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Associations of IL-18 and IL-9 expressions and gene polymorphisms with asthma

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Abstract. – OBJECTIVE: To explore the associations of interleukin-18 (IL-18) and IL-9 gene polymorphisms with susceptibility to asthma, and to study the associations between IL-18 and IL-9 expression levels and polymorphisms.

PATIENTS AND METHODS: A total of 200 asthma patients in our hospital were collected as disease group, while 200 healthy people were taken as control group. The deoxyribonucleic acid (DNA) was extracted from peripheral blood and sent to the company for the detection of IL-18 and IL-9 gene polymorphisms via sequencing. The levels of serum IL-18 and IL-9 were determined using enzyme-linked immunosorbent assay (ELISA), and arterial blood gas analysis was performed for patients.

RESULTS: The allele distributions at IL-18 gene loci rs189667 and rs360715 had no differences between control group and disease group. The allele distributions at IL-9 gene loci rs1859430 and rs2066758 were different between the two groups (p=0.001, p=0.022), among which the G allele frequency was the highest in disease group [245 (0.613)], and the T allele frequency was also the highest in disease group [240 (0.600)]. There was a difference in the genotype distribution at IL-9 gene locus rs1859430 between the two groups, and the GG genotype frequency in disease group [82 (0.410)] was significantly higher than that in control group (p=0.005). The CC genotype frequency at rs2066758 was significantly lower in disease group [27 (0.135), p=0.044]. In disease group, the frequency of heterozygous model CT (p=0.047) at IL-18 gene locus rs360715, and recessive model GA+AA (p=0.021) and heterozygous model GA (p=0.031) at IL-19 gene locus rs1859430 was significantly lower than that in control group. In disease group, the AC haplotype frequency at IL-18 gene loci rs189667 and rs360715 was evidently lower than that in control group (p=0.048). Disease group had evidently lower AT haplotype frequency (p=0.006) and evidently higher GT haplotype frequency (p=0.000) at IL-9 gene loci rs1859430 and rs2066758. Moreover, the level of serum IL-18 in patients with TT genotype at IL-18 gene locus rs360715 was higher than that in those with other genotypes in disease group (p<0.05), and the level of serum IL-9 in patients with AG genotype at IL-9 gene locus rs1859430 was also higher than that in those with other genotypes in disease group (p<0.05). There was a remarkable association between CT genotype at IL-18 gene locus rs360715 and partial pressure of oxygen (PaO_2) (p=0.035), and between CC genotype at IL-9 gene locus rs2066758 and partial pressure of carbon dioxide ($PaCO_2$) (p=0.041).

CONCLUSIONS: The expression levels of serum IL-18 and IL-9 and their gene polymorphisms are significantly associated with asthma.

Key Words:
Asthma, Gene polymorphism, IL-18, IL-9.

Introduction

Asthma is a respiratory system disease with severe symptoms, and it frequently occurs in adolescents, whose major pathogenesis is the re-exposure of susceptible people to such physical, chemical, and biological factors as pollen, dust, and mites[1]. During seizure of asthma, the pulmonary ventilation function will be affected, the blood oxygen concentration and partial pressure of oxygen (PaO_2) will decline, and the partial pressure of carbon dioxide ($PaCO_2$) will even rise, leading to dyspnea and wheezing in patients. In addition to genetic factors, the occurrence of asthma is associated with cell autophagy[2], smoking[3], and immune regulatory dysfunction. Both type 2 immune response[4] and Toll-like receptor-mediated innate immune response[4] are associated with the occurrence of asthma. It can be seen that changes in the body's immune function may have a great correlation with the onset of asthma. Therefore, studying the changes in the immune environment in asthma patients is significant for the occurrence, development, and treatment of the disease.

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Interleukins (ILs) are cytokines with complex functions produced by a variety of cells, which play regulatory roles in the differentiation and maturation of immune cells, and the immune function and inflammatory response in the body. ILs are involved in a variety of important physiological processes, and their disorders may result in immune disorders *in vivo*, thereby causing autoimmune diseases. IL-35 can inhibit the occurrence of autoimmune diseases, and IL-31 is associated with skin diseases caused by immune disorders[5,6]. Therefore, ILs may play important roles in immune diseases, such as asthma. At the same time, the IL gene polymorphism is associated with susceptibility to various diseases, such as chronic periodontitis[7], psoriasis[8], and prostate cancer[9]. Among them, the associations between polymorphisms of IL-18 and IL-9, as important factors promoting immune response, and asthma have not been reported yet.

