

MicroRNA-146a and Ets-1 Gene Polymorphisms Are Associated with Pediatric Uveitis

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Abstract

Background: MicroRNA-146a (miR-146a) was a key negative regulator of autoimmunity. V-Ets oncogene homolog 1 (Ets-1) was demonstrated to bind to the miR-146a promoter region and markedly affects miR-146a promoter activity. This study aimed to investigate the association of miR-146a and Ets-1 gene polymorphisms with pediatric uveitis in a Han Chinese population.

Methodology/Principal Findings: A total of 520 patients and 1204 healthy controls were included in the present study. Five single-nucleotide polymorphisms (SNPs), miR-146a/rs2910164, miR-146a/rs57095329, miR-146a/rs6864584, ets-1/rs1128334 and ets-1/rs10893872 were genotyped using a polymerase chain reaction-restriction fragment length polymorphism assay. The expression of Ets-1 in peripheral blood mononuclear cells from genotyped healthy controls was tested by real-time PCR. Two SNPs (rs2910164 and rs10893872) were associated with pediatric uveitis in this study. The frequencies of the rs2910164 GG genotype and G allele were significantly increased ($P_c = 3.11 \times 10^{-4}$; $P_c = 2.75 \times 10^{-6}$) while the CC genotype and C allele were significantly decreased ($P_c = 0.001$; $P_c = 2.75 \times 10^{-6}$) in patients compared with normal controls. The frequencies of the rs10893872 CC genotype and C allele were significantly increased ($P_c = 3.89 \times 10^{-4}$; $P_c = 0.01$) while the CT genotype and T allele were significantly decreased ($P_c = 0.004$; $P_c = 0.01$) in patients compared with normal controls. The SNP rs2910164 GG genotype and G/C allele were also associated with the presence of microvascular leakage as detected by fundus fluorescein angiography in pediatric uveitis ($P_c = 0.01$; $P_c = 0.005$, respectively). Ets-1 expression in rs10893872 CC carriers was significantly higher than in CT and TT individuals ($P_c = 0.013$). There was no association of the other three SNPs with pediatric uveitis.

Conclusions: This study shows that miR-146a and Ets-1 are both associated with pediatric uveitis in Han Chinese. SNP rs10893872 may affect the genetic predisposition to pediatric uveitis by modulating expression of Ets-1.

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Introduction

Uveitis is a potentially sight threatening disease and one of the important causes of blindness in the world. It may occur due to an infection or may be due to an autoimmune etiology [1–3]. Children account for 5%~10% of all uveitis patients [4,5]. Although uveitis is less common in children than in adults, patients with pediatric uveitis more often develop serious complications such as cataract and glaucoma that result in visual loss [4]. Epidemiologic studies indicate that idiopathic uveitis (28.8%) is the most common subtype of pediatric uveitis, followed by juvenile idiopathic arthritis (JIA)-associated uveitis (20.9%) and pars planitis (17.1%) [6]. Fundus fluorescein angiography (FFA), rheumatoid factor (RF) and anti-nuclear antibody (ANA) are

considered as important markers for pediatric uveitis [7]. Genetic factors have been reported to be involved in the pathogenesis of pediatric uveitis and a study of 316 Caucasian children demonstrated that human leukocyte antigen (HLA)-A19, HLA-B22 and HLA-DR9 increased the susceptibility of JIA-associated uveitis whereas HLA-DR1 was protective for uveitis development [8]. Other HLA alleles have also been reported to be associated with JIA-associated uveitis such as HLA-DRw5, HLA-DRB1*1104, HLA-DRB1*1301 [8–10]. It is suggested that the genetic factors play important role in pediatric uveitis and investigating proper genetic factors is imperative for pediatric uveitis.

MicroRNAs (miRNAs) are endogenous ~22 nt non-coding RNA molecules that function as negative regulators by targeting

mRNAs for cleavage or translational repression, playing critical roles in diverse biologic processes, such as infection, immune response, inflammation and tumorigenesis [11,12]. The microRNA-146a (miR-146a) was reported as a negative regulator of innate immunity in systemic lupus erythematosus (SLE) patients and a key negative regulator of inflammation [13,14]. It was also indicated to be a vital regulator during viral infection [15,16].

