

An asthma-associated genetic variant of STAT6 predicts low burden of ascaris worm infestation

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Th-2 immune mechanisms are involved in the pathology of asthma and in the protective immune response to parasitic worms. Common upregulating genetic variants of Th-2 immune signalling are risk factors for asthma, and we tested whether they may confer a counteradvantage in protecting against parasitic worms. We examined the intensity of infection by the parasitic worm, Ascaris lumbricoides, by microscopic counting of ascaris eggs in the stool of 614 schoolchildren from an area of endemic ascaris infection in China. We investigated the relationship between the intensity of ascaris infection and common, asthma-associated genetic variants of Th-2 and Th-1 immune signalling. Ascaris egg counts per gram of stool (epg), mean 1068 epg, ranged from barely detectable (< 240 epg) to heavy (~ 9600 epg) in a skewed distribution. Logistic regression, after exploratory discriminant analysis, showed a major association between a common genetic variant of the 3'-UTR regulatory elements of the signal transducer and transactivating factor (STAT6) ($P=0.0002$) and egg counts, at the 77th centile. Linear regression after log transformation of egg counts confirmed a highly significant association with this STAT6 variant ($P=0.001$). Thus, a common, asthma-associated, genetic variant of the pivotal transduction and transactivating factor for Th-2 immune signalling, STAT6, predicts increased resistance to ascaris worm infection. The evolution of enhanced resistance to parasitic worm infection, through human genetic variation in Th-2 immune signalling, may represent one origin for asthma.

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Introduction

Infestation with parasitic helminths is a natural condition for billions of humans in regions of the world where poor sanitation promotes rounds of infection and reinfection; children are notably susceptible to heavy infection when ill-health and poor growth result.¹ It has been repeatedly observed that there is a significant heterogeneity in the burden of helminth infestation in areas of endemic infection, in which only some 20% of any young population show a high intensity of infection and therefore carry most of the worms.^{1,2} Environmental, behavioural and genetic factors are considered to contribute to this heterogeneity—but the detailed nature of the genetic factors in particular is not known.^{3,4}

Asthma has become a highly prevalent chronic disease of children and young adults in developed countries.⁵ Environmental factors, including changing patterns of early childhood infection and diet, are considered candidate mechanisms for the rapid rise of asthma,^{6–8}

but genetic factors are also important in the disease. The heterogeneity of asthma's genetic background is emphasised by the many chromosomal linkage results in genome-wide studies.⁹

It is notable that the bronchial pathology in asthma and the protective host response to helminth include prominent elements of Th-2 immune activity, which lead to increased mucosal eosinophil activity, mucus hypersecretion and muscle hyperactivity.^{10,11} In the bronchus, these features underlie the chronic, episodic symptomatology of asthma.¹⁰ In the gut, these immune mechanisms promote the expulsion of helminths.¹¹ Observational studies in humans, and knockout experiments in mice, emphasise the key roles of IL-13^{12–14} and STAT6^{11,15–17} in mediating these mucosal changes.

We, and others have found that common genetic variants of Th-2 immune signalling are significant predictors of asthma—including variants of IL-13,^{18,19} IL-13R α 1,¹⁸ IL-4R α 1^{19,20} (which with IL-13R α 1 constitutes the IL-13 receptor) and STAT6.^{21,22} Biological, functional and molecular modelling studies point to upregulation of Th-2 immune signalling in two of the variants tested, those in IL-13 and IL-4R α 1.^{18,20,23}

We reasoned that the high prevalence of such variants, which promote asthma in early life, might derive from a counterbalancing evolutionary advantage of the variants in promoting protective Th-2 immune responses against high burdens of helminth infection. We therefore tested

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whether these common asthma-promoting genetic variants might limit helminth burden in an area of China, where infection with the helminth *Ascaris lumbricoides* is endemic.

Methods

Study population

In total, 614 schoolchildren (319 males, 295 females), aged 11–15 years (mean 12.42), from four schools in the Xing Chang district of Shanghai were the subjects of the study. In the Xing Chang district, the majority of the population is involved in subsistence agriculture, many farmers still use fresh night soil to fertilise green-leaf vegetables, and ascaris infection is endemic.

