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Association of a single nucleotide polymorphism in the CD209 (DC-SIGN) promoter with SARS severity

Introduction

Severe acute respiratory syndrome (SARS) is an acute respiratory disease resulting from infection by a novel coronavirus (SARS-CoV). Dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) is a C-type lectin expressed on the surface of subsets of human DCs and alveolar macrophages, and functions both as a cell adhesion and as a pathogen recognition receptor to facilitate infection. Pseudo-particles presenting SARS-CoV spike protein can bind to DC-SIGN. Homozygous genotypes of the tandem-repeat polymorphism of DC-SIGNR, which share 77% similarity with DC-SIGN, are protective against SARS-CoV infection.

As a pathogen recognition receptor, DC-SIGN allows DCs to capture and interact with numerous pathogens, such as *Mycobacterium tuberculosis*, HIV-1 and dengue viruses, etc. As an adhesion receptor, it plays an important role in many DC functions, such as the DC-T-cell interaction and DC migration. Host genetic factors have been shown to result in differences in host susceptibility to SARS infection and outcome of the disease. These include the genetic polymorphisms in L-SIGN, MBL, HLA-B and HLA-DRB1 for host susceptibility; and polymorphisms in ACE1, ICAM3, MXA and FcγRIIA for disease outcome.

In the DC-SIGN promoter region, a putative functional single nucleotide polymorphism (SNP), -336A>G, (rs4804803) affecting an Sp1-like transcription factor binding site has been described.¹ This SNP was found to affect the transcription activity of DC-SIGN *in vitro*. It has significant association with susceptibility for HIV-1 and *M tuberculosis* infections, and with the severity of dengue.¹⁻³

As the SARS-CoV spike proteins share great similarity with HIV envelope proteins, we hypothesised that the DC-SIGN promoter SNPs may also be associated with the severity of SARS-CoV infection. Therefore, we analysed the promoter SNPs of DC-SIGN for genetic association to SARS-CoV using a large case-control study, and performed *in vitro* functional studies to verify the effect of DC-SIGN promoter SNPs on promoter activity and its influence on DC-SIGN expression *in vivo*.

Methods

Study design

This case-control genetic association study was conducted from June 2006 to May 2008 to examine the contribution of DC-SIGN -336A/G SNP to SARS-CoV infection and/or association with SARS patients' clinico-pathological outcomes.

A total of 824 SARS patients confirmed by serology and/or RT-PCR for SARS were recruited from the Pamela Youde Nethersole Hospital, Princess Margaret Hospital, United Christian Hospital, Queen Mary Hospital, Alice Ho Miu Ling Nethersole Hospital, and Prince of Wales Hospital. The 471 controls included 281 household contacts (genetically unrelated household members of SARS patients) who remained unaffected and sero-negative, and 190 health care workers who had worked in SARS wards but remained disease-free and sero-

Key Messages

1. In this genetic risk-association study involving about 1300 subjects, SARS patients carrying the DC-SIGN promoter -336G variant had lower risk of having higher lactate dehydrogenase levels on admission, an independent prognostic indicator for severity of SARS-CoV infection.
2. *In vitro* functional studies demonstrated that the DC-SIGN -336G promoter provided a less effective binding site and lower promoter activity, which may lead to reduced DC-SIGN protein expression and hence may contribute to a reduced immune-response with reduced lung injury during the progression of SARS infection.

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negative. All the subjects and controls were Chinese.

Main outcome measures

The clinical data of the SARS cases were retrospectively obtained from the Hospital Authority, with permission from all attending clinicians. The data included age, sex, length of hospital stay, extent of intensive care unit treatment, whether patients received assisted ventilation, any form of steroid treatment or intravenous immunoglobulin, as well as final outcomes in terms of survival and death. Results of haematological and biochemical laboratory investigations on admission included the haemoglobin level, absolute lymphocyte count, platelet count, white blood cell count; and biochemical indices of alanine-aminotransferase, albumin, globulin, creatinine-kinase, lactate-dehydrogenase, urea, sodium, potassium and serum creatinine.