The Ets1 transcription factor is a member of the Ets gene family and is highly conserved throughout evolution. It is known to regulate a number of important biological processes in normal cells and in tumors and associated with regulation of immune cell function and with an aggressive behavior in tumors [19,20]. Moreover, Ets1 can bind to the miR-146a promoter region and markedly affects miR-146a promoter activity *in vitro*. Knockdown of Ets-1 impaired the induction of miR-146a, whereas overexpression of Ets-1 enhanced the induction of miR-146a [21].

Single nucleotide polymorphisms (SNPs), located either in the pre-miRNAs or within miRNA-binding sites, have been shown to affect miRNA target expression, thereby possibly contributing to disease susceptibility [17,18]. Luo et al. identified 12 variants of miR-146a, 9 of which were already reported in the dbSNP database Build 130 [21]. Three variants of these 9 had a minor allele frequency (MAF) >1% (rs2910164, rs57095329, and rs6864584). A recent study showed that SNPs rs1128334 and rs10893872 located in the 3'UTR of Ets-1 were on putative miRNA binding sites and were both associated with SLE in Asian populations [22].

Considering the role of miR-146a in the development of autoimmune diseases and the role of Ets-1 as a regulator of miR-146a expression, we investigated the association of miR-146a/rs2910164, miR-146a/rs57095329, miR-146a/rs6864584, ets-1/rs1128334 and ets-1/rs10893872 with pediatric uveitis in a Han Chinese population. We showed a significant association between both miR-146a and Ets-1 gene polymorphisms and pediatric uveitis in our study population.

Materials and Methods

Study population

The study group comprised 520 consecutive Han Chinese pediatric uveitis patients and 1204 unselected, consecutive, ethnically and geographically matched normal controls. The blood samples of patients and controls were obtained from the Uveitis Study Center of the Sun Yat-sen University (Guangzhou, P.R. China) and the First Affiliated Hospital of Chongqing Medical University (Chongqing, P.R. China). Pediatric uveitis was defined as uveitis first occurring in a child under 16 years old. Children with Behcet's disease, Vogt-Koyanagi-Harada syndrome or with definite infectious uveitis were excluded. JIA was defined as arthritis of unknown etiology presenting in children younger than 16 years old and persisting for at least 6 weeks according to the criteria of International League of Associations for Rheumatology [23]. The study was approved by the Local Ethics Research Committee of the First Affiliated Hospital of Chongqing Medical University. The written informed consent from the guardians on the behalf of minor participants and adult subjects involved in the study were obtained. We adhered to the tenets of the Declaration of Helsinki during all procedures of this study.

Clinical and laboratory observations

Pediatric uveitis patients underwent a uveitis screening protocol encompassing ANA (n = 34; positive rate was 17.8%), RF (n = 32; positive rate was 15.5%), and FFA (n = 192; microvascular leakage rate detected by FFA was found in 62.1% of tested patients). An

ANA titer above 1:100 and an RF level above 20.0 IU/ml were considered positive. FFA was considered a positive result when dye leakage from a retinal vessel was observed. All these tests were performed in the First Affiliated Hospital of Chongqing Medical University (Chongqing, P.R. China).

DNA extraction and genotyping

Genomic DNA of patients and healthy controls was extracted by the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Amplification of the target DNA sequence was analyzed by polymerase chain reaction (PCR) and the primers used herein are shown in Table 1. Each PCR reaction was carried out in a 10 μ l reaction volume containing 5 μ l Premix Taq (Ex Taq Version; TaKaRa Biotechnology Co. Ltd., Dalian, China), 20 pmol primers, 0.2 μ g genomic DNA and proper sterile purified water. Genotyping of the five tested SNPs was carried out by PCR-restriction fragment length polymorphism (RFLP) analysis. PCR products were digested with 2 U of restriction enzymes which are listed in Table 1. The digestion products were separated on 4% agarose gels and stained with GoldView (SBS Genetech Beijing, China). Moreover, 10% of the samples were randomly selected to confirm the PCR-RFLP results using direct sequencing (Invitrogen Biotechnology Co., Guangzhou, China).