In this paper, therefore, the gene polymorphisms at IL-18 gene loci rs189667 and rs360715, and at IL-9 gene loci rs1859430 and rs2066758 were studied in asthma patients and healthy people, combined with the haplotype analysis of the two genes, and detection of expression levels of serum IL-18 and IL-9, so as to explore the associations of IL-18 and IL-9 gene polymorphisms with susceptibility to asthma.

Patients and Methods

General Data

A total of 200 asthma patients treated in our hospital from January 2017 to the present were collected as disease group, while 200 healthy people in the physical examination center were taken as the control group. The clinical data of subjects in both groups were collected, including the patient's name, ID number, age, gender, body mass index (BMI), past medical history, disease history, family history, and drug allergy history. The mean age was (42.31±2.34) years old in control group and (41.24±3.82) years old in disease group. There were no statistically significant differences in such general data as age and gender between the two groups (p>0.05).

Diagnostic criteria for asthma in disease group: the disease is manifested as recurrent wheezing, shortness of breath, chest tightness, and cough. The incidence of the disease is related to exposure to such allergens as pollen, physical and chemical stimuli and upper gastrointestinal infection. During onset, the expiratory wheezing rale is dominated and the expiratory phase is prolonged. The typical symptoms can be treated or relieved spontaneously. Asthma caused by other diseases of the respiratory system is excluded. This investigation was approved by the Ethics Committee of The First People Hospital of Zhangjiagang City, Soochow University. Signed written informed consents were obtained from all participants before the study.

Sample Collection and Processing

About 7-8 mL of peripheral blood was collected from both groups by the on-duty nurse in the department, and centrifuged using a centrifuge at 3000 rpm for 5 min within 2 h. Then, the upper-layer serum and mid-layer nucleated cells were transferred into new centrifuge tubes. The upper-layer serum was stored in liquid nitrogen for later detection of IL levels, and the genomic deoxyribonucleic acid (DNA) was extracted from the mid-layer nucleated cells.

Genomic DNA Extraction

The genomic DNA was extracted from the peripheral blood in both groups using the blood genome extraction kit (Tiangen, Beijing, China) in strict accordance with the instructions of the kit, specifically as follows: according to the volume of samples, 200 μL of protease K solution was added into the centrifuge tube, and peripheral blood samples and 2 mL of buffer (GE) were also added. The mixture was mixed evenly using a vortex oscillator for 1 min, and placed at 65°C for 5 min. Then, 2 mL of absolute alcohol was added into the samples, mixed evenly, and transferred into an absorption column. 2 mL of buffer was added into the absorption column, followed by centrifugation at 4000 rpm for 1 min. Finally, 200 μL of elution buffer was added into the absorption column, and the resulting solution was the genomic DNA. The samples with the optical density (OD) $_{260}$/OD$_{280}$ of 1.8-2.0 were qualified for subsequent studies.

Polymerase Chain Reaction (PCR) Amplification and Analysis of IL-18 and IL-9 Gene Polymorphisms

The polymorphic regions at IL-18 gene loci rs189667 and rs360715, and IL-9 gene loci rs1859430 and rs2066758 were amplified using the PCR instrument. The total PCR system was 25 μL, including 1 μL of forward primers, 1 μL of reverse primers, 0.5 μL of DNA template,

