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Functional polymorphisms of the *CCL2* and *MBL* genes cumulatively increase susceptibility to severe acute respiratory syndrome coronavirus infection

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Summary *Objectives:* To assess associations between the functional polymorphisms G-2518A at the chemokine (C–C motif) ligand 2 gene (*CCL2*) and mannose binding lectin (*MBL*) codon 54 variant (A/B) and susceptibility to SARS.

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Severe acute respiratory syndrome;
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Methods: We genotyped the *CCL2* G-2518A and *MBL* codon 54 variant (A/B) in 4 case–control populations of Chinese descent, totally consisting of 932 patients with SARS and 982 control subjects.
Results: Both the high-CCL2-producing GG genotype and the low-MBL-producing B allele were consistently associated with increased risks of SARS-CoV infection in all 4 case–control populations (joint $P = 1.6 \times 10^{-4}$ and 4.9×10^{-8}, for CCL2 and MBL respectively), with no interaction between polymorphisms could be detected. Furthermore, all the 4 case–control studies demonstrated a cumulative effect on risk of SARS-CoV infection for the combination of polymorphisms (joint $P = 1.3 \times 10^{-10}$). However, tests using the area under the curve (AUC) indicated that at this stage, the polymorphisms were unlikely to be appropriate for risk prediction testing because of low AUC values (all <66%). Additionally, no association was observed between the polymorphisms and severity of SARS.
Conclusions: The *CCL2* G-2518A and *MBL* codon 54 variant have a significantly cumulative effect on increased risk of SARS-CoV infection.

Introduction

Severe acute respiratory syndrome (SARS) is a newly emerged infectious disease of humans caused by a novel coronavirus (CoV), the SARS-CoV.[1] Pathogenesis of SARS is complex and host genetic background is considered to be one of the factors in determining susceptibility to, and outcome of SARS.[2] Previous reports have shown that polymorphisms of several putatively important genes affect an individual's susceptibility to SARS-CoV infection or disease severity of SARS.[3–10] In particular, our previous two independent association studies have implicated that a functional polymorphism at codon 54 in exon 1 (rs1800450, G230A, denoted as A/B variant) of mannose binding lectin (*MBL*), which encodes a protein belonging to the family of collectin and plays a critical role in the innate immune response, conferred a significantly increased susceptibility to SARS-CoV infection.[3,9] However, on the basis of the fact that the susceptibility to infectious disease is determined at different functional levels of innate and adaptive immunity,[11] we hypothesize that an unknown number of other unidentified genes are likely to mediate the susceptibility to SARS, including SARS-CoV infection and disease severity.

Chemokines play important role in cells trafficking during immune responses. Among the chemokine family, the chemokine (C–C motif) ligand 2 (CCL2), also designated as monocyte chemoattractant protein-1 (MCP-1), is known as a potent chemoattractant for monocytes and macrophages, and is considered to be involved in several diseases characterized by intense macrophage infiltration.[12] CCL2 influences both innate immunity, through effects on monocytes and macrophages, and adaptive immunity, through control of T helper cell polarization.[13] In the case of SARS, our and other studies have shown that CCL2 was one of the earliest and most prominent chemokines upregulated in either lung epithelial cells or monocyte derived dendritic cells infected with SARS-CoV.[14,15] The upregulation of CCL2 mediate the migration of monocytes and macrophages, which were the infiltrating cells indeed observed in the lung tissues of patients with SARS.[16] Notably, the overexpression of CCL2 was consistently detected in plasma of patients with SARS in several independent studies.[17–19] Furthermore, after treatment with corticosteroid, which is an effective cytokine modulator and has a beneficial effect on SARS patients, the level of plasma CCL2 was reduced significantly from 5 to 8 days.[17] Additionally, it has also been reported that the higher level of serum CCL2 in patients is correlated with more advanced disease severity of SARS.[17] In the lungs of BALB/c mice, the PDZ-binding motif of recombinant SARS-CoV envelope protein is a determinant of viral pathogenesis and induces the deleterious exacerbated immune response including increased expression of *CCL2*.[20] On the basis of the above relevance of the CCL2 in the pathogenesis of SARS, we hypothesize that the *CCL2* may be the excellent biologic candidate susceptibility gene for SARS. It is expected that the genetic variation within *CCL2* could contribute to inter-individual differences in susceptibility to, and outcome of SARS.