Ascaris burden

Ascaris is a roundworm, in which there is faecal–oral spread of infection and reinfection, in approximately 1 billion people worldwide. Its lifecycle involves transient passage of blood-borne larvae across the lung, and the mature worms reside in the small intestine for their life span of 1 year, where the female worm excretes some 0.25 million eggs per day into the human faeces.¹ The number of eggs in the faeces is generally regarded as a reliable indicator of the burden of adult worm carriage in the intestine,^{4,24} and high worm burdens lead to poor health and growth in children.¹

The children we studied had stool samples examined by the Kato–Katz method²⁵ for the presence and numbers of ascaris eggs per gram of faeces (epg), in preparation for eradication treatment. In addition, it was recorded whether the eggs of any other parasitic worms were identified, including hookworm species, trichuris and enterobius. Fresh stool was drawn through a 100-mesh gauze into plastic microscopy wells (with volumes defining the weight of stool), and helminth eggs were identified and counted by microscopy and expressed for each child as ascaris epg.

Questionnaire on social factors and health status

Each child responded to a questionnaire documenting their area of residence in the district, whether their house was traditional wood-built or modern brick-built, and

whether their diet was based on foods of local origin or more mixed. In addition, the children responded to questions from a modified ISAAC questionnaire on symptoms of asthma, hay fever and eczema. The children were weighed, as an indicator of nutrition. The diameter of indurated skin response to tuberculin (2 tuberculin units, TU) was measured, as a marker of Th-1 immune response, and as a potential confounder of the helminth burden—genetic variant analysis.

Genetic analysis

Genetic analysis was conducted on DNA extracted from circulating white cells. Assays were performed for common genetic variants belonging to the Th-2 and Th-1 immune cytokine signalling pathway, which we had previously found to be associated with asthma in a Japanese or British population.^{20,21,23,24} The loci and variants tested, the number of successful genotypes completed, the allele frequencies and heterozygosities are shown in Table 1. The genotype distributions at all loci tested matched Hardy–Weinberg equilibrium. All were single-nucleotide polymorphisms (SNPs) and were assayed by the Taqman TM high-throughput SNP screening method.²⁵ Fluorescent signal intensity was optimised by testing an array of primer concentrations using standard assay conditions. The sub-set of concentration pairs with two primers was derived empirically to reduce the number of reactions that had to be run for each assay optimisation. PCR products, amplified in triplicate for each set of concentrations, were analysed in an ABI prism 7700 sequence detector.

Statistical analysis

We tested for relationships between ascaris egg burden and (a) the genetic variant genotypes and (b) the social and health status variables recorded by questionnaire. The distribution of ascaris burden (Table 2) showed a continuous skewed distribution, and we employed a set of different statistical methods and utilised the Statistical Package for Social Sciences (SPSS).

Egg counts were log transformed and a linear regression analysis was performed, directly followed by stepwise procedures.

Exploratory discriminant analysis was used to test for genetic associations at specific levels of egg burden: (a) at

Table 1 Variant loci tested against ascaris burden in the Chinese schoolchildren

Locus	Number of genotypes completed	Variant	Heterozygosity	Allele frequencies
IL-4	606	Promoter (+33 C/T)	0.29	0.163, 0.837
IL-5	599	Promoter (–703 T/C)	0.31	0.294, 0.706
IL-13	605	Promoter (–1055 C/T)	0.39	0.196, 0.804
IL-13	579	Gln110Arg	0.36	0.332, 0.668
IL-4Rα1	602	Ile50Val	0.53	0.457, 0.543
IL-13Rα1	608	3′-UTR (1499 G/A)	0.27	0.471, 0.529
STAT6	608	3′-UTR (4219 G/A)	0.45	0.490, 0.510
IL-5Rα	606	Promoter (–80 G/A)	0.28	0.205, 0.795
FcεRIα	604	Promoter (–66 T/C)	0.07	0.037, 0.963
FcεRIβ	578	Intron 2 (1798 G/A)	0.27	0.161, 0.839
IL-10	608	Promoter (–627 C/A)	0.45	0.348, 0.652
IL-10R	602	Intron 1 (520 G/A)	0.49	0.365, 0.635
IFNγ	590	Intron 1 (1384 A/T)	0.26	0.139, 0.861
IL-12β2	594	3′-UTR (1159 G/A)	0.49	0.421, 0.579
IL-12Rβ2	593	Promoter (–325 G/T)	0.18	0.112, 0.888

Table 2 Frequency of different ascaris egg counts (epg) in a total of 614 subjects

Egg counts	Number of individuals ^a	Percentage
<240	305	50.0
240–720	162	26.6
960–1680	86	14.3
1920–3360	32	5.2
3600–6000	12	2.0
6480–9600	13	2.1

^aFour cases were unclassified.

the 77th centile (≥ 960 epg), and matching the general recognition that 20% of most populations carry the highest worm burdens;^{1,2} (b) the 50th centile (≥ 240 epg, generally recognised in the Shanghai Health Programme as positive cases requiring eradication treatment); (c) the 90th centile (≥ 1920 epg) as a test at the very highest burdens. It was planned that any significant genetic effect within the discriminant analysis should be further investigated by logistic regression, employing direct and then step-wise procedures.

