

Association of Plasma Soluble CTLA-4 with Lung Function and Gene Polymorphism in Chinese Asthmatic Children

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Key Words

Aeroallergen · Asthma · CTLA-4 · Genetic polymorphism · Immunoglobulin E

Abstract

Background: Cytotoxic T lymphocyte antigen 4 (CTLA-4) is known to downregulate the T_H2 immune response. Recent studies have suggested an association of *CTLA-4* polymorphisms with allergic diseases. We investigated the effects of single nucleotide polymorphisms (SNPs) of *CTLA-4* on asthma traits and plasma sCTLA-4 in 298 Chinese asthmatic children and 175 controls. **Methods:** Plasma sCTLA-4, total and allergen-specific IgE concentrations were measured by enzyme immunoassay. Six SNPs, namely –1147CT, +49AG, CT60, JO31, JO30 and JO27_1, in *CTLA-4* were genotyped by restriction fragment length polymorphism. **Results:** Plasma sCTLA-4 was negatively associated with FEV₁/FVC ($r = -0.146$, $p = 0.036$) among our asthmatic patients. Analysis of locus-locus interaction by generalized multifactor dimensionality reduction showed that –1147CT was the best model for plasma sCTLA-4 with a cross-validation consistency of 10 out of 10 and a prediction error of 40.9% ($p < 0.001$). Multivariate regression analysis confirmed the association between plasma sCTLA-4 concentration with –1147CT among

the 6 SNPs tested ($p = 0.002$) after adjustment for gender and age. The plasma sCTLA-4 concentration was significantly lower in patients homozygous for the C allele than in T allele carriers ($p = 0.001$). There was also a significant association between the most common haplotypes with low sCTLA-4 in asthmatics. We could not find any significant association between plasma total IgE, atopy and lung function with the 6 SNPs after Bonferroni correction. **Conclusions:** Plasma sCTLA-4 is associated with lung function and –1147CT polymorphism in Chinese asthmatic children. This may help to identify CTLA-4 signaling as a potential therapeutic target in asthma.

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Introduction

T lymphocytes play a central role in atopy and asthma through the action of T_H2 cytokines generated in response to allergens. A cluster of T lymphocyte immune-regulating genes including *CD28*, *cytotoxic T lymphocyte*

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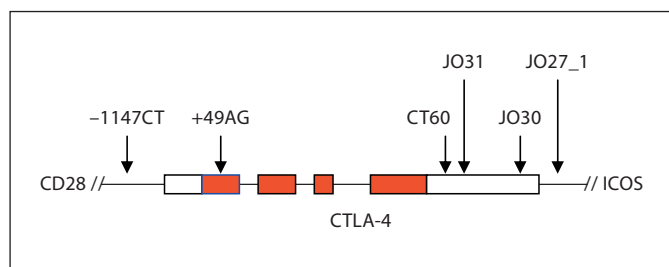


Fig. 1. Approximate map location of the tested SNPs in the CTLA-4 gene. Boxes indicate the locations of exons, and the dark areas indicate the translated regions (red in the online version).

antigen 4 (CTLA-4) and *ICOS* is located on chromosome 2q33 [1]. CTLA-4 shares sequence homology with CD28 and binds to the same ligands B7.1 (CD80) and B7.2 (CD86). However, these costimulatory molecules have antagonistic functions. CTLA-4 has a role in inducing a T_H1 response and suppressing T_H2 cytokines, an effect that is antagonized by CD28 [2]. CTLA-4 has been shown to be associated with asthma and atopy [3, 4]. Genetic association among IgE levels, atopic traits, and polymorphisms in the CTLA-4 gene has been studied; some show associations with asthma, bronchial hyperresponsiveness and atopic dermatitis [5–7], others show only associations with IgE levels [8, 9], and some do not show associations at all [10].

Ueda et al. [11] performed an extensive analysis on 330 kb of chromosome 2q33 with autoimmune diseases. From an original 108 single nucleotide polymorphisms (SNPs), the strongest associations of type 1 diabetes, autoimmune hypothyroidism, and Graves' disease were with the newly detected polymorphisms designated MH30, CT60, JO31, JO30, and JO27_1. These are located in the CTLA-4 gene. Munthe-Kaas et al. [12] also found associations between these newly discovered SNPs with serum IgE level, allergy, asthma, and reduced lung function, but not bronchial hyperresponsiveness in Caucasians. They also reported that those SNP alleles found positively associated with allergic phenotypes were shown to be associated negatively with autoimmune disorders. Thus, the aim of this study is to investigate the effect of six polymorphisms including a functional SNP, -1147CT in a consensus binding site for transcription factors [6], +49AG in exon 1 [9], CT60, JO31, JO30 and JO27_1 in the 3' UTR as shown in figure 1 on plasma soluble CTLA-4 (sCTLA-4), total and allergen-specific IgE concentrations as well as asthma phenotypes in Chinese asthmatic children.

