

Genetic association study between *mbi2* and asthma phenotypes in Chinese children

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Mannose-binding lectin (MBL), a member of the innate immune system, initiates complement deposition on microbial surfaces. MBL deficiency is associated with severe respiratory infections. Polymorphisms in the MBL gene (*mbi2*) were associated with the susceptibility and severity of autoimmune diseases. This study investigated whether *mbi2* polymorphisms at positions -550 and -221 and at codon-54 are associated with asthma phenotypes in Chinese children. Asthmatics aged 5–18 yr and non-allergic controls were eligible, and their plasma total and allergen-specific immunoglobulin E (IgE) concentrations were measured by micro-particle immunoassay and fluorescent enzyme immunoassay. *mbi2* polymorphisms were genotyped by restriction fragment length polymorphism. Three hundred and seventeen asthmatic children and 140 controls were recruited, with their mean (s.d.) log-transformed plasma total IgE being 2.61 (0.61) and 1.77 (0.77), respectively ($p < 0.0001$). Polymorphisms at -550 and codon-54 ($p < 0.0001$ for both) but not at -221 ($p = 0.534$) of *mbi2* were significantly associated with plasma MBL concentrations. *mbi2* genotypes were not associated with asthma, atopy, sensitization to individual aeroallergens or spirometric variable. Subjects with *LYB* haplotype had the lowest plasma MBL concentrations ($p < 0.0001$), but two- and three-loci *mbi2* haplotypes were also not associated with asthma diagnosis. However, patients with *LY* and *LYB* haplotypes were less likely to be atopic ($p = 0.006$ and 0.031). Subjects with *LY* and *LYA* were also less likely to be sensitized to cockroach ($p = 0.035$ and 0.047). The latter three associations became insignificant when adjusted for multiple comparisons. Despite the importance of MBL in innate immunity, our *mbi2* polymorphisms only show weak association with asthma and atopy in children.

Ting Fan Leung¹, Nelson L. S. Tang², Ying Man Sung², Chung Yi Li¹, Suk Ling Ma², Christopher W. K. Lam² and Gary W. K. Wong¹

Departments of ¹Paediatrics and ²Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong

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T. F. Leung, Department of Paediatrics, 6/F, Clinical Sciences Building, Prince of Wales Hospital, Shatin, Hong Kong

Tel.: 852 2632 2981

Fax: 852 2636 0020

E-mail: tfleung@cuhk.edu.hk

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Recent studies suggest protective roles of early-life infections against the susceptibility to asthma and atopy (1, 2). Microbial components such as endotoxin drive the immune system towards type 1 T-helper (Th1) lymphocyte-related cytokine production (3). Innate immunity plays a key role in this endotoxin recognition. Among the innate immune receptors, mannose-binding lectin (MBL) specifically recognizes the carbohydrate patterns found on infectious non-self surfaces, which leads to complement activation and the

orchestration of adaptive immune response. MBL is particularly important during the vulnerable period of infancy before adequate adaptive immunity is established (4). *mbi2*, the gene encoding human MBL and with four exons, is located at chromosome 10q21–24 (5, 6). Three single nucleotide polymorphisms (SNP) have been described in exon 1 of *mbi2* (7, 8). The normal wild-type allele is commonly designated *A*. These variants are due to three single-base pair substitutions at codon-54 (allele *B*), codon-

57 (allele *C*) and codon-52 (allele *D*), which independently cause lower serum MBL levels (7). The frequencies for codon-52 and codon-57 mutations were very rare in Chinese (9). The serum MBL concentration is further regulated by a number of SNP in the promoter region of the *mbi2* (10, 11). In particular, a polymorphism at -221*X/Y* has a significant effect on serum MBL concentration with the *Y* allele having higher MBL expressing activity than the *X* allele.

