



Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com



Association of *STAT6* genetic variants with childhood atopic dermatitis in Taiwanese population

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ARTICLE INFO

Article history:

Received 20 March 2015

Received in revised form 14 May 2015

Accepted 19 May 2015

Keywords:

Childhood

Atopic dermatitis

STAT6

Signal transducer and activator of transcription 6

Genetic variant

ABSTRACT

Background: Atopic dermatitis (AD) is the single most common allergic disease in children. *STAT6* has been noted as a hub molecule in IL-4 mediated response and AD pathogenesis. However, the association between *STAT6* genetic variants and childhood AD has never been thoroughly examined.

Objective: We investigate the association between *STAT6* genetic variants and childhood AD risk in Taiwanese population.

Methods: We used data from the Han Chinese in Beijing genome panel of International HapMap Project and the Taiwan Children Health Study cohort to investigate the association of *STAT6* genetic variants and childhood AD risks. Four tagged SNPs were selected from HapMap database and rs324011 was most significantly associated with childhood AD. Subsequently, deep sequencing around rs324011 and unconditional/conditional logistic models were applied.

Results: rs324011 showed statistical significance for the occurrence of childhood AD (OR: 1.23; 95% CI: 1.01–1.51) and rs167769 showed borderline statistical significance (OR: 1.21; 95% CI: 0.99–1.49). Likelihood ratio tests revealed that haplotypes (rs167769/rs324011) were associated with childhood AD (global $p = 0.0018$). T alleles of two *STAT6* intron2 SNPs, rs324011 and rs167769, increased *STAT6* promoter activity significantly in luciferase reporter assay.

Conclusion: T allele of rs324011 in *STAT6* would increase the risk of AD occurrence in children. Haplotypes of rs324011/rs167769 were also significantly associated with childhood AD in Taiwanese population.

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1. Introduction

Atopic dermatitis (AD) is the single most common allergic disease in children, which influences patients' quality of life and lays economic burdens on society [1,2]. The prevalence of

childhood AD has increased substantially in many countries during recent decades [1], as well as in Taiwan [2]. Inside the human body, dys-regulated Th2 predominant immune response and skin defects have been recognized as the two most important pathogenic mechanisms [3]. Th2 response triggers IgE production and Th2/IgE dominance may cause secondary defects in skin barrier integrity, which in turn initiates a vicious cycle of AD pathogenesis [4]. In addition, the elevated serum IgE has been proved to be strongly associated with childhood AD [5].

Signal transducer and activator of transcription 6 (*STAT6*) is a key molecule in Th2-induced responses, such as IgE production [6] and AD pathogenesis [7–11]. Basically, IL-4/IL-13 induces the associated Janus kinases (JaKs) and JaKs phosphorylate the conserved tyrosine residue on the cytokine receptors; then *STAT6*

Abbreviations: AD, atopic dermatitis; ETS, environmental tobacco smoke; FDR, false-discovery rate; LD, linkage disequilibrium; LRT, likelihood ratio test; MAF, minor allele frequency; *STAT6*, signal transducer and activator of transcription 6; TCHS, Taiwan Children Health Study.

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<http://dx.doi.org/10.1016/j.jdermsci.2015.05.006>

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is recruited to the cytokine receptor and activated [12]. Activated STAT6 may translocate to the nucleus and regulates gene transcription. *STAT6* knockout mice have been examined to be deficient in IL-4 mediated response, such as Th2 cell differentiation and IgE isotype switch [10]. In addition, STAT6 has been verified to regulate the production of IL-31, a pruritic factor of AD, and important skin barrier proteins under Th2 cytokine stimulation [7,11].

STAT6 genetic variants in rs1059513 [13], rs324011 [13–15], and rs4559 [16], have been reported to be significantly correlated with elevated serum IgE levels. However, studies concerning about the association between *STAT6* genetic variants and childhood AD were few and inconclusive. In German children, the risk of doctor-diagnosed AD was the highest in C allele homozygotes of rs324011 (20.6%), while 11.5% of heterozygotes and 4.4% of T allele homozygotes were diagnosed with AD ($p = 0.046$) [17]. Among Egyptians, children who carried T allele of rs324011 in *STAT6* had an increased risk of AD [18]. Results from these epidemiological studies and some biological evidence led us to speculate that *STAT6* is a pivotal gene in the development of childhood AD.