Materials and Methods

Study Population

This study recruited unrelated children aged 5–18 years with asthma, diagnosed according to the American Thoracic Society guidelines [13], from the pediatric outpatient clinics of a university teaching hospital in Hong Kong. Both parents of these subjects were ethnic Chinese. Age- and sex-matched control subjects were selected among children attending the hospital for minor complaints, who did not have allergic or immunological disease, and did not require any drug treatment at the time of this study. All subjects were free from any self-reported symptoms of infection for 4 weeks before the start of the study. Subjects or their parents gave written consent, and the Clinical Research Ethics Committee of our university approved this study.

Plasma Total IgE and Allergen-Specific IgE Assays

Plasma total IgE concentration was measured by microparticle immunoassay (IMx analyzer, Abbott Laboratories, Abbott Park, Ill., USA) and specific IgE antibodies to crude *Dermatophagoides pteronyssinus*, cat, dog, mixed cockroaches and mixed moulds were measured by fluorescent enzyme immunoassay (AutoCAP System, Phadia AB, Uppsala, Sweden). Plasma total IgE concentrations were analyzed as a quantitative trait following logarithmic transformation ($\text{IgE}_{\log}$). Specific IgE concentration ≥ 0.35 kIU/l was considered positive. Children were classified as atopic if they were positive with at least one type of allergen-specific IgE antibody [14].

Plasma sCTLA-4 Assay

Plasma sCTLA-4 concentration was measured by enzyme-linked immunosorbent assay (Bender MedSystems GmbH, Vienna, Austria). Samples were diluted 2-fold prior to the ELISA. The sensitivity of the assay was 0.2 ng/ml, and the intra- and interassay coefficients of variation were 4.8 and 7.5% at 8.6 ng/ml. Plasma sCTLA-4 results were classified into high and low sCTLA-4 levels using 40 percentiles cutoff for dichotomous analysis.

Spirometry

Patients aged 5 years and above underwent spirometric assessment (COMPACT II, Vitalograph, Buckingham, UK) of their forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC). The measured FEV_1 and FVC results were compared with local age- and sex-matched predicted values [15].

Analysis of CTLA-4 Polymorphisms

Genomic DNA was extracted from peripheral blood using the High Pure Viral Nucleic Acid Kit (Boehringer Mannheim, Mannheim, Germany). Table 1 summarizes the genotyping conditions for polymerase chain reaction and restriction fragment length polymorphism. The +49AG protocol was described in one of our earlier articles [16]. The digested polymerase chain reaction products were resolved on agarose gel containing ethidium bromide. All restriction enzymes used in this study were purchased from New England Biolabs (Hitchin, UK). Eight DNA samples of known genotypes were included in every restriction enzyme digestion as positive control together with 88 unknown samples in 96-well plates. Finally, the results of these restriction fragment length polymorphism assays were validated by direct sequencing of the polymorphisms using Big Dye Terminator Cycle sequenc-

Table 1. Polymerase chain reaction and restriction fragment length polymorphism conditions for the 6 polymorphic markers genotyped in this study