Results

Ascaris infection was common. The numbers of ascaris eggs in stool (geometric mean, 1068 epg) ranged greatly across the children, and the distribution matched the expectation that approximately 20% would show high burdens of worm infestation (Table 2). The geometric mean egg count (1068 epg) matches that in a recent study in Nepal²⁴ (1453 epg) and indicates a moderate worm burden. No hookworm or enterobius eggs were identified. In all, 99 of the children with ascaris infection also showed sparse numbers of trichuris eggs.

Linear regression analysis, after log transformation of the egg counts, identified a significant genetic association. After stepwise procedures, these genetic associations were attributable to the common genetic variants tested in STAT6 ($P=0.001$) and IL-4 ($P=0.008$).

The exploratory discriminant analysis showed significant genetic associations with egg burden at the 77th centile of egg counts ($X^2=52.549$, 24 degrees of freedom matching the number of genetic and environmental variables tested; $P=0.001$), but no significant association at either the 50th or 90th centiles. No associations were found between egg count at this centile with age, gender, body weight, tuberculin status, or any other behavioural or environmental variable. Table 3 shows the coefficients of discriminant analysis after a stepwise procedure, indicating that the variants of IFN- γ , STAT6 and IL-13 were the principal associating factors. The subsequent stepwise logistic regression analysis at this 77th centile of egg count (Table 4) supported and amplified these indications; the genetic associations with egg burden were attributable principally to the variant of STAT6—operating independently ($\chi^2=13.89$, $P=0.0002$), and operating through an interaction with the variant of IFN- γ ($\chi^2=7.65$, $P=0.006$). A variant of IL-13 ligand contributed a lesser, but significant contribution ($\chi^2=4.02$, $P=0.045$).

Table 3 Associations of genetic variants with ascaris egg count (≥ 960) at the 77th centile

Genetic variants	Coefficients of discriminant function after stepwise procedure
IL-13	−0.41972
STAT 6	−0.50814
Fc ϵ R2	+0.21755
IL-12 β	+0.21881
IFN- γ	−0.67165

The five most significant standardized canonical discriminant function coefficients (after the stepwise procedure of the discriminant analysis) are shown: the greatest coefficient values (positive or negative) were for IFN- γ , STAT6, and IL-13.

Table 4 Associations of genetic variants with ascaris egg count at the 77th centile: logistic regression analysis, stepwise procedure

Variants	χ^2	P-value
STAT6	13.89	0.0002
Interaction (STAT6-IFN γ)	7.65	0.0060
IL-13	4.02	0.0450
Constant	34.77	0.0000

Homozygous GG (*vs* AG and AA) at 4219 STAT6 predicted protection against high, ascaris egg counts (≥ 960 epg) with odds ratio 0.34, and 95% confidence intervals of 0.17–0.69. There was a significant trend ($P=0.004$) for decreasing mean egg counts according to genotype at 4219-AA (1417.22), AG (1195.09), GG (505.98). Figure 1 shows the trends for the STAT6 genotypes, by ascaris egg count.

Regular symptoms of asthma affected only seven of the 614 Chinese children studied, and therefore no analysis for associations with any of the genetic variable was attempted.

Discussion

The results of both linear regression and logistic regression at the 77th centile of egg counts indicate a strong association between allele G at nucleotide 4219 of STAT6, particularly in the homozygous state, with diminished ascaris burden in children living in an area of endemic infection. In our published study of asthma in an urban Japanese population, homozygous GG-4219 of STAT6 predicted chronic, regular treatment-requiring asthma in young adults with mild allergy at an odds ratio of 3.19, 95% confidence intervals 1.40–7.08.²¹

The data suggest that the G-4219 variant within the 3' regulatory elements of STAT6, or another variant in tight linkage disequilibrium, may potentiate STAT6 activity and hence Th-2 immune effects, in turn promoting asthma in urban environments (free of ascaris infection), but protecting against ascaris infection in environments where there is endemic infection. This possibility is consistent with the central role of STAT6 in mediating Th-2 cytokine-induced eosinophil, mucus and muscle activity in both asthma²⁶ and in helminth infections.¹¹