Taiwan Children Health Study (TCHS) is a nation-wide population-based cohort representing a wide range of genetic susceptibility [19,20], which offers a good opportunity to investigate the genetic effects on childhood AD. In this study, we aimed to investigate the functional relationship of *STAT6* genotypes/haplotypes with AD among Taiwanese children.

2. Methods

2.1. Study subjects

The Taiwan Children Health Study (TCHS) is a multipurpose nationwide study in which the subjects were recruited from school among 14 communities in Taiwan. Study design and setting have been previously described [20–22]. The research cohort consisted of 5915 children, 3618 seventh-grade children recruited in 2007 and 2297 fourth-grade children recruited in 2010. The informed consents and questionnaires were written and completed by the parents or guardians of each participating student. The questionnaire included

demographic information, child's health outcomes, family history of diseases, exposure information and household characteristics. AD status was assessed by standard questionnaire. Children were considered to have AD if there was a positive answer all of the following questions "Has a doctor ever diagnosed this child as having AD?" and "Has your child ever had an itchy rash which was coming and going for at least 6 months and affected any of the following places: the folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears or eyes?" [23] The study protocol of TCHS was approved by the institutional review board (National Taiwan University Hospital Research Ethics Committee) and complied with the principles outlined in the Helsinki Declaration [24].

2.2. Tagging variants selection and genotyping of *STAT6*

The information of *STAT6* SNPs was provided by the Han Chinese in Beijing genome panel of International HapMap Project. All Variants were downloaded and analyzed by Haploview to select tag variants and to investigate the linkage disequilibrium (LD) patterns for *STAT6* (Fig. 1). SNPs with minor allele frequency (MAF) less than 0.05 were excluded. Tag SNPs were selected due to pairwise tagging tests at the prescribed squared correlation (r^2) value ≥ 0.8 . There were four tagged variants of *STAT6* (rs703817, rs324015, rs3024974 and rs324011), which captured 88% of allele's variations in *STAT6* gene.

Genomic DNA was isolated from cotton swabs containing oral mucosa, which were kept at -80°C after collection. Genetic variants of *STAT6* were detected by TaqMan Allelic Discrimination assay on ABI PRISM™ 7900 Sequence Detector (Applied Biosystems). Approximately 1.0–5.0 ng of genomic DNA was used for real-time PCR. The call rates of genotyping were higher than 98% for each genetic variant.

2.3. Deep sequencing and the second association study

Deep sequencing was performed around the variant rs324011. We sequenced upstream and downstream 3.5 kb of this variant

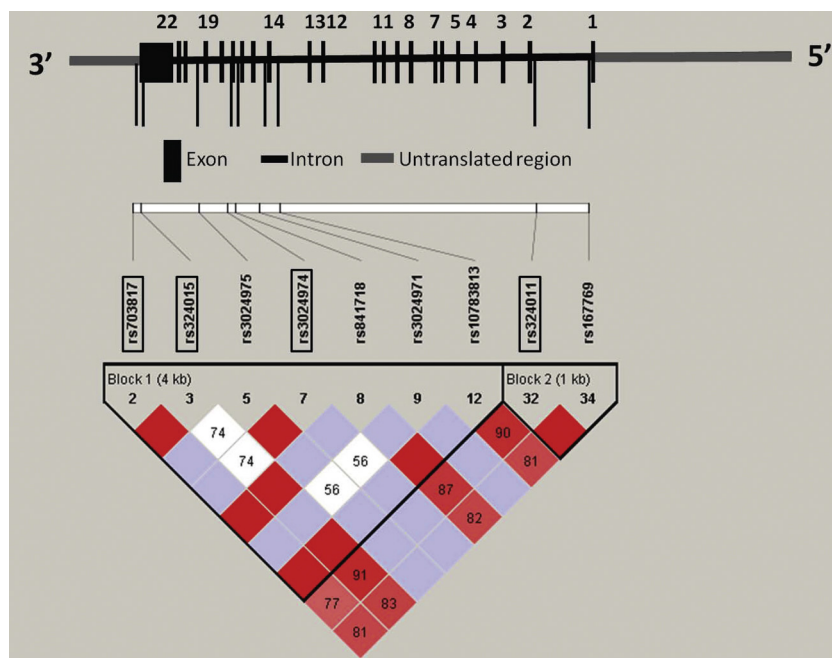


Fig. 1. Variants and pairwise linkage disequilibrium map of *STAT6* gene from HapMap database. D' is pairwise linkage disequilibrium using the Haploview program. The block structure of *STAT6* is defined by the confidence intervals of D' . D' values are displayed in the lozenge-shaped cells, and empty cells indicate $D' = 1$. Four tagged variants (rs703817, rs324015, rs3024974 and rs324011) captured 88% of allele's variations in *STAT6* gene.