Deficiency of MBL is a risk factor for viral, bacterial and fungal infections (12, 13). In particular, MBL deficiency is a significant cause of acute respiratory infection in early childhood (13). Patients with *mbi2* variant alleles had increased susceptibility to bacterial and viral infections (14, 15). *mbi2* polymorphisms were also linked to the susceptibility and prognosis in autoimmune diseases as rheumatoid arthritis and systemic lupus erythematosus (16, 17). On the other hand, there has only been one adult study that investigated the possible association between asthma and *mbi2* polymorphisms (18). In this study, we investigated the frequencies of three *mbi2* SNP and their relation to asthma phenotypes in Chinese children.

Methods

Study population

This study recruited unrelated Chinese patients aged 5–18 yr with asthma, diagnosed according to American Thoracic Society guideline (19), from the pediatric outpatient clinics of a university teaching hospital in Hong Kong. The controls were recruited from children of similar age attending the general pediatric clinics for minor non-allergic and non-immunologic complaints. All subjects were free from symptoms of infection for 4 wk before study. Subjects' parents gave written consent, and Clinical Research Ethics Committee of our University approved this study.

Spirometry

Asthmatic patients underwent spirometric assessment (Compact II; Vitalograph, Buckingham, England) to measure their forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC), which were compared with the local reference values (20).

Plasma total and allergen-specific immunoglobulin E concentrations

Plasma total IgE concentration was measured by micro-particle immunoassay (IMx analyzer; Abbott Laboratories, Abbott Park, IL, USA) and specific IgE to *Dermatophagoides pteronyssinus*, cat, dog, cockroaches and molds were measured by fluorescent enzyme immunoassay (Pharmacia Diagnostics AB, Uppsala, Sweden). Specific IgE concentration ≥0.35 kIU/l was positive, and atopy was defined as the presence of at least one type of allergen-specific IgE.

Plasma MBL concentrations

The concentrations of oligomerized MBL in 100-fold diluted plasma samples were measured by enzyme-linked immunosorbent assay (Antibody-Shop, Gentofte, Denmark) according to manufacturer's instructions. The inter- and intra-assay coefficients of variation were 4.3% and 3.8%, respectively. The lower detection limit for plasma MBL was 2 ng/ml.

Genotyping for *mbi2* polymorphisms

Polymorphisms at -550*H/L*, -221*X/Y* and codon-54 *A/B* of *mbi2* were genotyped by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Table 1 describes the primers, PCR conditions, restriction enzymes and digestion products. Briefly, PCR reactions were carried out in a total volume of 25 µl containing 60 ng of DNA in the presence of

Table 1. Primers, polymerase chain reaction (PCR) conditions and restriction enzymes for restriction fragment length polymorphism (RFLP)

Polymorphism	Primers	PCR conditions	Restriction enzyme (units)	RFLP products*
-550 <i>H/L</i>	F: 5'-GGA GGA GGA TTC AAG GCA AG-3' R: 5'-TGC TTC CCC TTG GTG TTG T-3'	60°C/35 cycles	<i>RsaI</i> (5)	<i>L</i> allele: 176-bp <i>H</i> allele: 156-bp, 20-bp
-221 <i>X/Y</i>	F: 5'-CAG ATG GAC CCG AAG AGG AC-3' R: 5'-TGG CCT CTA GCT GGG GAT TT-3'	60°C/35 cycles	<i>BtgI</i> (5)	<i>X</i> allele: 300-bp <i>Y</i> allele: 168-bp, 132-bp
Codon-54 <i>A/B</i>	F: 5'-ATT GTA GGA CAG AGG GCA AGC T-3' R: 5'-TGA ATA GGG GTC TGA CAT GAC A-3'	60°C/35 cycles	<i>BanI</i> (5)	<i>B</i> allele: 560-bp <i>A</i> allele: 312-bp, 248-bp

*. All PCR products were digested by the respective enzymes overnight at 37°C, and these digested products were visualized on 4% (w/v) agarose gel containing ethidium bromide.