Study instruments

Genotyping for DC-SIGN -336A/G promoter SNP was performed using Allelic Discrimination TaqMan Assay (Applied Biosystems). Proper controls and replicates were included for quality control. DC-SIGN -336A/G genotyping results were also integrated with the ICAM3 +443T/C genotyping results⁴ for combined genotype risk-association analysis.

In vitro functional studies of DC-SIGN -336A/G promoter SNP was performed using (1) an electrophoretic mobility shift assay (EMSA) and (2) a luciferase-reporter promoter activity assay. The EMSA was used to investigate the differential binding of nuclear extract to the -336A/G polymorphic site. Complementary oligonucleotide pairs harbouring either -336A or -336G of the DC-SIGN promoter were ³²P-end-labelled and were incubated with HeLa cell nuclear extracts. Consensus Sp1 and AP2 probes were used to compete with -336A or -336G probe for nuclear extract binding. Before incubation, unlabelled -336A or -336G oligonucleotide probes were added to compete for the radiolabelled probe reaction mixture at 25 or 50 times molar excess. The protein-DNA complexes were resolved using non-denaturing polyacrylamide gel electrophoresis.

The luciferase-reporter promoter activity assay was used to examine whether -336A or G allele would give rise to lower promoter activity. One µg of luciferase-reporter DC-SIGN promoter construct plasmid and 0.1 µg of Renilla plasmid were transfected into the HeLa cell lines using lipofectamine 2000 (Invitrogen). After transient transfection for 24 hours, the cells were harvested and the activity of the promoter constructs assayed using the dual-luciferase reporter assay system (Promega). To determine promoter activity, firefly luciferase-expression levels were normalised against Renilla-luciferase levels. The luciferase expression levels between pGL3-basic/DC-SIGN-336A and pGL3-basic/DC-SIGN-336G were compared. The pGL3-basic plasmid was used as a negative control. Light emission was measured; the experiments were performed as three sets of triplicates. Results were compared using the

Student's *t* test.

The expression level of DC-SIGN in the peripheral blood mononuclear cells (PBMCs) derived DCs (which carried different -336A/G genotypes) was compared. CD14⁺ monocytes were isolated from PBMCs and then cultured in the presence of GM-CSF and IL-4, and were harvested after 5 days of culture for DC-SIGN expression analysis by flow cytometry. The percentage and mean fluorescence level of positive cells were measured by counting the cells demonstrating a higher signal than the control.

Statistical analysis

For risk-association analysis, genotype distributions of the patients and controls were compared using the χ^2 test; strength of association was measured using an odds ratio (OR) and 95% confidence intervals (CI). Genotyping results were checked for Hardy-Weinberg equilibrium. For *in vitro* studies, statistical significance was calculated using the Student's *t* test. The χ^2 test was used to test for possible association with nominal clinical outcome measures. For numerical variables, each was first analysed by Student's *t* test. If significant, it was further stratified and then examined using the χ^2 test. Logistic regression analysis was applied to adjust for age and gender.

Results

Genetic susceptibility analysis

A total of 824 SARS cases and 471 controls were successfully genotyped. The SNP was in Hardy-Weinberg equilibrium in both patients and controls. There was no significant difference between patients and controls in terms of genotypes ($P=0.93$) or allele frequency ($P=0.81$). Trend of association was not observed. Therefore, further investigations with more healthy controls were not performed.