from 26 subjects with childhood AD and 26 healthy controls. Deep sequencing and variants screening were performed by ABI 3730XL. The variants among 7 kb were identified by using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI).

After getting deep sequencing results, the second association study was performed in TCHS population. A total of 488 asthmatic cases and 488 control subjects were chosen. Control subjects were selected by matching gender, grade and community from cases. We performed genotyping for five new targeted *STAT6* genetic variants which were rs167769, rs324012, rs3024948, rs117195019 and rs118014438. Genotyping was done by real-time PCR. The primers and probes were prepared by Applied Biosystems and Mission Biotech.

2.4. Luciferase vector construction and luciferase assay

It is widely believed that introns modulate the transcription of the gene containing them [25]. Since both rs324011 and rs167769 were located in the intron2 of *STAT6* gene, we examined the effects of these genetic variants on *STAT6* transcription. The influence of rs324011 and rs167769 on *STAT6* promoter activity was analyzed by luciferase reporter assay. *STAT6* plasmids for luciferase assay were constructed as previously described [26]. Briefly, *STAT6* promoter (1.1 kb) was cloned into upstream of luciferase gene in pGL3 vector (Promega) and *STAT6* intron2 carrying both rs324011 and rs167769 polymorphic alleles was cloned into downstream of luciferase gene. Luciferase vectors carrying different *STAT6* variants were constructed as illustrated in Fig. 2A. The polymorphic T allele