50 ng of each primer and 0.5 unit of Taq DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). Eight DNA samples of known -550H/L, -221X/Y and codon-54 A/B genotypes were included in every PCR reaction and restriction enzyme digestion as positive control together with 88 samples in 96-well plates. Finally, RFLP results for 40 randomly selected samples were confirmed by direct sequencing using BigDye Terminator Cycle sequencing kits with an ABI-3100 autosequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Results were expressed as proportions or mean and standard deviation (s.d.). Patient characteristics were compared using the student *t*-test or ANOVA for numerical data, and χ^2 or Fisher Exact test for categorical variables. The associations between *mbi2* genotypes and dichotomous asthma and related phenotypes were analyzed using logistic regression, and with quantitative traits using linear regression. Two alternative models (recessive and co-dominant) were fitted for each outcome, adjusting for age and gender as covariates. Finally, two- and three-loci haplotypes for *mbi2* -550/-221 and -550/-221/codon-54 were calculated as maximum likelihood estimates using EH program (Rockefeller University, New York, NY, USA). The frequencies of these haplotypes were analyzed in relation to different dichotomous asthma phenotypes by χ^2 or Fisher Exact test, and the distribution of plasma MBL concentrations among subjects with different *mbi2* haplotypes were analyzed by the non-parametric Kruskal-Wallis and Mann-Whitney *U*-tests. *p*-values less than 0.05 were considered significant.

Results

Subjects

Three hundred and seventeen asthmatic children and 140 age- and sex-matched controls were

Table 2. Summary of clinical and laboratory characteristics

Characteristics	Asthmatics (n = 317)	Controls (n = 140)
Mean (s.d.) age, years	10.7 (4.0)	11.1 (4.3)
Male gender (%)	205 (65)	81 (58)
Mean (s.d.) log-transformed plasma total IgE concentration	2.61 ± 0.61†	1.77 ± 0.77
Number of subjects with increased plasma total IgE level (%)	241 (76)†	41 (29)
Number of subjects with positive specific IgE in plasma*		
<i>D. pteronyssinus</i> (%)	270 (90)†	52 (40)
Cockroaches (%)	87 (29)‡	16 (12)
Cat (%)	52 (17)†	4 (3)
Dog (%)	6 (2)	0
Molds (%)	26 (9)§	1 (1)
Atopy (i.e., with ≥ one specific IgE) (%)	274 (91)†	54 (41)

s.d., standard deviation; IgE, immunoglobulin E.

*. Samples available in 300 patients and 131 controls for analysis. Odds ratios (95% confidence intervals) for asthma diagnosis were 13.67 (7.93–23.69) for *D. pteronyssinus*, 2.94 (1.59–5.47) for cockroaches, 6.66 (2.25–22.17) for cat and 12.34 (1.76–246.99) for molds.

†, *p* < 0.0001.

‡, *p* < 0.0005.

§, *p* < 0.005.

recruited. The mean (s.d.) FEV₁ and FEV₁/FVC in these patients were 93 (20)% of predicted and 80 (11)%, respectively. Table 2 summarizes the demographic data and laboratory investigations for these subjects.

mbi2 polymorphisms and plasma MBL concentrations

The frequencies for minor alleles -550H, -221X and codon-54B in our controls were 0.41, 0.22 and 0.17, respectively. The genotype distributions of these three *mbi2* SNP followed Hardy-Weinberg equilibrium. The mean (s.d.) plasma MBL concentrations for patients and controls were 2971 (2221) ng/ml and 2716 (2034) ng/ml, respectively (*p* = 0.266). Table 3 summarizes the relation between individual *mbi2* genotypes and plasma MBL concentrations. In brief, *mbi2* polymorphisms at -550 and codon-54 but not at -221 were highly significantly associated with plasma MBL concentrations in our children. However, plasma

Table 3. Relation between *mbi2* genotypes and plasma MBL concentrations

<i>mbi2</i> polymorphism	Plasma MBL concentrations (ng/ml)			<i>p</i>
	Homozygous WT	Heterozygous	Homozygous M	
-550H/L	1729 (1436–2023)	2980 (2709–3251)	4942 (4488–5396)	<0.00001
-221X/Y	3009 (2723–3295)	2842 (2504–3180)	2526 (1781–3271)	0.534
Codon-54A/B	3984 (3778–4189)	677 (555–799)	15 (9–21)	<0.00001

M, mutant; WT, wild-type; MBL, mannose-binding lectin.