Polymorphism	PCR primers	Hybridization temperature, °C	Restriction enzyme	References
-1147CT	F: 5'-AAA GAG GCA CGT GGA AGG AA-3' R: 5'-ATA TGC CCG GAA TTC TGT GC-3'	63.3	<i>FokI</i>	Howard et al., 2002 [6] (rs16840252)
+49AG	F: 5'-GTG CCT TCT GTG TGT GCA CA-3' R: 5'-ACT CAC CTT TGC AGA AGA CAG G-3'	59.6	<i>BbvI</i>	Chan et al., 2006 [16] (rs231775)
CT60	F: 5'-TTC ACC ACT ATT TGG GAT ATA CC-3' R: 5'-TGA TAG TTG AGC TGA GAA AGC A-3'	57.1	<i>NcoI</i>	Ueda et al., 2003 [11] (rs3087243)
JO31	F: 5'-GCA TCT CAG GTG ACC CAG C-3' R: 5'-TGT TTC TGA AAC ACT GTT AGT TGC-3'	64.5	<i>HpaII</i>	Ueda et al., 2003 [11] (rs11571302)
JO30	F: 5'-GTC ATT TTC TCT CTG CTT CCA C-3' R: 5'-TGC ACG CTA ACT ACA AAG AAG G-3'	63.3	<i>TaqI</i>	Ueda et al., 2003 [11] (rs7565213)
JO27_1	F: 5'-CTA CCA GAA GTT GAA GTG TAG GGA-3' R: 5'-CTT TCA CCC CTG CTT GGT G-3'	63.3	<i>EcoRV</i>	Ueda et al., 2003 [11] (rs11571297)

F = Forward primer; R = reverse primer.

ing kits with an ABI-3100 autosequencer (Applied Biosystems, Foster City, Calif., USA) of 30 randomly selected samples.

Statistical Analysis

The results on plasma total IgE and allergen sensitization as risk factors for the development of asthma between different groups were compared using Student's *t* test, χ^2 or Fisher exact test as appropriate. The associations between plasma sCTLA-4, FEV₁/FVC, total IgE concentration and atopy were analyzed using Spearman or Pearson correlation coefficients as appropriate. Locus-locus interaction for plasma sCTLA-4 was done by generalized multifactor dimensionality reduction [16]. Multivariate regression analysis was performed using SPSS version 15.0 (SPSS, Chicago, Ill., USA) for evaluating the effects of different covariates on plasma sCTLA-4. Student's *t* test was used to analyze sCTLA-4 in different groups. Because only 5 patients and 3 controls with the TT genotype of -1147 had plasma sCTLA-4 data available, CT and TT genotypes were combined into a single category for the statistical comparisons. Haplotype analysis was done by Haploview [17] and WHAP (<http://www.genome.wi.mit.edu/~shaun/whap/>). All comparisons were made two-sided. Multiple testing was corrected with Bonferroni correction, 5,000 or 10,000 permutations. *p* < 0.05 was considered significant.

Results

Clinical and Laboratory Characteristics

Two hundred and ninety-eight asthmatic children and 175 controls were recruited. Table 2 summarizes their demographic and clinical characteristics. The mean (standard deviation) ages at evaluation of patients and control children were 10.4 (3.7) and 11.0 (3.8) years, respectively. Two hundred and fifty-four (85%) patients and 106 (61%)

controls in this study were atopic (odds ratio 3.76 and 95% CI 2.42–5.84, *p* < 0.001). Sensitization to *D. pteronyssinus*, cat, mixed cockroaches and mixed moulds represented significant risk factors for asthma diagnosis.

Plasma sCTLA-4 Concentration

The mean (SD) plasma sCTLA-4 concentrations of our asthmatics and controls were 5.8 (3.1) and 5.5 (2.7) ng/ml, respectively (*p* = 0.315). No significant correlation was found between plasma sCTLA-4 concentration and total IgE_{log} (*p* = 0.253) and atopy (*p* = 0.811). However, plasma sCTLA-4 was negatively associated with FEV₁/FVC (*r* = -0.146, *p* = 0.036) among our asthmatic patients.

CTLA-4 Polymorphisms as a Predictor of Asthma and Atopy

The allele frequencies for the 6 SNPs tested, namely -1147CT, +49AG, CT60, JO31, JO30 and JO27_1, are shown in table 3. The distributions of genotypes and alleles for these 6 polymorphisms followed the Hardy-Weinberg equilibrium. There was no significant association between these polymorphisms and asthma diagnosis (table 3).

Significant associations were found between increased plasma total IgE and -1147CT (*p* = 0.038), atopy with JO31 (*p* = 0.015), JO30 (*p* = 0.027) and JO27_1 (*p* = 0.035). After the Bonferroni correction for multiple comparisons, none of the SNPs showed a significant association. We could not find any significant association of lung

Table 2. Clinical and laboratory characteristics in our 298 asthmatics and 175 controls