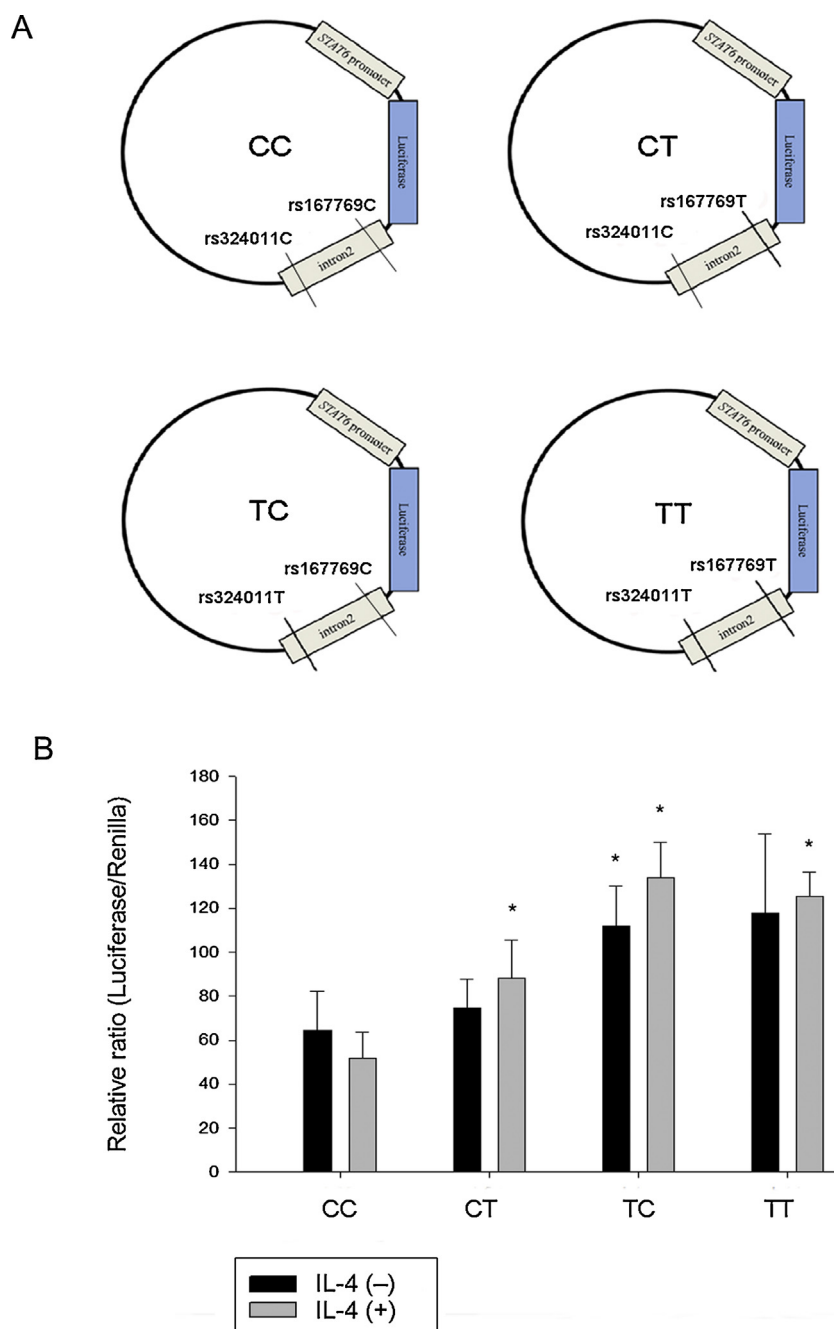


Fig. 2. Functional analyses of *STAT6* rs324011 and rs167769. A, Plasmid constructs used for luciferase assay. B, Transcriptionally regulatory effects measured by luciferase activity with or without IL-4 treatment. The statistical results are shown by comparison with CC group in each set. * $p < 0.05$. Haplotypes were shown by the order of rs324011 and rs167769.

of both rs324011 and rs167769 were created by site-directed mutagenesis (Stratagene) following the manufacturer's instructions.

HaCaT, an immortal human skin keratinocyte cell line, was maintained in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml Penicillin/Streptomycin [27]. HaCaT were transfected with different luciferase constructs (20 µg) and pRL-TK Renilla Luciferase Reporter Vectors (0.5 µg) (Promega) using Mirus TransIT2020 transfection reagent following the manufacturer's instructions. In each set of transfected HaCaT, cells were splitted into two dishes and starved 12 h. One dish of cells was treated with 100 ng/ml IL-4 (PEPROTECH 300-02) for 22 h before harvest while the other was control. Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega E2920) according to the manufacturer's protocol.

2.5. Statistical analysis

To analyze the effect of each *STAT6* variant selected from Haploview, unconditional logistic models were used to estimate the risks of genetic variants on childhood AD. To analyze the effect of each *STAT6* variant found in deep sequencing, unconditional/conditional logistic models were used to estimate the risk of childhood AD among matched cases and controls. Age, gender, and community were considered as a priori confounders in unconditional logistic regression. Environmental factors such as family income, family history of atopy, parental education, lifetime environmental tobacco smoke (ETS) exposure, cat ownership, and home dampness were all selected as confounding factors from the significance test method [28]. False-discovery rate (FDR) was used for multiple comparisons and 0.05 was set as a criterion. The haplotype frequencies of rs324011/rs167769 were estimated by Tag SNPs [29]. The global association of two variants of *STAT6* haplotypes with childhood AD was estimated by likelihood ratio test (LRT). The effect of *STAT6* haplotypes on childhood AD was estimated in logistic regression model. Each analysis was conducted by using SAS software version 9.1.3 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Characteristics of the TCHS population

A total of 5915 children with variants genotyping data were included in this study. All of participants were Han Chinese and the mean age was 11.7 ± 1.4 years. Among these children, 8.4% children have ever diagnosed as having AD (Table 1). The genotype frequencies of *STAT6* in the TCHS cohort were shown in Table 2. The minor allele frequencies of rs324011, rs324015, rs324974, and rs703817 were 0.25, 0.46, 0.21, and 0.24, respectively. All of the four selected variants distributions were in Hardy-Weinberg equilibrium.