Recently, a functional single nucleotide polymorphism (SNP) (rs1024611, G-2518A) in the distal regulatory region of the *CCL2* at position −2518 relative to the transcription start site has been well characterized.[21] Compared with the *CCL2* -2518A allele, the −2518G allele conferred a greater *CCL2* transcriptional activity mediated by differential protein-DNA interactions, an increased CCL2 protein production *in vitro* and *in vivo*, and an enhanced leukocyte trafficking to tissues.[21–23] Furthermore, prevalence of the high-CCL2-producing −2518G allele has been shown to be associated with increased susceptibility or severity of infectious diseases, including HIV-1 infection and AIDS dementia,[21,24] HCV infection,[22] HBV clearance,[25] HCMV reactivation,[26] and pulmonary tuberculosis.[27] The role of this functional polymorphism in SARS, however, has never been specifically evaluated.

In this study, we therefore investigated whether the functional polymorphism G-2518A in the *CCL2* gene have any bearing on the SARS. To this end, we genotyped the *CCL2* G-2518A polymorphism in 4 independent case–control populations of Chinese descent, totally including 932 patients with SARS and 982 control subjects. We also reassessed the *MBL* codon 54 variant (A/B) in susceptibility to SARS-CoV infection in the present study. Furthermore, we investigated whether a combination of these two functional polymorphisms of *CCL2* and *MBL* would have a cumulative effect on risk of SARS-CoV infection.

Materials and methods

Study population

Four independent case–control populations of Chinese ancestry were included in the present study. The details about enrollment criterion and demographic characteristics of these populations had been described previously,[3,4,9] and were also provided in the Supplementary Materials and Methods and Supplementary Table 1. The study was performed with the approval of the Medical Ethical Committee of Beijing Institute of Radiation Medicine (Beijing, China) and the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (Hong Kong, China).

Genotyping

The polymorphism *CCL2* G-2518A (rs1024611) was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.[28] The sequences of primers and conditions of PCR and enzyme digestion were provided in the Supplementary Materials and Methods.

The *MBL* codon 54 A/B variant (rs1800450, G230A) was genotyped by using PCR direct sequencing as described previously.[9] This polymorphism had been investigated in Beijing community population and Hong Kong population in our previous studies[3,9]; therefore, the present study genotyped this polymorphism only in Beijing health care worker (HCW) and Tianjin population.

Statistical analysis

The results of SNP genotyping were tested for deviations from Hardy–Weinberg equilibrium (HWE) by using the Markov chain method implemented in the GENEPOP software (available at: http://wbiomed.curtin.edu.au/genepop/). Associations between SNPs and risk of SARS were assessed by use of logistic regression analyses in SPSS software (version 9.0; SPSS Inc., Chicago, IL). For *CCL2* G-2518A, the reference category is the combination of the heterozygous genotype AG and the homozygous minor genotype AA, while for *MBL* codon 54 variant (A/B) the reference category is the homozygous major genotype AA. The odds ratios (ORs) and 95% confidential intervals (CIs) were calculated and adjusted for potential confounders including age (continuous; years), sex (categorical; female or male) and populations (categorical; Beijing community, Beijing HCW, Tianjin, or Hong Kong), where it was appropriate.

The meta-analysis of data generated from multiple stages was conducted to estimate pooled genetic effects using fix-effect model based on Mantel–Haenszel method. We calculated Cochran's Q statistic to test for between-group heterogeneity and the I^2 statistic to quantify the proportion of the total variation due to heterogeneity. The heterogeneity was considered significant for $P < .05$. For there was no significant heterogeneity for multiple populations, we further analyzed the pooled data by logistic regression with adjustment for potential confounders.

The *CCL2* gene resides on chromosome 17q11.2-q12, while the *MBL* gene resides on chromosome 10q11.2, meaning that *CCL2* G-2518A and *MBL* codon 54 variant (A/B) are not in linkage equilibrium with each other. To further test whether or not a SNP is dependent on the other, independence test was performed for a SNP with adjustment for the other and potential confounders. Logistic regression with the use of an interaction term was also performed to investigate potential gene–gene interaction between two SNPs. For there was no significant interaction detected, we then tested the cumulative effects of SNPs on SARS by counting the number of at-risk genotypes associated with SARS for these two SNPs in each subject. The odds ratio for patients carrying any combination of one or two at-risk genotypes was estimated by comparing them with those carrying none of the at-risk genotypes by logistic-regression analysis. The Cochran–Armitage trend test was used to assess the disease risk upon the increasing number of at-risk genotypes.