	Asthmatics (n = 298)	Controls (n = 175)
Age, years	10.4 ± 3.7	11.0 ± 3.8
Male	192 (64.4)	97 (55.4)
Plasma IgE _{log}	2.61 ± 0.62*	1.89 ± 0.73
Atopy (≥one specific IgE)	254 (85.2)*	106 (60.6)
IgE sensitization to aeroallergens		
<i>D. pteronyssinus</i>	256 (88)*	93 (53)
Cat	43 (15)*	8 (5)
Dog	6 (2)	0
Mixed cockroaches	86 (29)*	24 (14)
Mixed moulds	28 (10)**	4 (2)
Spirometric variables		
FEV ₁ , % predicted	93.8 ± 20.9*	83.0 ± 11.2
FVC, % predicted	111.4 ± 27.0*	93.3 ± 10.7
FEV ₁ /FVC, %	78.9 ± 10.9*	83.3 ± 9.4

Figures represent number with the percentage in parentheses or mean ± standard deviation. * $p < 0.001$; ** $p = 0.002$.

function with these SNPs. Analysis of locus-locus interaction among the 6 SNPs by generalized multifactor dimensionality reduction found that -1147CT alone was the best model for plasma sCTLA-4 with a cross-validation consistency of 10 out of 10 and a prediction error of 40.9% based on 5,000 permutations ($p < 0.001$, table 4). One of the 2 locus models which consisted of +49AG and CT60 was also marginally significant with plasma sCTLA-4 with a prediction error of 43.6% ($p = 0.051$, table 4). Multivariate stepwise regression analysis confirmed the association between plasma sCTLA-4 concentration with -1147CT polymorphism among the 6 SNPs ($p = 0.002$) after adjustment for gender and age. Table 5 summarizes the association between plasma sCTLA-4 levels with the -1147CT genotypes. The plasma sCTLA-4 concentration was significantly lower in those homozygous for the C allele than those T allele carriers in all subjects, asthmatic patients as well as atopic asthmatics ($p = 0.020$, 0.001 and 0.003, respectively).

Haplotype Analyses

We conducted haplotype reconstruction and linkage disequilibrium (LD) analysis incorporating all 6 SNPs in the *CTLA-4* gene by using Haploview 3.2. It was observed that all tested SNPs were located in one haplotype block and in moderate to strong LD with each other. The D' ranged from 0.49 to 0.98 and r^2 from 0.03 to 0.75. There

Table 3. Allele and genotype frequencies of the *CTLA-4* SNPs

Polymorphism	Asthmatics	Controls	p^a
-1147CT	n = 291	n = 175	
CC	216	129	
CT	68	43	
TT	7	3	0.855 ^b
q	0.14	0.14	0.970
+49AG	n = 272	n = 171	
AA	40	21	
AG	119	75	
GG	113	75	0.748
q	0.37	0.34	0.473
CT60	n = 293	n = 174	
GG	183	109	
GA	97	58	
AA	13	6	0.878
q	0.21	0.20	0.783
JO31	n = 295	n = 172	
GG	150	92	
GT	123	69	
TT	22	11	0.825
q	0.28	0.26	0.542
JO30	n = 296	n = 174	
GG	156	90	
GA	118	69	
AA	22	14	0.965
q	0.27	0.28	0.825
JO27_1	n = 294	n = 175	
TT	146	89	
TC	117	68	
CC	31	17	0.939
q	0.30	0.29	0.715

q = Minor allele frequency. ^a Compared by Pearson χ^2 test. ^b Compared by Fisher's exact test.

were no significant association of haplotypes with asthma diagnosed, plasma total IgE, allergen-specific IgE and lung function (data not shown).

However, significant associations were found in haplotypes with plasma sCTLA-4. Estimation of haplotype distribution and test statistics for testing the significance of differences between subjects with high sCTLA-4 and low sCTLA-4 for the haplotypes with frequency $>2\%$ in at least one group were listed in table 6. The p value estimated from 10,000 permutations was 0.005, which indicated that there was a significant haplotypic association. The most common haplotypes (-1147CT/C, +49AG/G, CT60/G, JO31/G, JO30/G and JO27_1/T) were associated with low sCTLA-4 in Chinese asthmatic children ($p = 0.001$). Upon analysis of haplotypes consisting of +49AG and CT60 alone, the most common haplotype (+49AG/G

Table 4. Multilocus interaction model for plasma sCTLA-4 concentration by GMDR method