RNA extraction and real-time RCR

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of healthy controls by Ficoll-Hypaque density-gradient centrifugation. Total RNA was extracted from PBMCs using TRIzol (Invitrogen, San Diego, California, USA), and then it was reverse transcribed by transcriptase kit (Applied Biosystems, ABI, Foster City, California, USA). Real-time PCR was performed to detect the quantity of Ets-1 mRNA using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following primers Ets-1F: 5'-GCAGCCAGTCATCTTTCAACAGCC-3'; Ets-1R: 5'-TC-AGCACGGTCCCGCACATA-3'. The β -actin (β -actinF: 5'-GGATGCAGAAGGAGATCACTG-3' β -actinR: 5'-CGATC-CACACGGAGTACTTG-3') and GAPDH (F: 5'-GGGTG-TGAACCATGAGAAGT-3'; R: 5'-GGCATGGACTGTGGT-CATGA-3') were chosen as dual internal reference genes to normalize the expression of Ets-1. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

A Hardy-Weinberg equilibrium (HWE) calculation was performed to ensure a balance in genotype and allele distribution between patients and normal controls. HWE was tested in the subjects using the χ^2 test. Genotype and allele frequencies were compared between patients and controls by χ^2 test using SPSS version 17.0 (SPSS, Inc., Chicago, IL). The P values were corrected (Pc) with the Bonferroni correction by multiplying with the number of analyses performed. The odds ratio (OR) was used to determine whether a particular SNP is a risk factor for pediatric uveitis patients, and to compare the magnitude of various SNPs for pediatric uveitis patients (OR = 1: Exposure does not affect odds of pediatric uveitis; OR > 1: Exposure associated with higher odds of pediatric uveitis; OR < 1: Exposure associated with lower odds of pediatric uveitis) [24]. The independent-samples T test was used to compare Ets-1 expression among three genotype groups. P values < 0.05 were considered to be statistically significant.

Table 1. Primers and restriction enzymes used in RFLP analysis.

SNP	Primers	Tm (°C)	Restriction enzymes
rs2910164	5'-ATGGGTTGTGTCAGTGTCCAGACAT-3' 5'-TGCCTTCTGTCTCCAGTCTCCAA-3'	58	HSP92II
rs17057381	5'-GTGCTCAGTTACTGTCCATGCACTT-3' 5'-CAGGCATAGAGGAAGGGGAGATTA-3'	60	AflIII
rs57095329	5'-GGGGCTGCGGAGAGTACCG-3' 5'-GGACCTCTTGACGACGTGTC-3'	60	MspI
rs6864584	5'-CGATAAAGCTCTCGGATTTTC-3' 5'-TCTTATTTGCTGGGTAGAGGA-3'	56	Apal
rs1128334	5'-TATTGTGTTGACTATTTTCCAACAT-3' 5'-ACTTACATCGCTACATCTCT-3'	55	HSP92II
rs10893872	5'-ATCCCAGACCAACCCAGTA-3' 5'-TGGGCAGTAACAGGCTCTTT-3'	60	TSP509I

RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.
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Results

Clinical features of pediatric uveitis patients and normal controls

Detailed clinical findings of the enrolled pediatric uveitis patients and normal controls were presented in Table 2. Age and gender distribution in pediatric uveitis patients and normal controls were also described in Table 2. Only category of JIA-associated uveitis (n = 70) can be confirmed in our study. Other cases cannot be confirmed in which category of pediatric uveitis.

Genotype and allele frequencies of SNPs in patients and controls

A total of 5 SNPs of miR-146a and Ets-1 were successfully genotyped in 520 pediatric uveitis patients and 1204 normal controls. The distribution of genotype frequencies of 5 SNPs were

Table 2. Age, gender and clinical features distribution in pediatric uveitis patients and controls.

Clinical features	%	
Pediatric uveitis patients	n = 520	
Age	9.6 ± 3.6	
Male	242	46.5
Female	278	53.5
Uveitis	520	100
Pediatric uveitis with JIA	70	13.5
FFA(+)	192(309 tested)	62.1
ANA(+)	34(191 tested)	17.8
RF(+)	32(206 tested)	15.5
Controls	n = 1204	
Mean age	34.8 ± 11.4	
Male	545	45.3
Female	659	54.7