12.5 μL of Taq DNA polymerase, and 10 μL of dH_2O. The PCR conditions are as follows: 95°C for 5 min, (95°C for 35 s, 57°C for 40 s and 72°C for 30 s) × 45 cycles, 72°C for 5 min, and insulation at 4°C. The primers of polymorphic loci are as follows: IL-18 gene locus rs189667: forward (5'→3'): AGAGAGGGGTCGCATGAACT [temperature of melting (Tm) =61.5), reverse (5'→3'): TCATCTGTTAGACTGTCTGCCTT (Tm=61.8); IL-18 gene locus rs360715: forward (5'→3'): ATGTTGGTGACATACATCCTTGC (Tm=62.3), reverse (5'→3'): TGACGGTGGATCATCCTTCAG (Tm=60.8). IL-9 gene locus rs1859430: forward (5'→3'): CTTGTGTCTCTCCGTCCCAAC (Tm=59.3), reverse (5'→3'): ACTATCCTTTTCACCCGATGGA (Tm=60.4); IL-9 gene locus rs2066758: forward (5'→3'): ATGTTGGTGACATACATCCTTGC (Tm=62.1), reverse (5'→3'): TGACGGTGGATCATCCTTCAG (Tm=59.4). The PCR products were sent to Shandong Biotechnology Co., Ltd. (Jinan, China) for sequencing, and the polymorphisms at IL-18 and IL-9 gene loci were analyzed in both groups.

Detection of Lvels of Serum IL-18 and IL-9

The levels of serum IL-18 and IL-9 were determined using enzyme-linked immunosorbent assay (ELISA): the serum samples stored in liquid nitrogen were taken out, and slowly thawed in a refrigerator at 4°C. Then, the levels of serum IL-18 and IL-9 in both groups were determined using the kits (Thermo Fisher Scientific, Waltham, MA, USA) and Luminex 300 system (Luminex Corporation, Austin, TX, USA) according to the instructions. The mean sensitivity in the test was <0.51 pg/mL, and the inter-batch coefficient of variation was 6.1%.

Arterial Blood Gas Analysis

The arterial blood gas analysis was performed for the two groups using the bedside arterial blood gas analyzer. After the arterial blood was drawn by the on-duty nurse, the pH, PaO_2, and $PaCO_2$ were immediately detected.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 23.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Enumeration data were compared using χ^2-test, and the Hardy-Weinberg equilibrium test was performed. Haplotype analysis was conducted on the SHEsis website. $p<0.05$ suggested a statistically significant difference.

Results

Allele Distributions at IL-18 Gene Loci rs189667 and rs360715, and IL-9 Gene Loci rs1859430 and rs2066758

As shown in Table I, the allele distributions at IL-18 gene loci rs189667 and rs360715 had no differences between control group and disease group. Allele distributions at IL-9 gene loci rs1859430 and rs2066758 were different between the two groups (p=0.001, p=0.022), among which the G allele frequency was the highest in disease group [245 (0.613)], and the T allele frequency was also the highest in disease group [240 (0.600)].

Genotype Distributions at IL-18 Gene Loci rs189667 and rs360715, and IL-9 gene loci rs1859430 and rs2066758

As shown in Table II, the genotype distributions at IL-18 gene loci rs189667 and rs360715 had no differences between the two groups. There was

Table I. Allele distributions at IL-18 gene loci rs189667 and rs360715, and IL-9 gene loci rs1859430, and rs2066758.

Gene	Locus	Allele	Control group	Disease group	OR	95% CI	χ^2	*p*-value
IL-18	rs189667	A	187 (0.468)	191 (0.477)	1.04	0.78-1.37	0.08	0.776
		G	213 (0.532)	209 (0.522)				
	rs360715	C	206 (0.515)	218 (0.545)	1.12	0.85-1.48	0.72	0.141
		T	194 (0.485)	182 (0.455)				
IL-9	rs1859430	G	201 (0.502)	245 (0.613)	0.63	0.48-0.84	9.81	0.001
		A	199 (0.497)	155 (0.388)				
	rs2066758	T	208 (0.520)	240 (0.600)	0.72	0.54-0.95	5.19	0.022
		C	192 (0.480)	160 (0.400)				

Table II. Genotype distributions at IL-18 gene loci rs189667 and rs360715, and IL-9 gene loci rs1859430, and rs2066758.