Severity association analysis

Because only three subjects were homozygous for GG polymorphism, we combined GG and GA heterozygous genotypes for severity association analysis. The mean±standard error of the mean of lactate dehydrogenase (LDH) levels in the AA group was significantly higher than that in GG/GA group (1.14 ± 0.03 vs 1.03 ± 0.04 , $P=0.019$), suggesting that LDH levels were associated with the DC-SIGN-336A/G SNP. All other parameters tested (ie ALT, albumin, creatinine kinase, haemoglobin, lymphocyte, platelet, white blood cell and globulin) showed no significant association with the genotype groups by independent *t* test (Table 1). To examine the risk level, patients were categorised into two subgroups, namely high and low LDH levels (Table 2). The frequency of G-carriers in persons with low LDH levels was significantly higher than in those with high LDH levels (18.46% vs 8.33%, $P=0.015$, OR=0.40, 95% CI=0.19-0.85); the DC-SIGN-336G allele was overrepresented in the low LDH level group than in the high LDH level group (9.49% vs 4.17%,

P=0.014, OR=0.41, 95% CI=0.20-0.86). These results suggested that G carriers had a 2.44-fold greater chance of having lower LDH levels. The association remained significant after adjustment for age and gender as well as genetic relationships of the subjects.

DC-SIGN and ICAM3 combined genotype analysis

The genotyping results of DC-SIGN -336A/G SNP of the SARS patients was combined with our previously reported genotyping results of ICAM3 Asp143Gly (+443T/C) SNP⁴ (Table 3). The wild-type genotypes of both SNPs (ie ICAM3+443TT/DC-SIGN-336AA genotypes) were of highest frequency in the cohort. Analysis of the mean LDH level on admission of the combined genotypes showed that combined genotype +443CC/-336AA was the highest. Non-parametric t-test of +443CC/-336AA genotype and the wild-type genotype groups showed significant difference in the LDH level (P=0.033). The frequency of patients with

high LDH levels in each combined genotype was compared to that of the wild-type +443TT/-336AA genotype using the χ^2 test (Table 3). Significant association of the overall genotypes was observed (P=0.024). Comparison of the wild-type genotype with each combined genotype also showed association of the combined +443CC/-336AA genotype with higher LDH levels (P=0.021, OR=4.34, 95% CI=1.34-14.12), although the number of cases having the combined genotype was small (n=12). Multivariate logistic regression analysis for synergistic effect between the SNPs indicated that interaction between the two SNPs was not significant (P=0.845).

Electrophoretic mobility shift assay and promoter activity assays

DC-SIGN -336A/G was found to harbour the Sp-1 transcription factor binding site, with the - 336G allele harbouring the binding site, but being abolished with the

Table 1. Univariate association between laboratory parameters on admission and genotypes of DC-SIGN-336A/G among SARS patients

Parameters	G-carrier (GG/GA)			Non G-carrier (AA)			P value*
	No.	Mean	SEM	No.	Mean	SEM	
Alanine aminotransferase	127	0.66	0.05	656	0.82	0.04	0.093
Albumin	127	40.19	0.38	655	39.78	0.17	0.337
Creatine kinase	133	133.22	16.76	659	162.03	14.47	0.384
Haemoglobin	134	13.53	0.12	687	13.28	0.07	0.090
Lymphocyte	134	0.94	0.03	683	0.92	0.02	0.501
Platelet	134	169.79	4.64	687	180.16	2.51	0.051
White blood cell	134	5.63	0.18	687	5.67	0.09	0.869
Globulin	93	34.76	0.48	537	33.87	0.21	0.107
Lactate dehydrogenase level (ratio of upper normal reference)	116	1.03	0.04	565	1.14	0.03	0.019

* Two independent sample comparison between patients being heterozygous and homozygous for the least prevalent allele (G-carrier, GA/GG) versus patients being homozygous for the most prevalent allele (non G-carrier, AA)

Table 2. Frequencies of DC-SIGN-336A/G genotypes and alleles in subgroups of SARS patients