JIA, juvenile idiopathic arthritis; FFA, fundus fluorescein angiography; ANA, anti-nuclear antigen; RF, rheumatoid factor.
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accorded with HWE in the controls. Altogether we found two SNPs (rs2910164 and rs10893872) that were associated with pediatric uveitis (Table 3). The frequencies of the rs2910164 CC genotype and C allele in pediatric uveitis patients were significantly lower than that in normal controls ($P = 8.93 \times 10^{-5}$, $P_c = 0.001$, OR = 0.646; $P = 5.50 \times 10^{-7}$, $P_c = 2.75 \times 10^{-6}$, OR = 0.686, respectively). While the frequencies of the rs2910164 GG genotype and G allele were significantly higher in pediatric uveitis patients compared to normal controls ($P = 2.07 \times 10^{-5}$, $P_c = 3.11 \times 10^{-4}$, OR = 1.773; $P = 5.50 \times 10^{-7}$, $P_c = 2.75 \times 10^{-6}$, OR = 1.457, respectively). In SNP rs10893872, the frequencies of the CC genotype and C allele were significantly increased ($P = 2.59 \times 10^{-5}$, $P_c = 3.89 \times 10^{-4}$, OR = 1.582; $P = 0.002$, $P_c = 0.01$, OR = 1.259) in patients compared to controls while the CT genotype and T allele were significantly decreased in patients compared to controls ($P = 2.63 \times 10^{-4}$, $P_c = 0.004$, OR = 0.680; $P = 0.002$, $P_c = 0.01$, OR = 0.794). There was no statistically significant difference concerning the genotype and allele of the other three SNPs between pediatric uveitis patients and controls (Table 3).

We subsequently studied the associations of the 5 SNPs with clinical and laboratory observations of pediatric uveitis including FFA, RF and ANA. An association was found between rs2910164 and patients with microvascular leakage as detected by FFA (FFA⁺). The GG genotype and G allele frequencies of rs2910164 were significantly increased in FFA⁺ patients compared to controls ($P = 6.84 \times 10^{-4}$, $P_c = 0.01$, OR = 1.899; $P = 9.41 \times 10^{-4}$, $P_c = 0.005$, OR = 1.441) while the C allele frequency was significantly decreased ($P = 9.41 \times 10^{-4}$, $P_c = 0.005$, OR = 0.694) (Table 4). No statistically significant association was found between the other four SNPs and FFA⁺ patients. Moreover, there was no statistically significant association between the five SNPs and patient groups that were subdivided according to their RF or ANA status or whether they had JIA or not (data not shown).

The influence of rs10893872 on Ets-1 expression

Because a significant association was found between SNP rs10893872 and pediatric uveitis patients, we further tested the expression of Ets-1 in PBMCs obtained from 32 healthy individuals with a known genotype for this SNP. The mean

Table 3. Frequencies of genotypes and alleles of microRNA (miR)-146a and Ets-1 polymorphisms in pediatric uveitis patients and controls.

SNP	Genotyp Allele	Pediatric uveitis (n = 520)	Controls (n = 1204)	χ^2	p value	pc value	OR (95% CI)
rs2910164	GG	113(0.217)	163(0.135)	18.127	2.07×10^{-5}	3.11×10^{-4}	1.773 (1.359–2.313)
	GC	248(0.477)	553(0.459)	0.453	0.501	NS	1.073(0.873–1.319)
	CC	159(0.306)	488(0.405)	15.350	8.93×10^{-5}	0.001	0.646(0.519–0.805)
	G	474(0.456)	879(0.365)	25.080	5.50×10^{-7}	2.75×10^{-6}	1.457(1.257–1.688)
	C	566(0.544)	1529(0.635)	25.080	5.50×10^{-7}	2.75×10^{-6}	0.686(0.592–0.796)
rs57095329	AA	317(0.609)	801(0.665)	4.937	0.026	NS	0.786(0.635–0.972)
	AG	184(0.354)	372(0.309)	3.347	0.067	NS	1.225(0.985–1.522)
	GG	19(0.037)	31(0.026)	1.502	0.220	NS	1.435(0.803–2.564)
	A	818(0.787)	1974(0.820)	5.206	0.023	NS	0.810(0.676–0.971)
	G	222(0.213)	434(0.180)	5.206	0.023	NS	1.234 (1.030–1.479)
rs6864584	CC	2(0.004)	5(0.004)	0.008	0.927	NS	0.926(0.179–4.788)
	CT	39(0.075)	112(0.093)	1.476	0.224	NS	0.791(0.541–1.156)
	TT	479(0.921)	1087(0.903)	1.466	0.226	NS	1.257(0.867–1.823)
	C	43 (0.041)	122(0.051)	1.384	0.239	NS	0.808(0.566–1.153)
	T	997(0.959)	2286(0.949)	1.384	0.239	NS	1.237(0.867–1.766)
rs1128334	AA	64(0.123)	145(0.120)	0.024	0.877	NS	1.025(0.749–1.403)
	AG	253(0.487)	544(0.452)	1.760	0.185	NS	1.150(0.936–1.413)
	GG	203(0.390)	515(0.428)	2.085	0.149	NS	0.857(0.694–1.057)
	A	381(0.366)	834(0.346)	1.273	0.259	NS	1.091(0.938–1.270)
	G	659(0.634)	1574(0.654)	1.273	0.259	NS	0.916(0.788–1.066)
rs10893872	CC	209(0.402)	359(0.298)	17.695	2.59×10^{-5}	3.89×10^{-4}	1.582(1.277–1.960)
	CT	218(0.419)	620(0.515)	13.320	2.63×10^{-4}	0.004	0.680(0.552–0.837)
	TT	93(0.179)	225(0.187)	0.156	0.693	NS	0.948(0.726–1.238)
	C	636(0.612)	1338(0.556)	9.270	0.002	0.01	1.259(1.085–1.460)
	T	404(0.388)	1070(0.444)	9.270	0.002	0.01	0.794(0.685–0.921)