Gene	Locus	Genotype	Control group	Disease group	OR	95% CI	χ^2	*p*-value
IL-18	rs189667	AA	42 (0.210)	42 (0.210)	0.45	0.23-0.67	0.22	0.890
		AG	103 (0.515)	107 (0.535)				
		GG	55 (0.275)	51 (0.255)				
	rs360715	CC	51 (0.255)	67 (0.335)	0.69	0.41-0.78	4.46	0.107
		CT	104 (0.520)	84 (0.420)				
		TT	45 (0.225)	49 (0.245)				
IL-9	rs1859430	GG	52 (0.260)	82 (0.410)	1.22	1.03-1.46	10.38	0.005
		GA	97 (0.485)	81 (0.405)				
		AA	51 (0.255)	37 (0.185)				
	rs2066758	TT	51 (0.255)	67 (0.335)	1.11	0.98-1.21	5.92	0.044
		TC	106 (0.530)	106 (0.530)				
		CC	43 (0.215)	27 (0.135)				

a difference in the genotype distribution at IL-9 gene locus rs1859430 between the two groups, and the GG genotype frequency in disease group [82 (0.410)] was significantly higher than that in control group (p=0.005). The CC genotype frequency at rs2066758 was significantly lower in disease group than that in control group [27 (0.135), p=0.044].

Analysis of Polymorphisms at IL-18 Gene Loci rs189667 and rs360715, and IL-9 Gene Loci rs1859430 and rs2066758 and Modeling

As can be seen from Table III, in disease group, the frequency of heterozygous model CT (p=0.047) at IL-18 gene locus rs360715, and recessive model GA+AA (p=0.021) and heterozygous model GA (p=0.031) at IL-19 gene locus rs1859430 was significantly lower than that in control group.

Haplotype Analysis of IL-18 Gene Loci rs189667 and rs360715, and IL-9 Gene Loci rs1859430 and rs2066758

In disease group, the AC haplotype frequency at IL-18 gene loci rs189667 and rs360715 was evidently lower than that in control group (p=0.048). Disease group had evidently lower AT haplotype frequency (p=0.006) and evidently higher GT haplotype frequency (p=0.000) at IL-9 gene loci rs1859430 and rs2066758 (Table IV).

Associations of Serum IL-18 and IL-9 Levels With Genotypes at IL-18 Gene Loci rs189667 and rs360715, and IL-9 Gene Loci rs1859430 and rs2066758

Except the AG genotype at IL-18 gene locus rs189667, the levels of serum IL-18 and IL-9 were higher in patients with other genotypes in disease group than those in control group. The level of serum IL-18 in patients with TT genotype at IL-18 gene locus rs360715 was higher than that in those with other genotypes in disease group (p<0.05), and the level of serum IL-9 in patients with AG genotype at IL-9 gene locus rs1859430 was also higher than that in those with other genotypes in disease group (p<0.05; Figures 1-4).

Associations Between Clinical Indexes and Genotypes at IL-18 Gene Loci rs189667 and rs360715, and IL-9 Gene Loci rs1859430 and rs2066758

As shown in Table V, there was a remarkable association between CT genotype at IL-18 gene locus rs360715 and PaO_2 (p=0.035), and between CC genotype at IL-9 gene locus rs2066758 and $PaCO_2$ (p=0.041).

Discussion

The occurrence process of asthma is that after the body's exposure to allergens, immune cells such as mastocytes will secrete inflammatory factors, so that the severe immune dysfunction occurs in patients, leading to serious respiratory symptoms[10,11]. During the onset of asthma, the cytokines playing key regulatory roles such as ILs will have expression dysregulation, which may be the root cause of aggravation of the disease. Studies[12-14] have demonstrated that IL-5, IL-33, etc. are highly correlated with the occurrence of asthma. The specific roles of IL-18 and IL-9 in asthma and their mechanisms have not been studied, but both IL-18[15] and IL-9[16] have been proved to be related

Table III. Analysis of polymorphisms at IL-18 gene loci rs189667 and rs360715, and IL-9 gene loci rs1859430, and rs2066758.