Results are expressed in means and 95% confidence intervals.

*, Analyzed by anova.

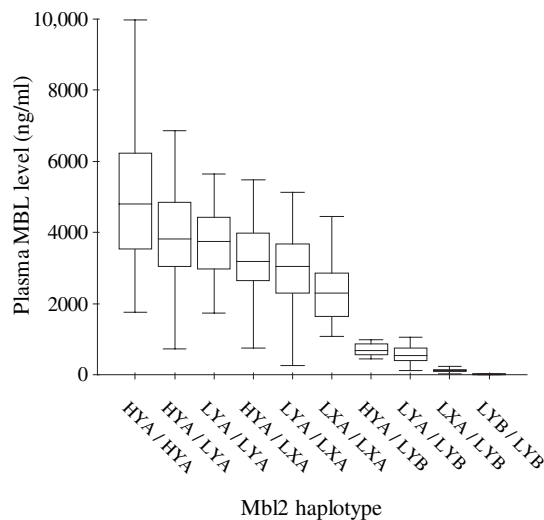


Fig. 1. Box plots showing the relation between *mbi2* three-loci haplotypes and plasma mannose-binding lectin (MBL) concentrations. Analyses included only the four major *mbi2* haplotypes: *HYA*, *LXA*, *LYA* and *LYB*, whereas the other minor haplotypes with <5% frequencies were omitted. Subjects with at least one *LYB* haplotype (i.e., in the last four columns) had significantly lower plasma MBL concentrations as compared to those with other haplotypes [median (interquartile range): 567 (249–781) ng/ml vs. 3590 (2788–4857) ng/ml; $p < 0.0001$].

MBL concentrations did not show significant correlation with any demographic factor, plasma total IgE or spirometric variable (results not shown). Fig. 1 reveals box plots for the distribution of plasma MBL concentrations among subjects with different *mbi2* haplotypes. In particular, subjects carrying at least one *LYB* had lowest plasma MBL concentrations as compared with the others ($p < 0.0001$).

Asthma traits and individual *mbi2* polymorphisms

The *L/L*, *L/H* and *H/H* genotypes of *mbi2* -550H/L were found in 33%, 49% and 18% of asthmatics and 33%, 52% and 14% of controls ($p = 0.451$ and 0.746 under recessive and co-dominant models, respectively). Similarly, we could not observe any excess percentage in patients vs. controls of the homozygous mutants

for -221X/Y (6% vs. 5%; $p = 0.775$ and 0.567) or -54A/B (3% vs. 4%; $p = 0.879$ and 0.972). These individual SNPs were also not significantly associated with atopy or the presence of allergen-specific IgE. Linear regression models were used to analyze the transformed continuous outcome variables, namely logarithm of plasma total IgE and spirometric indices (i.e., FEV₁ percentage and FEV₁/FVC). Table 4 summarizes the corresponding results, and there was no significant association between *mbi2* polymorphisms and these quantitative traits in our population.

Asthma traits and *mbi2* haplotypes

Concerning the analyses between asthma-related phenotypes and two-loci *mbi2* haplotypes, *HY*, *LY* and *LX* were detected in 41%, 38% and 19% of patients and 40%, 37% and 23% of controls ($p > 0.3$), respectively. Asthmatic patients with *LY* haplotype had less atopy as compared with *HY* (38% vs. 42%; $p = 0.006$). Besides, the former group was associated with lower rate of sensitization to cockroach (34% vs. 48%; $p = 0.035$) but not other allergens. When codon-54 was included in the haplotype analysis (i.e., three-loci), patients with *LYB* were again less likely to be atopic as compared with the wild-type *HYA* (16% vs. 42%; $p = 0.031$). The haplotype *LYA* was associated weakly with less cockroach-specific IgE among asthmatics (19% vs. 48%; $p = 0.047$). However, except for the relationship between atopy and *LY* haplotype, all other associations became insignificant after adjusted for multiple statistical comparisons. Among our patients, *mbi2* haplotypes were also not associated with increased plasma total IgE (as compared with in-house references), reduced FEV₁ (<80% predicted) or subnormal FEV₁/FVC (<80%) (results not shown).