The population attributable fractions (PAFs) were estimated for the at-risk genotypes of *CCL2* G-2518A and *MBL* codon 54 variant (A/B) with the use of the formula $PAF = f(OR-1)/[1+f(OR-1)]$, where f is the prevalence of at-risk genotype associated with SARS and OR is used in the place of relative risk.[29] The PAF value indicates percentage of the increase in the risk of developing SARS attributed to at-risk genotypes.

The specificity and sensitivity of the regression model were calculated by constructing receiver-operating-characteristic (ROC) curves, and then statistics for the area under the curve (AUC) were calculated to estimate the ability of models to distinguish case subjects from control subjects. The values for AUC range from 50% (no case–control discrimination) to 100% (perfect discrimination of cases and controls).

An association was considered significant at a *P* value of less than .05 in individual populations, and with more stringent threshold in the pooled samples based on the Bonferroni correction for multiple testing of two SNPs. All statistical tests were two-sided. All analyses were performed using the SPSS (version 9.0, SPSS Inc., Chicago, IL, USA) and Stata 9.2 software (StataCorp LP, College Station, TX, USA).

Results

Initially, we estimated the effect of *CCL2* G-2518A on susceptibility to SARS-CoV infection in Beijing community population consisted of 352 patients with SARS and 392 controls. The observed genotype frequencies for this polymorphism conformed to the HWE in both patients and controls ($P > .05$). On the basis of logistic regression analysis with adjustment for age and sex, the subjects carrying the GG genotype had a significantly increased susceptibility to SARS-CoV infection, compared with ones carrying the −2518A allele (i.e., AG plus AA genotype) (OR 1.41, 95% CI, 1.03 to 1.92, $P = .031$; Table 1).

To test for replication of the association, we then genotyped the G-2518A in three additional case–control sample sets. The genotype distributions of all groups did not deviate from the HWE. Again, there was an excess of

Table 1 Association between susceptibility to SARS-CoV infection and the *CCL2* G-2518A and *MBL* codon 54 variant (A/B).

Populations	No. of subjects (%)					
	Genotypes at *CCL2* G-2518A			Genotypes at *MBL* codon 54 variant (A/B)		
	GG	AG	AA	AA	AB	BB
Beijing community population						
Community cases (N = 352)	129 (36.6)	159 (45.2)	64 (18.2)	229 (65.6)	112 (32.1)	8 (2.3)
Random controls (N = 392)	114 (29.1)	195 (49.7)	83 (21.2)	301 (76.8)	85 (21.7)	6 (1.5)
OR (95% CI)	1.41 (1.03–1.92)			1.73 (1.25–2.39)		
P value	.031			8.6×10^{-4}		
PAF (%)	12.8			19.4		
Beijing HCW population						
HCW cases (N = 42)	25 (59.5)	16 (38.1)	1 (2.4)	18 (42.9)	21 (50.0)	3 (7.1)
HCW controls (N = 40)	15 (37.5)	22 (55.0)	3 (7.5)	28 (70.0)	12 (30.0)	0 (0.0)
OR (95% CI)	2.91 (1.14–7.45)			3.01 (1.20–7.53)		
P value	.025			.019		
PAF (%)	53.1			53.3		
Tianjin population						
Community cases (N = 60)	28 (46.7)	21 (35.0)	11 (18.3)	37 (61.7)	21 (35.0)	2 (3.3)
All controls (N = 129)	41 (31.8)	62 (48.1)	26 (20.2)	103 (79.8)	24 (18.6)	2 (1.6)
OR (95% CI)	2.09 (1.04–4.19)			2.61 (1.23–5.55)		
P value	.038			.012		
PAF (%)	33.9			38.0		
Hong Kong population						
Community cases (N = 478)	138 (28.9)	225 (47.1)	115 (24.1)	320 (66.9)	147 (30.8)	11 (2.3)
Random controls (N = 421)	95 (22.6)	213 (50.6)	113 (26.8)	315 (74.8)	98 (23.3)	8 (1.9)
OR (95% CI)	1.57 (1.12–2.22)			1.67 (1.20–2.33)		
P value	.010			.0023		
PAF (%)	14.8			18.8		
All four populations						
All cases (N = 932)	320 (34.3)	421 (45.2)	191 (20.5)	604 (65.0)	301 (32.4)	24 (2.6)
All controls (N = 982)	265 (27.0)	492 (50.1)	225 (22.9)	747 (76.1)	219 (22.3)	16 (1.6)
OR (95% CI)	1.48 (1.21–1.82)			1.79 (1.45–2.21)		
P value	1.6×10^{-4}			4.9×10^{-8}		
PAF (%)	14.6			21.9		

CI, confidence interval. HCW, health care worker. OR, odds ratio. PAF, population attributable fraction. For each population, the associations were performed by logistic regression analysis adjusted for age and sex. For pooled population, the associations were performed by logistic regression analysis adjusted for age, sex and population. For *CCL2* G-2518A, the AA plus AG genotype is combined as referent. For *MBL* codon 54 variant (A/B), the AA genotype is referent.