	Gene	Locus	Genotype	Control group	Disease group	OR	*p*-value
Dominant model	IL-18	rs189667	AA+AG	145 (0.725)	149 (0.745)	3.87	0.231
			GG	55 (0.275)	51 (0.255)		
		rs360715	CC+CT	155 (0.775)	151 (0.755)	2.57	0.428
			TT	45 (0.225)	49 (0.245)		
	IL-9	rs1859430	GG+GA	149 (0.745)	163 (0.815)	3.58	0.145
			AA	51 (0.255)	37 (0.185)		
		rs2066758	TT+TC	157 (0.785)	173 (0.865)	4.61	0.056
			CC	43 (0.215)	27 (0.135)		
Recessive model	IL-18	rs189667	AA	42 (0.210)	42 (0.210)	0.23	0.874
			AG+GG	158 (0.790)	158 (0.790)		
		rs360715	CC	51 (0.255)	67 (0.335)	2.14	0.457
			CT+TT	149 (0.745)	133 (0.665)		
	IL-9	rs1859430	GG	52 (0.260)	82 (0.410)	7.44	0.021
			GA+AA	148 (0.740)	118 (0.590)		
		rs2066758	TT	51 (0.255)	67 (0.335)	3.71	0.089
			TC+CC	149 (0.745)	133 (0.665)		
Heterozygous model	IL-18	rs189667	AA	42 (0.210)	42 (0.210)	0.68	0.861
			AG	103 (0.515)	107 (0.535)		
		rs360715	CC	51 (0.255)	67 (0.335)	4.83	0.047
			CT	104 (0.520)	84 (0.420)		
	IL-9	rs1859430	GG	52 (0.260)	82 (0.410)	6.48	0.031
			GA	97 (0.485)	81 (0.405)		
		rs2066758	TT	51 (0.255)	67 (0.335)	3.78	0.211
			TC	106 (0.530)	106 (0.530)		
Homozygous model	IL-18	rs189667	AA	42 (0.210)	42 (0.210)	0.67	0.823
			GG	55 (0.275)	51 (0.255)		
		rs360715	CC	51 (0.255)	67 (0.335)	2.85	0.341
			TT	45 (0.225)	49 (0.245)		
	IL-9	rs1859430	GG	52 (0.260)	82 (0.410)	4.23	0.065
			AA	51 (0.255)	37 (0.185)		
		rs2066758	TT	51 (0.255)	67 (0.335)	3.92	0.087
			CC	43 (0.215)	27 (0.135)		

to the occurrence of asthma. It can be inferred that IL-18 and IL-9 may also exert a key immunomodulatory effect in asthma.

The IL-18 and IL-9 gene polymorphisms are associated with susceptibility to a variety of diseases, and they may have important effects of promoting immune cell differentiation and proliferation, and regulating secretion of other key cytokines in these diseases, such as metabolic syndrome[17], type 2 diabetes[18], and Grave's disease[19]. In this study, it was found that the allele distributions at IL-18 gene loci rs189667 and rs360715 had no differences between control group and disease group. The allele distributions at IL-9

Table IV. Analysis of polymorphisms at IL-18 gene loci rs189667 and rs360715, and IL-9 gene loci rs1859430, and rs2066758.

Gene	Haplotype	Control group	Disease group	OR	95% CI	χ^2	*p*-value
IL-18	AC	97.75 (0.244)	106.48 (0.266)	1.122	0.816-1.542	4.502	0.048
	AT	89.25 (0.223)	84.52 (0.211)	0.933	0.666-1.305	0.165	0.685
	GC	108.25 (0.271)	111.52 (0.279)	1.042	0.764-1.421	0.067	0.796
	GT	104.75 (0.262)	97.48 (0.244)	0.908	0.660-1.250	0.349	0.555
IL-9	AC	94.46 (0.236)	82.54 (0.206)	0.841	0.602-1.175	1.031	0.310
	AT	104.54 (0.261)	72.46 (0.181)	0.625	0.446-0.877	7.466	0.006
	GC	97.54 (0.244)	77.46 (0.194)	0.745	0.532-1.043	2.949	0.086
	GT	103.46 (0.259)	167.54 (0.419)	2.066	1.532-2.786	22.915	0.000

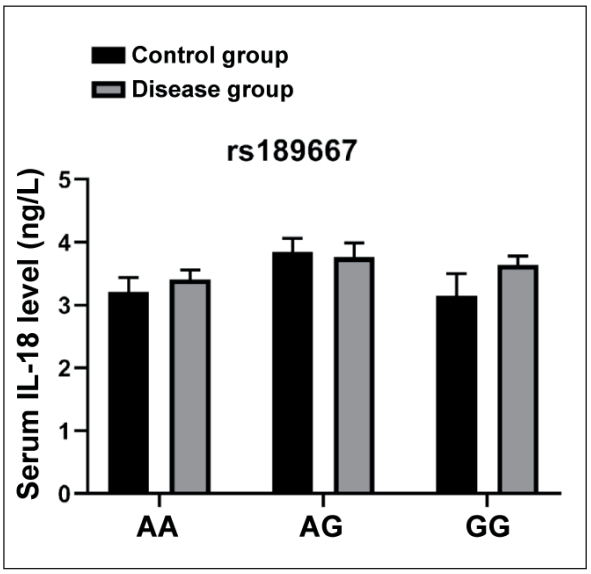

Figure 1. Association between genotype at IL-18 gene locus rs189667 and serum IL-18 level.