Discussion

In this genetic association study, we genotyped three *mbi2* SNPs using RFLP in 457 southern

Table 4. Relation between *mbi2* genotypes and quantitative traits

	<i>mbi2</i> -550*		<i>mbi2</i> -221*		<i>mbi2</i> codon-54*	
	Recessive	Co-dominant	Recessive	Co-dominant	Recessive	Co-dominant
IgE _{log}	0.097	0.099	0.238	0.083	0.800	0.254
FEV ₁ percent†	0.359	0.512	0.155	0.355	0.260	0.950
FEV ₁ /FVC†	0.740	0.957	0.059	0.629	0.987	0.687

IgE_{log}, log-transformed plasma total immunoglobulin E concentration; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

*, p values were obtained by linear regression with recessive and co-dominant models, fitting age and gender as covariates.

†, Spirometric results could be successfully obtained from 269 patients.

Chinese children. The minor allele frequencies for -550*H/L*, -221*X/Y* and codon-54*A/B* in the controls were 41%, 22% and 17%, respectively. Polymorphisms at -550 and codon-54, but not the one at -221, were highly significantly associated with plasma MBL concentrations. There was no significant association between any one of these SNP and asthma and related phenotypes. *mbi2* haplotypes significantly influenced plasma MBL concentrations, with the lowest level being found among subjects with *LYB*. However, haplotype analyses did not show any significant association with asthma diagnosis. However, asthmatic patients with *LY* haplotype suffered less from atopy in our Chinese children. Subjects with *LY* were less likely to be sensitized to cockroach.

Our finding of a weak yet protective effect of *mbi2* haplotypes against the susceptibility for atopy and cockroach sensitization is consistent with the hygiene hypothesis (21), which attributed the recent upsurge of the prevalence rates of atopic disorders to reduced exposure to infectious agents in childhood. Population studies suggested a negative association between early respiratory tract infections and the later development of asthma and atopy (1, 2). An inverse relationship was also observed between farm contact and early-life endotoxin exposure and the risk of having allergic sensitization and asthma in children (3). Microbial agents interact with innate immune receptors, such as CD14 and Toll-like receptors to skew the Th1/Th2 balance away from the allergy-driven Th2 cytokine profile. Asthma and related phenotypes were also associated with polymorphisms in *CD14* and Toll-like receptor genes (22, 23). As MBL also plays crucial roles in protecting against various microbes (12, 13), subjects with low-producing *mbi2* variants can have more frequent early infections and a stronger Th1 cytokine profile. This latter in turn may lead to less atopic disorders.

In contrary to our findings, Aittoniemi et al. reported recently that *mbi2* -221*X/Y* polymorphism was significantly associated with asthma in non-atopic males and a decrease in FEV₁ during follow-up among asthmatics in Caucasian adults (18). On the other hand, neither this nor the three SNP in exon 1 of *mbi2* was associated with atopy as assessed by skin-prick testing. These authors analyzed many asthma phenotypes and subject groups. However, they did not attempt to adjust for multiple statistical comparisons. Besides, that study did not investigate any relation between *mbi2* polymorphisms and MBL levels in peripheral blood. In the present study, SNP at -221 of *mbi2* was not associated with any change in

plasma MBL levels. These discrepant findings may be because of the differences in the patho-physiologic basis of asthma and atopy between children and adults, or that *mbi2* shows linkage disequilibrium to other loci that are associated with asthma and atopy. It would be prudent for future studies to try to replicate our findings and those from this published one.