pc, Bonferroni corrected p value; OR, odds ratio; NS, not significant; SNP, single nucleotide polymorphism.
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Table 4. Genotype and allele frequency analysis of rs2910164 polymorphism in FFA positive pediatric uveitis patients.

SNP	Genotyp Allele	FFA ⁺ pediatric uveitis (n = 192)	Controls (n = 1204)	χ^2	p value	pc value	OR (95% CI)
rs2910164	GG	44(0.229)	163(0.135)	11.533	6.84×10^{-4}	0.01	1.899(1.305–2.762)
	GC	86(0.448)	553(0.459)	0.086	0.769	NS	0.955(0.703–1.297)
	CC	62(0.323)	488(0.405)	4.709	0.030	NS	0.700(0.506–0.967)
	G	174(0.453)	879(0.365)	10.941	9.41×10^{-4}	0.005	1.441(1.160–1.791)
	C	210(0.547)	1529(0.635)	10.941	9.41×10^{-4}	0.005	0.694(0.558–0.862)

pc, Bonferroni corrected p value; OR, odds ratio; NS, not significant; SNP, single nucleotide polymorphism.
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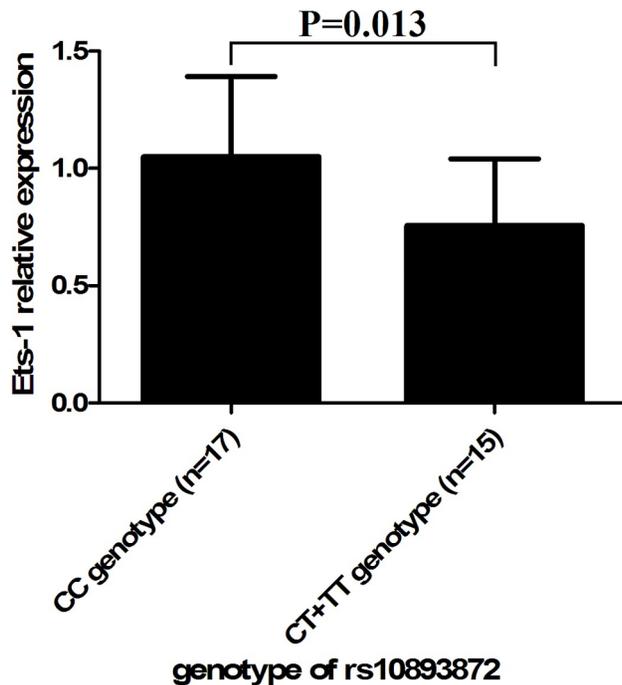


Figure 1. The relative expression of Ets-1. Ets-1 expression with three different genotypes of single nucleotide polymorphism (SNP) rs10893872 in peripheral blood mononuclear cells obtained from genotyped healthy controls (PBMCs). Real-time PCR analysis of Ets-1 expression in PBMCs derived from healthy individuals of SNP rs10893872 CC, CT and TT genotypes. The y axis represents relative Ets-1 expression level by real-time PCR of each genotype of SNP rs10893872. Data are shown as mean \pm SD. doi:10.1371/journal.pone.0091199.g001

expression of Ets-1 in CC carriers was 1.39-fold higher than that in CT and TT carriers ($P = 0.013$) (Figure 1).

Discussion

In the present study, we investigated the association of five SNPs of miR-146a and the transcription factor Ets-1 with pediatric uveitis patients in a Chinese Han population. To the best of our knowledge, this is the first study to investigate the possible associations of miR-146a and Ets-1 polymorphisms with pediatric uveitis. It was found that rs2910164 of miR-146a and rs10893872 of Ets-1 were both associated with pediatric uveitis. The expression of Ets-1 in individuals carrying the rs10893872 CC genotype was increased compared to the CT and TT genotype, suggesting a functional explanation for the observed association.