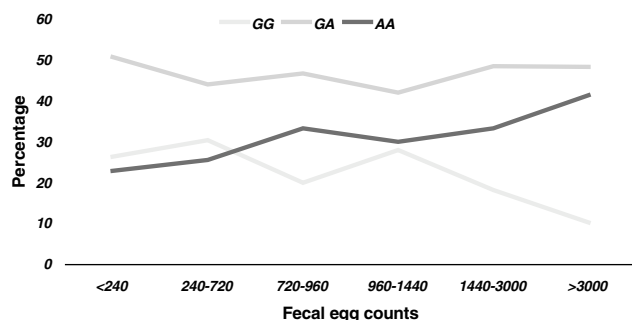


Figure 1 Plots of STAT6 variant genotypes—percentage prevalence at different faecal ascaris egg counts among 614 Chinese children.

Our data also indicate the action of other genetic variants of immune signalling in relation to ascaris burden—a variant of IL-4 in the linear regression, a variant of IFN- γ in an interaction with STAT6, and a variant of IL-13 operating at the 77th centile of burden in the logistic regression (Table 4). A polygenic interaction, with a major effect from the pivotal Th-2 transduction molecule (STAT6), in limiting worm burden also accords with the strong evolutionary pressure that must be exerted by endemic helminth infection in billions of humans, and the helminths' many evasive and subversive tactics, directed at diverse elements of the human's protective responses to the parasite.^{1,27} The chromosomal location of STAT-6 (chromosome 12) does not match chromosomal linkages recently observed between ascaris burden and chromosomes 1 and 13 in a Nepalese genetic isolate (Jirels).²⁸ Genetic differences between the Chinese and Jirels may explain this discrepancy, but we have no information on STAT6 variation in the Jirels. With respect to our own discriminant and logistic regression analysis, it is notable that the relationship we observe between the STAT6 variant and ascaris burden in the Chinese population operates close to the well-recognised boundary of infection intensity for many helminths; in this ~20% of a human population is heavily infected and carry most of the worms, and are also most likely to incur reduced biological fitness due to ill-health and poor growth.²

Our data, based on one population, are essentially hypothesis generating. Further epidemiological studies are needed on genetic variants of STAT6, and variants of other Th2/Th1 signalling components, in relation to ascaris burden in other geographical areas of endemic infection and also in relation to the varying burden of other parasitic worm infections.

It is of interest that the 4219 variant of STAT6 lies in a C-rich region of the 3'UTR regulatory elements, since there is increasing documentation that these elements are major modulators of protein expression, through diverse actions including the determination of mRNA transcript half-life.^{29,30} No natural coding sequence variants of STAT6 have been described—and no data are available on the functional impact of its noncoding variants and how these might underlie the association with asthmatic²¹ and atopic phenotypes,^{22,31} and now helminth burden. It is plausible that their actions should be on mRNA or protein quantity, and hence impact on the degree of STAT6-mediated transcriptional activation of many genes encoding the effector mechanisms of IL-4

and IL-13 signalling in Th-2 cells and at mucosae. Coding sequence variants of STAT6 have been induced by mutagenesis, and have been shown to influence the structure and stability of monomeric and dimeric STAT6 protein, and enhance STAT6 activity.³² The epidemiological interaction of a variant of IFN- γ (the major cytokine of Th-1 immunity) with STAT6 in limiting worm burden raises different functional possibilities; that the IFN- γ variant may either up- or downregulate Th-1 activity, resulting in Th-2/Th1 cooperation or diminished antagonism by Th-1 of Th-2 mechanisms in limiting worm burden, or that high Th-1 function and low Th-2 activity promote high worm burdens. Detailed functional studies are now needed to clarify these various points.

Our data, recording the association between a common genetic variant of STAT6 with more asthma but less ascaris infection, may be relevant to the long-studied and complex relationship between helminth infections and asthma and allergy. Recent studies have recorded both inverse^{33,34} and positive relationships³⁵ between helminth infection and asthma, but have not taken into account pertinent molecular genetic variants. Our data suggest that one element may be an inverse relationship, through genetic variation in Th-2 immune signalling, in which those with heavy worm burdens are least likely to manifest asthma. Other relationships must also exist, as suggested by recent studies in which helminth infections may inhibit allergy and asthma, through the induction of immune controlling factors, for instance the enhanced secretion of IL-10.³⁶ Future epidemiological studies into the relationship between worm infection and asthma/allergy therefore need to take into account genetic variants in Th-2 signalling.

Our findings suggest that one mechanism for asthma may be humans' evolutionary adaptation to ubiquitous helminth infection—operating in this instance through enhanced STAT6 and Th-2 activity. Further studies are now needed.

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