Variable	Lactate dehydrogenase levels on admission*		P value	Odds ratio (95% CI)
	Low (n=585)	High (n=96)		
Genotype				
GG	3 (1%)	0 (0%)	-	-
GA	105 (18%)	8 (8%)	0.047	-
AA	477 (82%)	88 (92%)	-	-
Non G-carrier (AA)	477 (82%)	88 (92%)	Reference	
G-carrier (GA/GG)	108 (18%)	8 (8%)	0.014	0.40 (0.19-0.85)
Alleles				
A	1059 (91%)	184 (96%)	Reference	
G	111 (9%)	8 (4%)	0.015	0.41 (0.20-0.86)

* Cutoff value of normalised LDH level is 1.6 fold of upper normal reference

Table 3. χ^2 tests for lactate dehydrogenase (LDH) level and combined ICAM3/DC-SIGN genotyping of all patients

ICAM3 Asp143Gly (+443T/C)	Combined genotype DC-SIGN -336A/G	LDH level*		P value [†]	Odd ratio (95% CI)
		High (n=95)	Low (n=582)		
TT	AA	62 (65%)	377 (65%)	-	Reference
TT	AG	6 (6%)	89 (15%)	0.039	0.41 (0.17-0.98)
TT	GG	0 (0%)	3 (1%)	1.000	-
TC	AA	20 (21%)	90 (15%)	0.286	1.35 (0.78-2.35)
TC	AG	2 (2%)	15 (3%)	1.000	-
CC	AA	5 (5%)	7 (1%)	0.021	4.34 (1.34-14.12)
CC	AG	0 (0%)	1 (0%)	1.000	-

* Cutoff value of normalised LDH level is 1.6 fold of upper normal reference

† P=0.024 for overall combined genotype, df=6

-336A allele.¹ Our EMSA results showed that the G probe (lane 13) had stronger binding capacity to the nuclear extract compared to the A probe (lane 8) [Fig 1]. The binding capacity of the G probe was effectively competed by the Sp1 consensus probe (lane 17) and by the unlabelled cold G probe (lane 15). This suggested that the DC-SIGN -336A/G SNP affected the binding of Sp1 present in the nuclear extracts. Results from luciferase reporter assays on

the DC-SIGN promoter constructs containing the -336A/G SNP showed that the G allele promoter construct had significantly lower promoter activity compared to the A allele promoter construct (-336G:-336A=3.8:1, P=0.011) [Fig 2].

Our EMSA and promoter activity results suggested that transcription factor binding sites, such as Sp1, may span