3.2. Association of *STAT6* SNPs from HapMap database with childhood AD

The association between these four variants and childhood AD were analyzed by co-dominant model, dominant model and additive genetic model (Table 3). After adjustment for a priori confounders and environmental factors, children who carried rs324011 TT genotype showed an increased risk of childhood AD in comparison with those with CC genotype in co-dominant model (OR: 1.65; 95% CI: 1.18–2.23; FDR $\leq$ 0.05). When compared with CC genotype in rs324011, CT or TT genotypes were borderline significantly associated with an increased risk of AD in dominant model (OR: 1.17; 95%CI: 0.97–1.42). T allele of rs324011 were associated with higher risks of childhood AD compared with C

Table 1

Characteristics of TCHS study population.

	N	(%)
Age (mean $\pm$ SD)	11.7 $\pm$ 1.4	
Gender		
Male	2337	44.0
Female	2978	56.0
Family income ^a		
<50,000	2943	52.9
50,000–100,000	1797	32.3
>100,000	823	14.8
Family history of atopy		
Yes	1724	29.8
No	4053	70.2
Parental education, years		
$\leq$ 12	3305	56.4
13–15	1272	21.7
$\geq$ 16	1278	21.8
In utero smoke exposure to maternal smoking		
Yes	188	3.2
No	5655	96.8
Lifetime ETS exposure		
Yes	2711	46.4
No	3136	53.6
Incense burning at home		
Yes	3477	60.5
No	2273	39.5
Dog ownership		
Yes	1555	26.6
No	4299	73.4
Cat ownership		
Yes	280	4.8
No	5543	95.2
Home dampness		
Yes	3212	54.5
No	2682	45.5
Atopic dermatitis		
Yes	491	8.4
No	5350	91.6

Number of subjects may not equal to total *n* due to missing data.

^a New Taiwan Dollar per month.

allele in additive genetic model (OR: 1.20; 95% CI: 1.04–1.40; FDR: 0.06).

3.3. Identification of genetic variants near rs324011 and results of the second association study

The single variant analyses results showed that rs324011 was associated with childhood AD. Therefore, we performed deep sequencing to identify novel variants around rs324011 (in intron2) in *STAT6* gene. A total of seven variants were identified (Table 4).

Table 2

Genotype frequencies of 4 tagging SNPs in *STAT6*.

STAT6 genotypes	N	%	MAF
rs324011			
CC	3343	56.5	
CT	2180	36.9	0.25
TT	392	6.6	
rs324015			
AA	1296	21.9	
AG	2850	48.2	0.46
GG	1769	29.9	
rs324974			
CC	3729	63	
CT	1930	32.6	0.21
TT	256	4.3	
rs703817			
GG	3403	57.5	
GA	2184	36.9	0.24
AA	328	5.5	

MAF: minor allele frequency.

Table 3Association of 4 tagging SNPs in *STAT6* with childhood AD.

	OR	95%CI	P value	FDR
rs324011				
CC	1.00			
CT	1.09	(0.89,1.33)	0.40	0.64
TT	1.65	(1.18,2.32)	<0.01	0.03
CT/TT	1.17	(0.97,1.42)	0.10	0.21
T allele	1.20	(1.04,1.40)	0.01	0.06
rs324015				
AA	1.00			
AG	1.22	(0.95,1.58)	0.12	0.45
GG	1.21	(0.92,1.60)	0.17	0.45
AG/GG	1.22	(0.96,1.55)	0.11	0.21
G allele	1.09	(0.95,1.24)	0.21	0.43
rs324974				
CC	1.00			
CT	1.10	(0.90,1.35)	0.34	0.64
TT	0.94	(0.57,1.54)	0.81	0.81
CT/TT	1.09	(0.89,1.32)	0.41	0.55
T allele	1.05	(0.89,1.23)	0.58	0.63
rs703817				
GG	1.00			
GA	1.04	(0.85,1.26)	0.73	0.81
AA	1.09	(0.72,1.65)	0.68	0.81
GA/AA	1.04	(0.86,1.26)	0.67	0.67
A allele	1.04	(0.89,1.22)	0.63	0.63

Among these seven variants, one variant was dismissed hereafter due to low minor allele frequency (MAF) with only one heterozygous subject. We calculated *R* square for the rest six variants and found that rs324011 was highly associated with rs167769 ($r^2 = 0.71$).