GG genotype in patients than in controls in Beijing HCW ($P = .025$), Tianjin ($P = .038$) and Hong Kong population ($P = .010$), respectively.

Meta-analysis on pooled data from all the four Chinese populations provided unequivocal evidence for a relationship between *CCL2* G-2518A and susceptibility to SARS-CoV infection (Fig. 1). OR associated with GG genotype was 1.58 (95% CI, 1.28 to 1.96, $P = 2.3 \times 10^{-5}$, $P_{heterogeneity} = .43$, $I^2 = 0\%$; Fig. 1a). Given there was no significant heterogeneity for the four Chinese populations, we further analyzed the pooled data by logistic regression, and a strong support for an association between the G-2518A and susceptibility to SARS-CoV infection was observed ($P = 1.6 \times 10^{-4}$), with the OR being 1.48 (95% CI, 1.21–1.82) for the at-risk GG genotype, compared with the AG plus AA genotype. On the basis of the ORs combined with the frequencies of GG genotype, PAFs were estimated to account for 12.8–53.1% of SARS cases in the 4 individual populations, and 14.6% in the overall population (Table 1).

We also reassessed the association between SARS and the codon 54 variant (A/B) in *MBL*, which were previously reported to be associated with SARS in both Beijing community and Hong Kong population (Table 1).[3,9] Again, we replicated that the subjects bearing the variant B allele (BB plus AB genotype), which is associated with low serum MBL,[30] have a significantly increased risk of SARS-CoV infection in both Beijing HCW ($P = .019$) and Tianjin population ($P = .012$), compared with those homozygous for the wild-type A allele. Combined analysis of the 4 case–control populations by meta-analysis yielded a stronger support for the association (OR 1.82, 95% CI, 1.47 to 2.25, $P = 5.3 \times 10^{-8}$, $P_{heterogeneity} = .50$, $I^2 = 0\%$; Fig. 1b). Logistic regression gave a similar result (OR 1.79, 95% CI, 1.45 to 2.21, $P = 4.9 \times 10^{-8}$; Table 1). The PAFs were 18.8–53.3% for the 4 individual populations, and the pooled PAF was 21.9% for the overall population (Table 1).

We further evaluated whether the effects for the *CCL2* G-2518A and *MBL* codon 54 variant (A/B) on risk of SARS-

CoV infection were independent of each other. Both the signals from *CCL2* G-2518A and *MBL* codon 54 variant (A/B) survived after adjustment for each other across all case–control populations and the pooled samples, except for Beijing HCW population (Supplementary Table 2). Furthermore, no consistent significant interaction between these two SNPs (*P* for interaction, .91 to .040; Table 2), or between SNPs and confounding factors (data not shown), on risk of SARS-CoV infection was observed across all case–control populations and in the pooled samples. Thus, the *CCL2* G-2518A and *MBL* codon 54 variant (A/B) were independent risk factors of SARS-CoV infection in most of the Chinese. However, these two SNPs appeared to have a cumulative association with increased risk for SARS-CoV infection (Table 2). For instance in the pooled population, the individuals who carried one at-risk genotype (OR 1.51, 95% CI, 1.24 to 1.85, $P = 5.8 \times 10^{-5}$) or carried two at-risk genotypes (OR 2.88, 95% CI, 2.03 to 4.07, $P = 2.3 \times 10^{-9}$) were at an increased risk for SARS-CoV infection, as compared with those who lacked both at-risk genotypes (OR 1.62, 95% CI, 1.40 to 1.88, *P* for trend $= 1.3 \times 10^{-10}$; Table 2), which is similar to the results given by meta-analysis (OR 1.69, 95% CI, 1.45 to 1.97, $P = 5.3 \times 10^{-8}$, $P_{heterogeneity} = .50$, $I^2 = 0\%$;