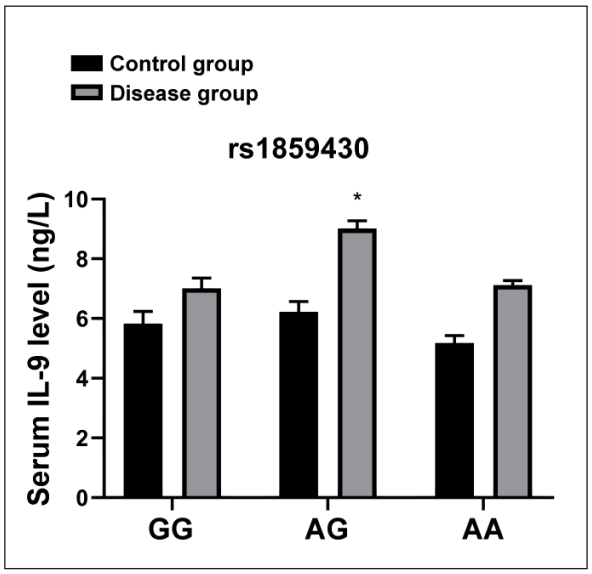

Figure 3. Association between genotype at IL-9 gene locus rs1859430 and serum IL-9 level (*p<0.05 *vs.* GG and AA genotypes).

gene loci rs1859430 and rs2066758 were different between the two groups (p=0.001, p=0.022), among which the G allele frequency was the highest in disease group [245 (0.613)], and the T allele frequency was also the highest in disease group [240 (0.600)], indicating that G allele at IL-9 gene locus rs1859430 and T allele at rs2066758 may be the susceptibility factors for asthma. Besides, there was a difference in the genotype distribution at IL-9 gene locus rs1859430 between the two groups, and the GG genotype frequency in disease group [82 (0.410)] was significantly higher than that in control group (p=0.005). The CC genotype frequency at rs2066758 was significantly lower in disease group [27 (0.135), p=0.044]. The above results suggest that the IL-9 gene loci rs1859430 and rs2066758 are important susceptibility factors for asthma. People with GG

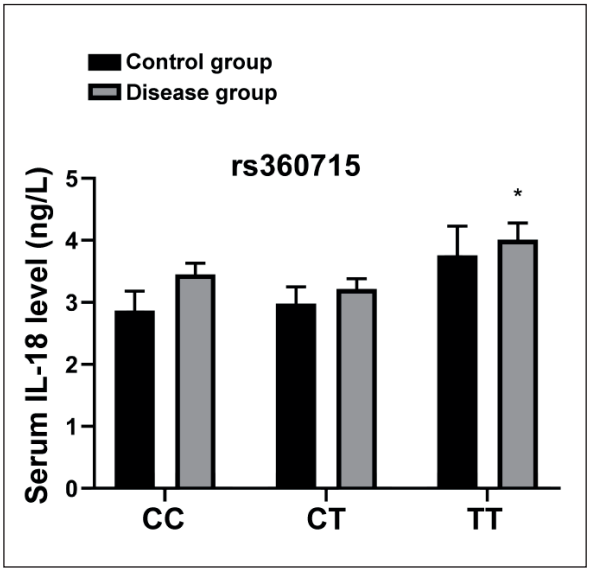

Figure 2. Association between genotype at IL-18 gene locus rs360715 and serum IL-18 level (*p<0.05 *vs.* CC and TT genotypes).