The frequencies of the rs2910164 CC genotype and C allele were significantly decreased in patients as compared to normal controls while the GG genotype and G allele were significantly increased. It was suggested that miR-146a might be a susceptibility factor for pediatric uveitis. Recently, we studied the miR-146a rs2910164 polymorphisms in acute anterior uveitis patients with ankylosing spondylitis and found a similar association as shown in the present work for pediatric uveitis patients [25]. Several similar results were also reported in ocular Behçet's disease [26], asthma [27] and various types of carcinoma [28–30]. Nevertheless, three SNPs of miR-146a/rs2910164, ets-1/rs1128334, and ets-1/rs10893872 were genotyped in Han Chinese patients with Fuchs uveitis syndrome (FUS), and no significant difference could be found between patients and the normal controls [31]. This is

different with results found herein. Moreover, a functional analysis performed in our previous study showed that carriers of the protective CC genotype showed a decreased expression of miR-146a and certain proinflammatory cytokines such as IL-17, TNF- α and IL-1 β [26]. These results suggest that the rs2910164 CC genotype and C allele and GG genotype and G allele are common predisposing factors for pediatric uveitis despite the fact that the pathogenic mechanisms underlying cancer and pediatric uveitis are totally different.

We also identified an association between SNP rs10893872 of Ets-1 and pediatric uveitis in the present study. The CC genotype and C allele frequencies of this SNP were significantly increased in patients compared to controls while the frequencies of the CT genotype and T allele were significantly decreased, indicating that the CC genotype and C allele were predisposing factors for pediatric uveitis. Based on this association, we further designed a study to investigate whether this SNP (rs10893872) affected the expression of Ets-1. Our study demonstrated that the Ets-1 expression was significantly upregulated in healthy individuals carrying the CC genotype of rs10893872. We performed this assay in healthy individuals since immunosuppressive treatment in the patient group could possibly alter gene expression. SNP rs10893872 of Ets-1 was found to be highly associated with SLE [22,32]. Differently, there was no significant association of SNP rs10893872 of Ets-1 found in ocular Behçet's disease, Vogt-Koyanagi-Harada syndrome, and Fuchs uveitis syndrome in Chinese Han patients with previous works [26,31].

The transcription factor Ets-1, originally discovered as an oncogene (*v-ets*) within the genome of the avian leukemia virus [33], shows a dual nature in autoimmune diseases [34]. It was reported to be overexpressed in rheumatoid arthritis (RA) synovial membrane and to be involved in the destructive pathway of RA [35]. Nevertheless, the expression of Ets-1 in SLE was lower in PBMCs as compared with that of healthy subjects [36]. Ets-1 also plays important roles in regulating the differentiation of T helper cell subsets, cytotoxic T cells, B cells and other cell types, and controlling the expression of cytokine and chemokine genes in a wide variety of different cellular contexts [37]. The regulatory T cell (Treg) lineage is a key player maintaining peripheral self-tolerance and modulating almost any type of immune responses. Julia K. Polansky *et al.* have proposed that Ets-1 binds to the CpG-rich Treg specific demethylated region only in its demethylated state, thereby restricting stable Foxp3 expression to the Treg lineage [38]. Concerning the important roles of Ets-1, the increased frequency of the rs10893872 CC genotype in patients and the increased expression of Ets-1 in CC genotype carriers suggest that Ets-1 is a predisposing factor in pediatric uveitis.

It is worthwhile to mention that there are several limitations in the present study. The sample of patients in our study is relatively small and only Han Chinese cohorts are included. Therefore, the results observed in this study need to be confirmed using a large sample size and should include other ethnic populations. We have not investigated whether the SNP rs10893872 influencing Ets-1 expression can also affect the production of cytokines importantly involved in pediatric uveitis development. Further studies are needed to clarify this issue.

In conclusion, our study has identified the associations of rs2910164 (miR-146a) and rs10893872 (Ets-1) with pediatric uveitis. Furthermore, our study has suggested that SNP rs10893872 may affect the genetic predisposition to this disease possibly through modulating the expression of Ets-1.

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Author Contributions

Conceived and designed the experiments: LW QZ PY. Performed the experiments: LW LB YL. Analyzed the data: LW SH JQ. Contributed reagents/materials/analysis tools: YZ QX. Wrote the paper: LW PY AK.