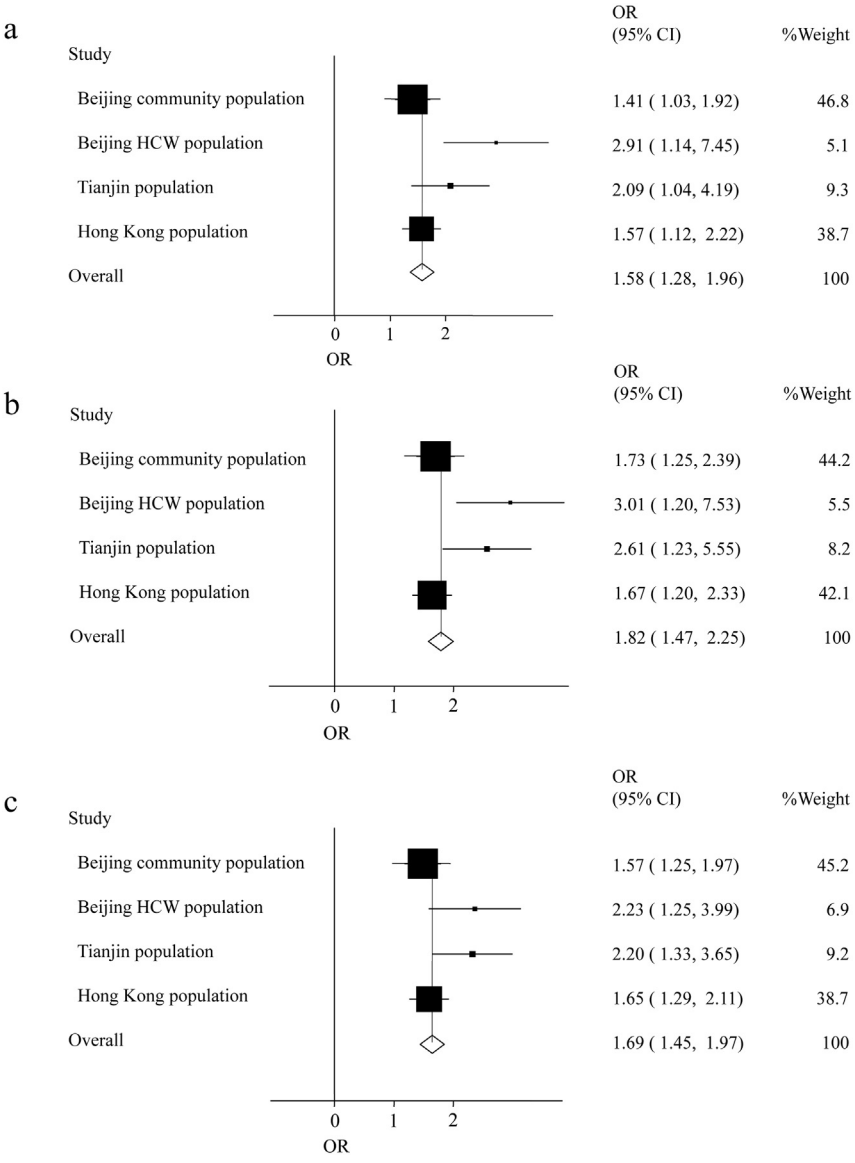

Figure 1 Forest plots for *CCL2* G-2518A and *MBL* codon 54 variant (A/B) across all four studies. We plot the odds ratio (OR; square) and the 95% CI (horizontal line) for each study. A vertical dashed line indicates the final OR across all four studies. The top four bars represent data from four studies and the open diamond below them summarizes their meta-analyzed effect. The area of each square is proportional to the weight of each study in the meta-analysis. Overall, the meta-analysis gave a joint OR of 1.58 (95% CI, 1.28 to 1.96, $P = 2.3 \times 10^{-5}$, $P_{heterogeneity} = .43$, $I^2 = 0\%$; panel a) for *CCL2* G-2518A, 1.82 (95% CI, 1.47 to 2.25, $P = 5.3 \times 10^{-8}$, $P_{heterogeneity} = .50$, $I^2 = 0\%$; panel b) for *MBL* codon 54 variant (A/B), and 1.69 (95% CI, 1.45 to 1.97, $P = 5.3 \times 10^{-8}$, $P_{heterogeneity} = .50$, $I^2 = 0\%$; panel c) for *CCL2* G-2518A and *MBL* codon 54 variant (A/B) in combination, respectively.

Table 2 Cumulative effect on risk of SARS-CoV infection for the combination of *CCL2* G-2518A and *MBL* codon 54 variant (A/B).