To understand the association of SNPs near rs324011 with childhood AD, we performed second association study for variants noted by deep sequencing. The rs324011 showed statistical significance for the occurrence of childhood AD (OR: 1.23; 95% CI:

1.01–1.51), and the rs167769 showed borderline statistical significance (OR: 1.21; 95% CI: 0.99–1.49) (Table 5). We performed genetic association study by two types of logistic models and the results were of great concordance, indicating that rs324011 and rs167769 were important variants for the pathogenesis of childhood AD.

3.4. Association of rs324011/rs167769 haplotypes with childhood AD

Both rs324011 and rs167769 showed significantly increased risks of childhood AD, so we performed haplotype analysis to study the loci effects. Haplotype frequencies were shown in Table 6. Haplotypes were shown by the order of rs324011(C/T) and rs167769(C/T). The haplotypes showed significant associations with childhood AD (global *p* value = 0.0018).

3.5. Alleles of rs324011 and rs167769 influence *STAT6* promoter activity in vitro

Luciferase vectors carrying different haplotypes were illustrated in Fig. 2A. Each plasmid contains *STAT6* promoter, luciferase gene and intron2 harboring rs324011(C/T) and rs167769(C/T). In CC group, the promoter activities were not significantly different between IL-4(-) and IL-4(+), which is congruent with previous report [30]. In the absence of IL-4, only T allele of rs324011 can significantly enhance *STAT6* promoter activities. However, when HaCaT were treated with IL-4, different haplotypes in intron2 significantly increase *STAT6* promoter activity ($p < 0.05$), though no obvious synergistic effects was observed when intron2 harbors both T alleles of rs324011 and rs167769 (Fig. 2B). These results suggest that T allele of rs324011 per se is sufficient to facilitate transcription of *STAT6* gene with or without IL-4 stimulation, and T allele of rs167769 can significantly increase *STAT6* promoter activities when HaCaT was treated with IL-4. Moreover, IL-4 would significantly enhance the effects brought by the 2 SNPs in terms of *STAT6* promoter activities.

Table 4

Location of variants around rs324011 by deep sequencing.

Variant no.		Position ^a	Location	Sequence	Minor allele frequency (%)	rs No.
1	C/T	1422	Intron2	CTTCTCTGGA(C/T)GTTTCTGCTC	0.154	rs167769
2	C/T	2216	Intron2	AGAGACTCTG(C/T)CTCAAAATAA	0.221	rs324012
3	C/T	2444	Intron2	GTGATCTGCC(C/T)GCCTCGGCCT	0.077	rs3024948
4	C/T	3015	Intron2	TCCTAGGGAC(C/T)GTCCCCACCA	0.135	rs324011
5	T/C	3117	Exon3	ACTTGGAGGT(T/C)GCCTGGAGGA	0.01	–
6	T/C	5089	Exon8	TCTCCTGCAG(T/C)AGCATGGCCA	0.067	rs117195019
7	C/T	5939	Exon9	TACCTGGTGA(C/T)GAGGGTTCTC	0.067	rs118014438

^a Numbering according to genomic sequence of *STAT6* (NG_021272.1). Position 1 is the A of the initiation codon.

Table 5Association of new targeted variants of *STAT6* gene with childhood AD by additive genetic model.

	Unconditional logistic model			Conditional logistic model		
	OR	95%CI	P value	OR	95%CI	P value
rs167769						
T allele	1.21	(0.99, 1.49)	0.07	1.22	(0.99, 1.51)	0.06
rs324012						
T allele	1.16	(0.96, 1.41)	0.13	1.16	(0.96, 1.40)	0.13
rs3024948						
T allele	1.00	(0.20, 4.97)	1.00	1.00	(0.20, 4.96)	1.00
rs324011						
T allele	1.23	(1.01, 1.51)	0.04	1.23	(1.01, 1.51)	0.04
rs117195019						
C allele	0.78	(0.50, 1.21)	0.27	0.78	(0.50, 1.21)	0.27
rs118014438						
T allele	0.79	(0.51, 1.21)	0.28	0.78	(0.50, 1.21)	0.27

Conditional logistic model were matched by age, gender and community.

Unconditional model was adjusted for age, gender, community, family income, family history of atopy, parental education, lifetime ETS exposure, cat ownership, and home dampness.

Table 6

Association of rs324011/rs167769 haplotypes with childhood AD.