Among Turkish asthmatic children (n = 72) and controls (n = 30), a recent study found that serum MBL levels were significantly higher among the former group (median: 3.6 mg/l vs. 2.8 mg/l; p < 0.0001) (24). There was no difference in MBL levels between stable patients and those with asthma exacerbation. The age ranges of their subjects were similar to ours, and that study as well as the present one obtained very similar results of MBL levels (i.e., in the magnitude of about 3 mg/l). However, their sample size was relatively small as compared with the present study involving 317 asthmatic children and 140 controls. We believe, thus, that this study should be more powerful in assessing any association between MBL levels and various asthma and atopy phenotypes.

In contrary to the weak associations found between *mbi2* and atopic disorders, promoter and structural variants of *mbi2* are more significantly and consistently related to complex human diseases with Th1 predominance. Tsutsumi et al. reported that Japanese adults with autoimmune disorders had a significantly higher frequency of having homozygous codon-54 mutation compared with controls. In particular, patients with systemic lupus erythematosus or Sjögren syndrome showed higher probabilities of being homozygous for this mutation (25). Patients with rheumatoid arthritis had significantly lower serum MBL levels compared with controls, and there were significant associations between rheumatoid arthritis and *mbi2* -550*H/L* and codon-54 mutation (26).

The minor allele frequencies for *mbi2* -550*H/L*, -221*X/Y* and codon-54*A/B* in our controls were 41%, 22% and 17%, and the frequencies for Japanese were 48%, 7% and 17% for alleles *H*, *X* and *B* (25), respectively. These figures were very much different from those in Caucasians and Eskimos for -221*X* (19–33% and 10%) and codon-54*B* (14% and 8%) (13, 18). South Americans had a very high frequency for codon-54 variant (42–46%), which accounted for the high prevalence (up to 16%) of MBL deficiency in this population (11). For *mbi2* haplotypes, the frequency of *LY* decreased from Africans (65%) to Caucasians (38%) to Chinese (30–37%) to Eskimos (14%) (10). This disparity in the allele frequencies of

atopy candidate genes between Chinese and other ethnic groups was also observed for *CD14* C-159T and *IL13* R130Q but not *TARC* C-431T (23, 27–29). Thus, it is important to perform genetic studies in other ethnic groups to confirm our results of an association between *mbi2* haplotypes and asthma phenotypes.

There are several limitations in this study. The present study is cross-sectional in nature, and did not provide any data on early-life events, such as frequencies of infective episodes, usage of antibiotics and daycare exposure. Prospective cohort studies are necessary to determine the gene–environment interaction between *mbi2*, early microbial exposure and the susceptibility to asthma and IgE sensitization. Second, this study includes only three *mbi2* SNP. Although these markers were carefully selected among the most relevant and widely studied ones in the Chinese, we cannot exclude the possibility that asthma and atopy may be associated with other *mbi2* polymorphisms in our population. Another limitation arises from the sample size of this study. There is no published data on any relation between *mbi2* SNP and asthma. Thus, we could not reliably calculate the numbers of asthmatic patients and controls that were required to show any significant association. Retrospectively, given the allele frequencies for *mbi2* SNP in this study, our sample size of 317 patients and 140 controls is sufficient to detect odd ratios of 0.53, 0.46 and 0.40 of *mbi2* -550H/L, -221X/Y and codon-54 A/B for asthma diagnosis. We try to overcome this by performing haplotype analyses. Nonetheless, in determining *mbi2* haplotypes, we found commonly that only small numbers (e.g., ≤ 5%) of subjects carried ‘at-risk’ haplotypes (e.g., *HX*). When haplotype frequencies were low in patients and controls, it would be less reliable for our analyses to detect any significant difference in the outcomes between the two groups.

In conclusion, low-producing, *LY*-containing haplotypes in *mbi2* promoter and structural variants were weakly associated with less atopy and cockroach sensitization in Chinese children. Thus, *mbi2* does not appear to be a major candidate gene for asthma and atopy.

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