	No. of subjects (%)									
	Beijing community population		Beijing HCW population		Tianjin population		Hong Kong population		All four populations	
	Community cases (N = 352)	Random controls (N = 392)	HCW cases (N = 42)	HCW controls (N = 40)	Community cases (N = 60)	All controls (N = 129)	Community cases (N = 478)	Random controls (N = 421)	All cases (N = 932)	All controls (N = 982)
No. of at-risk genotypes[a]										
0	144 (41.3)	214 (54.6)	13 (31.0)	18 (45.0)	22 (36.7)	71 (55.0)	226 (47.3)	242 (57.5)	405 (43.6)	545 (55.5)
1	161 (46.1)	151 (38.5)	9 (21.4)	17 (42.5)	25 (41.7)	49 (38.0)	208 (43.5)	157 (37.3)	403 (43.4)	374 (38.1)
2	44 (12.6)	27 (6.9)	20 (47.6)	5 (12.5)	13 (21.6)	9 (7.0)	44 (9.2)	22 (5.2)	121 (13.0)	63 (6.4)
Odds ratio (95% CI)[b]	1.58 (1.17–2.16)		0.75 (0.25–2.27)		1.44 (0.67–3.09)		1.69 (1.23–2.33)		1.51 (1.24–1.85)	
P value[b]	.0033		.61		.34		.0012		5.8×10^{-5}	
Odds ratio (95% CI)[c]	2.47 (1.46–4.20)		5.92 (1.71–20.47)		6.52 (2.13–19.89)		2.71 (1.46–5.06)		2.88 (2.03–4.07)	
P value[c]	7.7×10^{-4}		.0050		9.9×10^{-4}		.0017		2.3×10^{-9}	
Odds ratio (95% CI)[d]	1.57 (1.25–1.97)		2.23 (1.25–3.99)		2.20 (1.33–3.65)		1.65 (1.29–2.11)		1.62 (1.40–1.88)	
P value[d]	9.4×10^{-5}		.0067		.0023		6.3×10^{-5}		1.3×10^{-10}	
$P_{interaction}$	.90		.040		.20		.91		.29	
PAF (%)	29.1		40.8		41.0		29.7		28.5	

CI, confidence interval. HCW, health care worker. PAF, population attributable fraction. $P_{interaction}$, *P* value testing for interaction. For each population, the associations were performed by logistic regression analysis adjusted for age and sex; For pooled population, the associations were performed by logistic regression analysis adjusted for age, sex and population.

[a] 0 denotes *MBL* AA genotype plus *CCL2* AG or AA genotype; 1 denotes *MBL* AA genotype plus *CCL2* GG genotype, or *MBL* AB or BB genotype plus *CCL2* AG or AA genotype; 2 denotes *MBL* AB or BB genotype plus *CCL2* GG genotype.

[b] Value is for comparison of the at-risk genotype group 1 with the at-risk genotype group 0.

[c] Value is for comparison of the at-risk genotype group 2 with the at-risk genotype group 0.

[d] Value is for trend test.

Fig. 1c). The joint PAFs for the combination of at-risk genotypes of *CCL2* and *MBL* were 29.1—41.0% in the 4 individual populations, and the joint PAF was 28.5% in the pooled population (Table 2).

We next calculated statistics for AUC to estimate the ability of each of three models to distinguish case subjects from control subjects. In the four individual populations, the AUC was 53.2—61.0 for model 1 (sex, age, and the number of at-risk genotypes at *CCL2*), 53.9 to 63.6 for model 2 (sex, age, and the number of at-risk genotypes at *MBL*), and 55.7 to 65.8 for model 3 (sex, age, region, and the number of at-risk genotypes at both *CCL2* and *MBL*) with all *P* values equal or less than .10 (Supplementary Fig. 1). In the pooled population, the AUC was 53.7 (95% CI, 51.1 to 56.3; $P = 4.8 \times 10^{-3}$) for model 1, 55.5 (95% CI, 52.9 to 58.1; $P = 2.9 \times 10^{-5}$) for model 2, and 57.0 (95% CI, 54.5 to 59.6; $P = 1.0 \times 10^{-7}$) for model 3 (Supplementary Fig. 1).

We also assessed whether there was an association between the *CCL2* G-2518A and *MBL* codon 54 variant (A/B) and severity of SARS, but found that the two SNPs were associated with the severity of SARS neither individually nor jointly (Supplementary Table 3).

