roles in lung diseases (i.e. lung cancer, acute and/or chronic asthma, airway remodeling). For example, increased expression of mir-499-5p was significantly associated with inhibition of non-small cell lung cancer (NSCLC) proliferation and metastasis[35]. These miRNAs regulate multiple signal transduction pathways related to the pathogenesis of asthma[1,23-26], such as miR-133a[18,19]. Studies have demonstrated that IL-13 induces AHR[27] and expression of RhoA augments the contractility of BSM[19,28,29]. Both IL-13 and RhoA are regulated by miR-133a[18]. The result shows that miR-133a plays an important role in the pathogenesis of asthma.

Wang et al[20] reported that the SNP of pre-miRNA, hsa-mir-499 rs3746444 was associated with the risk of respiratory system disease. However, our result concurs with Su et al[32], who reported that there was no association between SNP of hsa-mir-499 rs3746444 and the risk of asthma in a Chinese Han population. In our investigation, we confirmed that there was no association between SNP of hsa-mir-499 rs3746444 and the risk of asthma in a Chinese Han population. Tan et al[21] reported that a SNP in the 3'untranslated region of HLA-G (an asthma-susceptibility gene) influences the targeting of three miRNAs, including miR-152, to this gene. Another miRNA, miR-155 directly targets interleukin (IL) 13 receptor α1 to modulate the response of human macrophages to IL-13 in determining phenotypes of macrophages[22]. IL-13, a crucial cytokine in the programming of Th2 responses[39], plays a central role in asthma[40,41].

Conclusions

Our study provided the evidence that polymorphisms of mir-133a-1 rs8089787 and rs9948906, and pre-miR-152 rs1707 might contribute to the risk of asthma. These results imply that the genetic factors may play important roles in the pathogenesis of asthma. However, the sample size needs to be enlarged to verify these findings. Also, a future study could include functional evaluations consisting of more rigorous study designs of other ethnic populations.

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Authors' contributions

Yuxin Jiang, lead investigator of the study, participated in its design and coordination and helped to draft the manuscript; Yang Li and Zhida Ma finished MALDI-TOF-MS experiments and wrote the manuscript; Zhiyang Li recruited patients and collected blood samples; Fangyuan Chen performed statistical analyses. All authors approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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