Locus number and combination	Cross-validation consistency	Prediction error, %	p value based on 5,000 permutations
Locus 1: -1147CT	10	40.1	<0.001
Locus 2: +49AG, CT60	5	43.6	0.051
Locus 3: -1147CT, +49AG, JO30	7	40.0	0.005
Locus 4: -1147CT, +49AG, JO30, JO27_1	9	38.5	0.002
Locus 5: -1147CT, +49AG, CT60, JO30, JO27_1	9	36.4	0.001
Locus 6: -1147CT, +49AG, CT60, JO31, JO30, JO27_1	10	36.2	0.001

Table 5. Association between plasma sCTLA-4 concentration and -1147CT genotypes in the *CTLA-4* gene

Phenotypes of asthma	-1147CT genotypes ^a			
	n	CC	CT/TT ^b	p value ^c
All subjects	388	5.42 ± 2.95	6.19 ± 2.83	0.020
Physician-diagnosed asthmatic patients	225	5.32 ± 3.10	6.90 ± 2.77	0.001
Control children	163	5.55 ± 2.73	5.29 ± 2.64	0.584
Atopic asthmatics	208	5.40 ± 3.14	6.81 ± 2.76	0.003
Nonatopic asthmatics	17	4.24 ± 2.36	7.07 ± 3.37	0.060

^a Results expressed as mean ± standard deviations for plasma sCTLA-4 concentration.

^b Since only 5 patients and 3 controls with the TT genotype of -1147 had plasma sCTLA-4 data available, CT and TT genotypes were combined into a single category for the statistical comparisons.

^c Compared by Student's t test.

Table 6. Haplotype frequency estimation and test statistics between subjects with high and low plasma sCTLA-4 concentration

Haplotype ^a	Subjects with high sCTLA-4	Subjects with low sCTLA-4	Odds ratio (95% CI)	p value ^b
CGGGGT	132 (44.8)	175 (59.0)	1.78 (1.28–2.46)	0.001
CAATAC	37 (12.6)	52 (17.5)	1.48 (0.94–2.34)	0.909
TAGGGT	33 (11.3)	26 (8.7)	0.76 (0.44–1.31)	0.323
CGGTAC	7 (2.4)	14 (4.7)	2.04 (0.81–5.12)	0.124
CAGGGT	15 (5.2)	7 (2.5)	0.45 (0.18–1.12)	0.079
CGGGGC	13 (4.4)	6 (2.1)	0.45 (0.17–1.19)	0.100
CGGGAT	11 (3.9)	3 (1.0)	0.26 (0.07–0.95)	0.057
CGATAC	8 (2.7)	0	NA ^c	0.004 ^d
TGGGGT	11 (3.6)	0	NA	0.001 ^d
CAATGC	6 (2.2)	0	NA	0.015 ^d
Others	20 (6.9)	13 (4.5)	1.59 (0.78–3.26)	0.203
Total				0.002

Values represent chromosomes with the percentage in parentheses. p value of 10,000 permutations: 0.005. CI = Confidence interval; NA = not available.

^a The order of the SNPs from left to right is -1147CT, +49AG, CT60, JO31, JO30 and JO27_1.

^b Compared by χ^2 test.

^c Odds ratio cannot be calculated by χ^2 as one of the cells was zero.

^d Compared by Fisher's exact test.

and CT60/G) was also marginally associated with low sCTLA-4 with an odds ratio of 1.39 (95% CI: 1.0–2.0; $p = 0.052$).

Discussion

The gene encoding *CTLA-4* is located on chromosome 2q33. Polymorphisms in this gene have been linked to asthma and atopy in several populations [6–9, 12]. However, whether there is an association of plasma sCTLA-4 concentration with these polymorphisms remains unknown. In this study, we showed that plasma sCTLA-4 was negatively associated with FEV₁/FVC. The analysis of locus-locus interactions showed that –1147CT alone was the most significant locus in affecting plasma sCTLA-4 level among the 6 SNPs. Patients homozygous for the C allele of –1147 as well as the most common haplotypes (–1147CT/C, +49AG/G, CT60/G, JO31/G, JO30/G and JO27_1/T) were associated with low sCTLA-4 in Chinese asthmatic children.