Discussion

This study includes 932 patients with SARS totally, which accounts for about 12% of the SARS cases worldwide during the SARS outbreak, being so far the largest cohort in the field of genetic association studies of SARS. Additionally, given whether the patients were infected in the hospital or community is a potential confounding factor, we matched the cases and controls for this factor in each case—control series. Furthermore, we performed statistical adjustment for age and sex to minimize other potential biases. Taken together, the large size of the investigation, the consistency of the observations in 4 independent case—control series and the low *P* values distinguish our study from previous studies investigating the influence of different other polymorphisms on the development of SARS, and strengthen the association between the *CCL2* G-2518A and *MBL* codon 54 variant (A/B) and susceptibility to SARS-CoV infection. To our best knowledge, this is the first report that functional polymorphisms of the *CCL2* and *MBL* genes cumulatively increase susceptibility to SARS-CoV infection, confirming the initial hypothesis that these genes may play a role in the pathogenesis of this disorder.

Several previous studies have consistently demonstrated that the carriers of the −2518G allele confer up-regulated transcriptional activity, higher CCL2 mRNA and protein levels *in vitro* and *in vivo*, and more infiltration of leukocytes into tissues in comparison with A carriers.[21—23] Therefore, one might expect that the individuals who carry the at-risk GG genotype, and thus have higher expression of CCL2 and successively more attraction of monocytes and macrophages, may have an increased susceptibility to SARS-CoV infection.

This hypothesis is biologically plausible. Previous studies have consistently reported an overexpression of CCL2 in the lung tissues and sera of SARS patients.[17—19] The pulmonary tissues of SARS patients were also well characterized by pronounced infiltration of monocytes and macrophages.[17,31,32] Furthermore, SARS-CoV was shown to infect the monocyte-derived macrophages *in vitro*[15,33] and, to be readily detectable in macrophages in lungs and other target organs of SARS patients.[31,32] Based on these observations, Gu et al. have argued that the SARS virus infects resident, infiltrating, and circulating immune cells, such as macrophages and monocytes; and then, these infected circulating cells may carry SARS-CoV to various target organs as manifested by widespread dissemination.[31,32] Notably, this kind of spread of infection by infected phagocytes (so-called "Trojan horses effect") has been implicated before in the infection of HIV, measles virus and other intracellular microorganisms.[34,35] Considering the known pathophysiologic role of the CCL2 in these disorders, these examples further suggest the high-CCL2-producing GG genotype may be the at-risk genotype conferring increased susceptibility to SARS-CoV infection.

Consistent with our previous findings,[3,9] this study further confirmed the significant association between the low-MBL-producing B allele and increased risk of SARS-CoV infection in two additional independent sample sets, suggesting that MBL deficiency may be a susceptibility factor for the acquisition of SARS. The independent confirmation of associations of these two SNPs with SARS-CoV infection supports the validity of genetic association studies in complex diseases.

This finding promoted us to investigate the role of gene—gene interaction in the genotype-to-phenotype relationship of SARS-CoV infection. However, no consistent significant genetic interaction between these two SNPs on risk of SARS-CoV infection was observed across all case—control populations and in the pooled samples. Furthermore, no functional studies reported for the interactive effect of MBL and CCL2 on the susceptibility to SARS-CoV infection. Several previous studies have implicated that MBL could increase the secretion of CCL2 from human monocytic U937 cell lines, peripheral blood mononuclear cells and umbilical vein endothelial cells mediated by a variety of bacterial microbes.[36,37] Whether or not there exit biological interactions between MBL and CCL2 under the condition of SARS-CoV infection remains to be investigated in future studies.

It has to be noted that many chemokines not only take part in the process of viral infection, but are also involved in cell damage and organ dysfunction. Indeed, several lines of evidence have indicated that CCL2 secretion is up-regulated during the development of SARS and correlates with intensifying immuno-mediated damage to the lungs and other target organs, resulting in acute lung injury and, subsequently, multi-organ dysfunction.[38] Therefore, we speculate that the high-CCL2-producing GG genotype may enhance the inflammation and associate with more severe clinical outcomes of SARS. However, we did not find a genetic association between *CCL2* polymorphism and admission to intensive care units or deaths due to SARS. This result may be due to the limited number of patients with severe SARS or died from SARS in the present study. For example, as for disease severity this study has merely powers of 14.6% (two-sided test of significance, $\alpha = 0.05$) to detect ORs of >1.66 in Beijing community, and 9.5% to detect ORs of <0.73 in Hong Kong population, for carriers

of the GG genotype relative to the carriers of the AA plus AG genotypes at the position −2518 of the *CCL2* gene (Supplementary Table 3). In contrast, as for SARS-CoV infection this study has powers of 56.2% to detect ORs of >1.41, and 82.7% to detect ORs of >1.57, in Beijing community and Hong Kong populations, respectively, for the same SNP (Table 1). It certainly warrants confirmation in additional studies with larger collections of SARS patients.