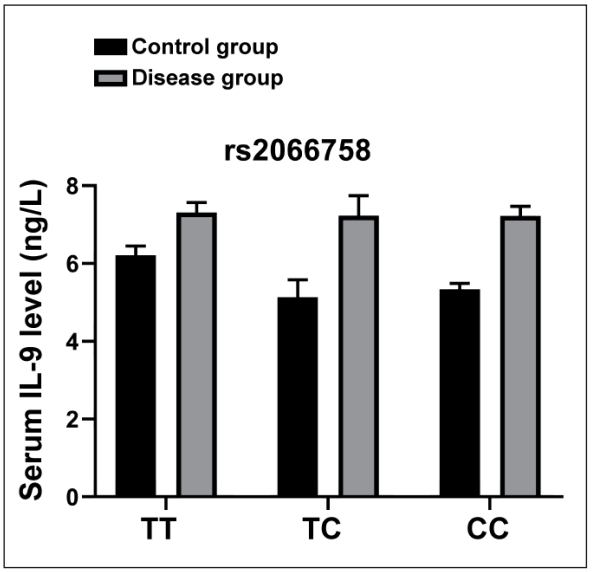

Figure 4. Association between genotype at IL-9 gene locus rs2066758 and serum IL-9 level.

Table V. Associations between clinical indexes and genotypes at IL-18 gene loci rs189667 and rs360715, and IL-9 gene loci rs1859430, and rs2066758.

Gene	Locus	Genotype	pH			PaO_2 (mmHg)			$PaCO_2$ (mmHg)		
			Control group	Disease group	*p*-value	Control	Disease group	*p*-value group	Control	Disease group	*p*-value group
IL18	rs189667	AA	7.45	7.45	0.231	94	84	0.241	40	41	0.251
		AG	7.42	7.35		95	82		36	45	
		GG	7.42	7.4		94	84		42	35	
	rs360715	CC	7.35	7.45	0.341	92	89	0.035	35	44	0.421
		CT	7.37	7.45		91	81		38	38	
		TT	7.36	7.37		95	87		36	39	
IL-9	rs1859430	GG	7.37	7.38	0.087	92	83	0.215	39	42	0.512
		GA	7.41	7.39		94	83		43	38	
		AA	7.36	7.43		94	87		36	35	
	rs2066758	TT	7.35	7.44	0.176	94	85	0.261	35	47	0.041
		TC	7.38	7.42		90	82		41	45	
		CC	7.41	7.39		93	87		44	49	

genotype at rs1859430 are more susceptible to asthma, and they are high-risk groups and should regularly receive physical examination to prevent asthma. People with CC genotype at rs2066758 are less susceptible to asthma, and they are low-risk groups.

According to polymorphism analysis, in disease group, the frequency of heterozygous model CT (p=0.047) at IL-18 gene locus rs360715, and recessive model GA+AA (p=0.021) and heterozygous model GA (p=0.031) at IL-19 gene locus rs1859430 was significantly lower than that in control group. The combinatory analysis of multiple genotypes is of great help for understanding the effects of IL-18 and IL-9 gene polymorphisms on asthma. Moreover, it was observed in haplotype analysis that in disease group, the AC haplotype frequency at IL-18 gene loci rs189667 and rs360715 was evidently lower than that in control group (p=0.048). Disease group had evidently lower AT haplotype frequency (p=0.006) and evidently higher GT haplotype frequency (p=0.000) at IL-9 gene loci rs1859430 and rs2066758. Haplotype analysis is significant in clarifying the effects of polymorphic loci on the susceptibility to asthma.

The expression levels of IL-18 and IL-9 may also be correlated with their single nucleotide polymorphisms. In this study, the level of serum IL-18 in patients with TT genotype at IL-18 gene locus rs360715 was higher than that in those with other genotypes in disease group (p<0.05), and the level of serum IL-9 in patients with AG genotype at IL-9 gene locus rs1859430 was also higher than that in those with other genotypes in disease group (p<0.05). The above findings demonstrate that IL-18 and IL-9 gene polymorphisms may affect the onset of asthma by affecting the levels of serum IL-18 and IL-9, which help the subsequent study on the pathogenesis of asthma.

Conclusions

Arterial blood gas analysis was performed for subjects. The results showed that there was a remarkable association between CT genotype at IL-18 gene locus rs360715 and PaO_2 (p=0.035), and between CC genotype at IL-9 gene locus rs2066758 and $PaCO_2$ (p=0.041), which indicate that IL-18 and IL-9 may facilitate the progression of the disease by affecting PaO_2 and $PaCO_2$, worsening the symptoms.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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