CTLA-4 plays a critical role in the regulation of T cell response through transmission of inhibitory signals upon interaction with B7 molecules on antigen-presenting cells. Among the 6 polymorphisms analyzed in this study, –1147CT showed a significant association with plasma sCTLA-4. The plasma sCTLA-4 concentration was significantly lower in those homozygous for the C allele than in the T allele carriers of –1147 in all subjects, asthmatic patients as well as atopic asthmatics. Such findings were not observed in control and nonatopic asthmatics. This could be explained by asthmatic patients suffering from acute bronchoconstriction and mucus formation directly after inhalation of the allergen. Chronic symptoms include airway hyperresponsiveness to broncho-spasmogenic stimuli, inflammation and airway remodeling. Central to the process of airway inflammation is the T lymphocyte [18]. Therefore, T cell recruitment and differentiation are critical elements in the evolution of the asthmatic state. Besides, CTLA4 is a member of the immunoglobulin superfamily and is a costimulatory molecule expressed by activated T cells [12]. Shi et al. [19] showed that serum sCTLA-4 level was positively correlated with the numbers of lymphocytes. Moreover, Qin et al. [20] found that serum sCTLA-4 concentrations increased after allergen inhalation in sensitized asthmatic subjects. These may explain why the functional SNP –1147CT affects sCTLA-4 production in asthmatics but not controls in our study. Patients with the T allele had a higher concentration of plasma sCTLA-4 and a high con-

centration of plasma sCTLA-4 was associated with poorer lung function. These were in line with the findings of Howard et al. [6] reporting that the T allele was associated with increased bronchial hyperresponsiveness and Shi et al. [19] reporting a higher concentration of sCTLA-4 in asthmatic patients and a negative association of its concentration with lung function. However, promoter luciferase assay found that the T allele of –1147 was expressed at half the level of the C allele [5]. This needs further investigations with other cell lines as analysis of the *CTLA-4* sequence using TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=SEA-FR-QueryS>) showed that the –1147 substitution of T for C creates a consensus binding site for SRF and CBF. These two transcription factors are involved in promoter activation [21, 22]. This supported our findings that T allele of –1147 was associated with high plasma sCTLA-4 concentration. Increased sCTLA-4 concentration has been found in autoimmune diseases such as autoimmune thyroid disease [23], systemic lupus erythematosus [24] and myasthenia gravis [25]. Here, we should be the first group to report the significant correlation of sCTLA-4 with *CTLA-4* polymorphism and lung function in asthmatic children. This implies that sCTLA-4 may play a role in promoting bronchoconstriction.

CTLA-4 is about 6.2 kb in size and consists of four exons. It has 2 alternative splice forms: a full-length protein (fICTLA-4) with a transmembrane domain encoded by exon 3, and a soluble protein (sCTLA-4) without the transmembrane domain [26]. Ueda et al. [11] found a disease-associated 6.1-kb region which consisted of +49AG and CT60 determining the splicing efficiency and production of sCTLA-4. Their data also demonstrated that the disease susceptibility haplotype (+49AG/G and CT60/G) produces less sCTLA-4 transcript. These were consistent with our findings that the interaction between +49AG and CT60 had a significant effect on sCTLA-4 levels. Our results showed that subjects with the +49AG/G and CT60/G haplotype had an odds ratio of 1.4 associated with a low plasma sCTLA-4 level. Therefore, our findings suggested that plasma sCTLA-4 was not only affected by the functional SNP –1147CT, but also by haplotypes such as +49AG and CT60 determining its splicing efficiency [11].

sCTLA-4 is thought to play an important role in the regulation of immune response as it can compete with the membrane-bound CTLA-4 (mCTLA-4) and CD28 for binding with the B7 molecules [23, 25–27]. sCTLA-4 could therefore either inhibit or enhance the immune response depending on the activation status of T lympho-

cytes [23–27]. Thus, at the initiation of the immune response when T cells do not express mCTLA-4, sCTLA-4 may block B7-CD28 interactions and interfere with T cell activation. This is consistent with the observation that the recombinant sCTLA-4 can inhibit mixed leukocyte reactions [24]. On the other hand, during the ongoing immune responses when T cells express mCTLA-4, sCTLA-4 may block B7-mCTLA-4 interactions, resulting in augmented and sustained T cell responses. Moreover, since mCTLA-4 on T cells induces immunological anergy, sCTLA-4 may inhibit induction anergy, leading to the enhanced autoreactivity [28].