The allele and genotype frequencies of the *CCL2* G-2518A polymorphism vary with ethnicity (according to the HapMap Project database). Indeed, in this study with 982 control subjects, we found that the frequency of the −2518G allele and GG genotype was 52.0% and 27.0%, compared with around 34.7% and 12.0%, respectively, among Caucasians from the Iceland.[39] Therefore, although the highly significant associations between *CCL2* G-2518A and susceptibility to SARS-CoV infection were biologically plausible, and strengthened by our 4 independent case−control studies, it remains interesting to investigate that whether there exist population-specific differences for this polymorphism to SARS susceptibility between Chinese and Europeans.

Interestingly, a significant cumulative effect on the susceptibility to SARS-CoV infection was observed for the combination of two functional SNPs in *CCL2* and *MBL* across the four independent case−control series ($P_{trend} = 1.3 \times 10^{-10}$ in the pooled samples). We estimated that individuals who have both of the at-risk genotypes have an odds ratio of 2.88 for SARS-CoV infection (Table 2). Therefore, PAFs can be calculated to be 14.6% for *CCL2*, 21.9% for *MBL*, and 28.5% for both in the pooled samples, indicating that, for example, 28.5% of the increase in the risk of SARS-CoV infection can be attributed to carrying 1 or 2 at-risk genotypes of the two polymorphisms. If the associations are real, then at-risk genotypes of the two polymorphisms are associated with a low to moderate fraction of SARS-CoV infection among Chinese, suggesting that there exist unrevealed genes conferring susceptibility to such an infection. In fact, previous reports have shown that polymorphisms of several genes affect an individual's susceptibility to SARS-CoV infection or disease severity of SARS,[3−10] although most of these susceptibility genes need to be confirmed in independent sample sets. In addition, it is notable that at this stage, *CCL2* G-2518A and *MBL* codon 54 variant (A/B) are unlikely to be appropriate for risk prediction testing individually or in combination, because of low AUCs (53.7% for *CCL2*, 55.5% for *MBL*, and 57.0% for both) in pooled population. However, as further susceptibility polymorphisms are identified and confirmed, and interaction effects among such polymorphisms together with other risk factors are taken into account, prediction of the susceptibility of SARS-CoV infection may become more accurate and clinically usable.

This study has several potential limitations. First, the small sample size of subjects homozygous for the minor allele B of *MBL* codon 54 variant (A/B) did not allow calculation of separate ORs for the BB genotype (the genotype with the greatest risk). Second, the small sample sizes likely meant that there was low power to detect genetic interaction between polymorphisms. Third, the small sample sizes of the two new case−control populations (Beijing HCW and Tianjin) meant that the effect estimates for these groups were larger in magnitude and had wider CIs (that is, they were less stable as shown in Table 1 and Fig. 1) than the effect estimates generated from the other two larger case−control populations. Last, there was no adjustment for co-morbid conditions (such as chronic diseases) that could affect susceptibility to SARS.

In summary, our findings indicate that individuals who are genetically predisposed to produce greater amounts of CCL2 protein seem to be more susceptible to SARS-CoV infection. We also confirmed our previous findings that low-MBL-producing allele is a susceptibility factor for the acquisition of SARS. Furthermore, a combination of *CCL2* and *MBL* polymorphisms has a stronger genetic association with the susceptibility to SARS-CoV infection. However, 1) no interaction between polymorphisms could be detected; 2) no association was observed between the polymorphisms and severity of SARS; and 3) although there were statistically significant associations between the two polymorphisms and SARS-CoV infection, tests using the AUC curve do not indicate that the at-risk genotypes have clinical usefulness (that is, the at-risk genotypes cannot discriminate cases from controls). Since both the CCL2 and MBL are important components in innate immunity system, our findings further support the hypothesis that variability in the innate immune response can be involved in susceptibility to SARS-CoV infection during the vulnerable period before the production of specific antibodies.[9]

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Conflict of interest

No potential conflict of interest relevant to this article was reported.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jinf.2015.03.006.

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