Haplotype	Frequency	RR	95% CI
CC	0.718	Ref	
CT	0.015	0.85	(0.49, 1.47)
TC	0.036	1.00	(0.61, 1.64)
TT	0.232	1.21	(0.75, 1.97)
Global <i>P</i> value		0.0018	

Models were adjusted for age, gender, community, family income, family history of atopy, parental education, lifetime ETS exposure, cat ownership, and home dampness.

Haplotypes were shown by the order of rs324011 and rs167769.

4. Discussion

To our best knowledge, this is the first study demonstrating that rs324011 in *STAT6* was significantly associated with childhood AD in Taiwanese population. We also found that haplotypes of rs324011/rs167769 were significantly associated with childhood AD. In IL-4 treatment condition, T alleles of both rs324011 and rs167769 may increase *STAT6* promoter activity significantly in functional genetic assay.

Children who carried T allele of *STAT6* rs324011 may have a lower risk on AD than those who carried homozygous or heterozygous C allele of rs324011 in previous German study [17]. However, we found that T allele of rs324011 was associated with an increased risk of developing childhood AD in Taiwanese population (Table 5). The differences may be due to ethnicity, age and study approaches. In the German study, Casaca and colleagues purposely chose IgE-level associated *STAT6* variants, rs1059513 and rs324011. This approach limited the possibility of identifying novel SNPs of childhood AD. On the other hand, our approach can lead us explore new childhood AD-associated *STAT6* SNPs regardless of IgE or other prerequisites.

Another study from Egypt revealed that there was a strong correlation between haplotypes (rs2243250 in *IL-4*, rs1805010 in *IL-4R*, rs324015 and rs324011 in *STAT6*) and childhood AD [18]. In concordance with their results, our in vitro study demonstrated that T alleles of both rs324011 and rs167769 have stronger effect on *STAT6* promoter activity in the presence of IL-4 (Fig. 2B). We have also checked if these two SNPs would influence IL-4 level in HaCaT cells and the results were all negative. Through different approaches, all these data agreed that IL-4 and *STAT6* signaling pathway is crucial in AD pathogenesis.

Several previous reports revealed that rs167769 and rs324011 was related to atopy. T allele of rs167769 was reported to be associated with childhood asthma in Mexicans (OR: 1.20; 95% CI: 1.00–1.40) [31]. T allele of rs324011 was noted to significantly associated with elevated total serum IgE levels in German schoolchildren [13] and Chinese children [15]. Additionally, homozygous T/T genotype of rs324011 was also reported to link with atopic asthma in Saudi population [32]. A previous functional genetic study showed that T rather than C allele of rs324011 might be bound by NF- κ B and would contribute to the enhancement of *STAT6* promoter activity [26]. Our study, which provided solid data linking rs324011 and haplotypes of rs324011/rs167769 to childhood AD, further substantiated the importance of these two SNPs in atopic diseases.

Confounders, such as age, gender, community, family income, family history of atopy, parental education, lifetime ETS exposure, cat ownership, and home dampness were adjusted by multiple regression models [33]. In utero smoke exposure to maternal smoking and other environmental factors such as incense burning at home were also considered in our study [19]. However, these covariates were not included in the final models because of not reaching selection criteria. We used unconditional/conditional

logistic models to estimate the risk of childhood AD among matched cases and controls (Table 5). The results from both models showed that rs324011 was significantly associated with childhood AD, confirming validity of the association. Another possible limitation was recall bias of AD outcomes. AD phenotypes in our study were ascertained by parental-reported questionnaire, so misclassification might have arisen from imperfect parental recall of events. However, the experimental design and statistical approaches still provided solid results implicating the strong association of rs324011 and haplotypes (rs324011/rs167769) with childhood AD.

In conclusions, *STAT6* genetic variants in rs324011 and haplotypes of rs324011/rs167769 were significantly associated with childhood AD in Taiwanese population. T alleles of both rs324011 and rs167769 could increase *STAT6* promoter activity significantly in the luciferase assay and IL-4 further increased aforementioned effect. Identification of novel childhood AD associated SNPs may lead to the development of personalized medicine in the treatment of childhood AD.

Conflicts of interest

The authors state no conflict of interest.

Acknowledgments

The authors thank the field workers, teachers, and other school staff who supported the data collection, and all the parents and children who participated in this study. We thank Taiwan National Science Council for grants # 98-2314-B-002-138-MY3 and 101-2314-B-002-113-MY3.

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