CTLA-4 polymorphisms have been reported to be associated with serum IgE levels, allergy, asthma, and/or reduced lung function in Caucasians [12], Japanese [8] and Taiwanese [9]. However, in our Southern Chinese asthmatic children, we could not find any significant association of *CTLA-4* polymorphisms with asthma, lung function, plasma total and specific IgEs. Allergy and asthma are polygenic and are also under environmental influence [29, 30]. Apart from gene-environment interactions, gene-gene interactions are also crucial for the initiation and development of asthma [16, 31]. The epigenetic effect of certain genes may mask the expression of other genes. Thus, it is not surprising to observe the associations of *CTLA-4* with asthma in some populations but not in others [10]. Besides, these polymorphism associations are with asthma-related phenotypes rather than with a protein level of *CTLA-4* gene. Indeed, in our study, we found a direct association of plasma sCTLA-4 with –1147CT polymorphism and its concentration affected lung function in asthmatic Chinese children.

Though our 6 SNPs genotyped were not tagSNPs for covering full LD pattern of *CTLA-4*, we analyzed the LD patterns reflected by these 6 SNPs using the HapMap phase II genotyped data in Chinese and Japanese. Our principle component analysis [32] covering the *CTLA-4* data from upstream 5,000 bp to downstream of 35,000 bp found that by genotyping –1147CT, +49AG and CT60 had already explained 78% variance of the gene, not to mention JO31, JO30 and JO27_1 which were in the region but not genotyped in the HapMap project. Therefore, our 6 genotyped SNPs were able to capture most of the LD pattern around the *CTLA-4* gene.

To control potential population stratification that may cause spurious association results, we used the software Structure 2.2 [33] to investigate the population structure of our cohort. With 11 unlinked SNPs genotyped in our previous studies [16, 31], all the 473 subjects were tightly clustered together under all the three assumed numbers

of population strata (i.e. $k = 2, 3$ or 4), suggesting no significant population substructure in this study.

This study investigated the polymorphisms on *CTLA-4* in a rather small sample size of 298 patients and 175 controls. However, by using HapMap phase II genotype data on the Chinese population, the allele frequency for the 6 SNPs was in the range of 0.122–0.322. Based on the International Study of Asthma and Allergies in Childhood (ISAAC) phase III result [34], the prevalence rate of asthma in Hong Kong was 10%. Therefore, in this study 116–201 subjects are required in each of the patient and control groups in order to detect a difference in allele frequency of 0.09–0.15 and an odds ratio of 2 for this gene on asthma, with 95% confidence, 80% study power and 5% type I error.

Previous studies have suggested that there were significant interethnic differences in the allele frequencies of a number of asthma predisposition genes in Asians as compared with the Caucasian population [14, 35]. With regard to the –1147CT polymorphism, the T allele frequency was 0.14 in our Chinese subjects while it was 0.08–0.23 in the Caucasians [5]. For other SNPs such as +49AG, the A allele frequency was 0.34 in our subjects. This frequency is similar to 0.35 in the Taiwanese [9] and 0.37 in the Japanese [8], and these allele frequencies are in accordance with the reported frequencies of 0.24–0.44 in the Caucasian populations [5].

The spirometric index of FEV₁/FVC in our asthmatic children was lower than that measured in the controls. In contrast, the former group had higher FEV₁ and FVC percentages. These differences are not unexpected, as a number of published studies showed that individual parameters of prebronchodilator spirometry were normal in the majority of children and adolescents with asthma. On the other hand, FEV₁/FVC as an indicator of airflow limitation is more reliable as it declines with increasing asthma severity [36, 37]. However, it is still possible that the observed differences were due to technical errors in performing spirometric measurements in the controls. It is widely appreciated that spirometry is effort-dependent, although much less so when compared with peak expiratory flow.

One major drawback of this study is that our asthmatic patients and control children were selected from a hospital cohort, and this approach generally identifies a more severe group of patients compared with the general population as shown by several epidemiological studies [38–40]. Leung et al. [40] showed in a study involving 1,062 schoolchildren in Hong Kong that 58% of the subjects were atopic as defined using the skin prick test. In our

study, atopy was found in 85% of asthmatic patients and 61% of age-matched controls. Although our findings may not be generalized to the general population, this study suggested that *CTLA-4* -1147CT polymorphism and plasma sCTLA-4 may be important predictors of allergy in Chinese children. Larger population-based studies are necessary to confirm these associations.

In summary, although the role of sCTLA-4 in the development of asthma remains obscure, our results suggest that there is a clinical correlation between the

sCTLA-4 level and lung function. Besides, its concentration is associated with -1147CT polymorphism and haplotypes among the 6 SNPs.

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