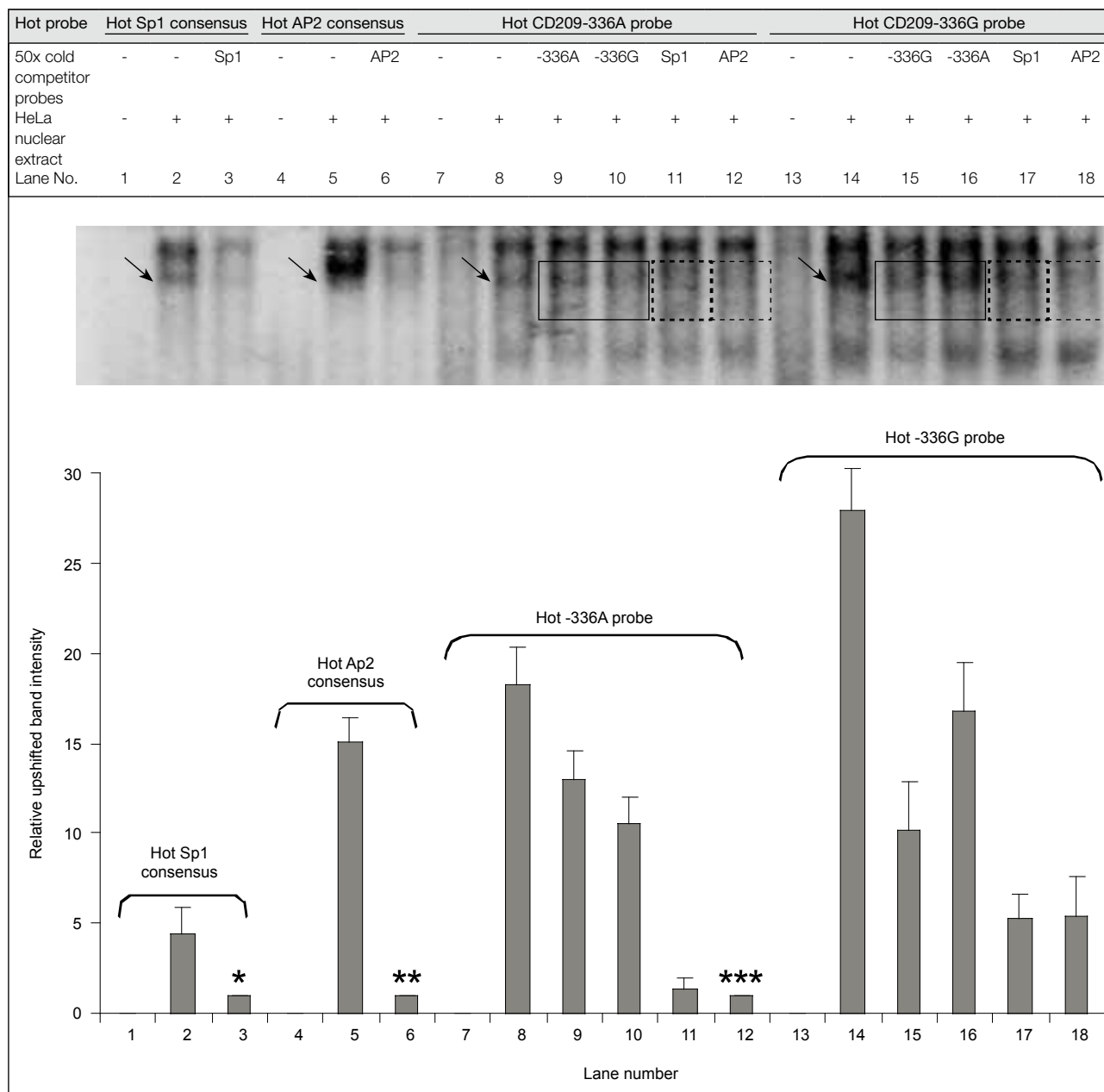


Fig 1. Electrophoretic mobility shift assay of Sp1 consensus, AP2 consensus, CD209 -336A and -336G probes using HeLa nuclear extract

The upshifted bands are indicated by arrows. The relative intensity of upshifted bands is plotted. The band intensity in lanes 3 and 6 are used to normalise against that in lanes 2 and 5 respectively, whereas the band intensity in lane 12 is used to normalise against that in lanes 8 to 11 and 13 to 18. Hence, Lanes 3, 6, and 12 are assigned an arbitrary relative value of 1. Bars denote standard deviations. The upshifted bands could be competed by respective cold competitor probes. The intensity of the upshifted band of DC-SIGN -336G probe (lane 14) is stronger than that of -336A probe (lane 8). The upshifted band of -336G probe is competed by cold -336G probe more effectively than by cold -336A probe (lanes 15 and 16), suggesting that -336G allele has stronger binding affinity to HeLa nuclear proteins than -336A allele

over the -336A/G SNP and hence modulate transcriptional activity. Our findings are in concordance with that reported by Sakuntabhai et al,¹ supporting the transcription regulatory role of the DC-SIGN -336A/G SNP.

DC-SIGN expression in PBMC derived DCs

On screening 20 consecutive PBMC samples, only two were found to carry the -336AG genotypes, whereas the rest were the homozygous -336AA genotype. The homozygous -336GG genotype was difficult to obtain as it contributed <1% in our population. As a result, two homozygous AA

and two heterozygous AG genotypes PBMC samples were used for deriving DCs, which were subsequently used for detection of DC-SIGN expression by flow cytometry. Our results showed that the expression of DC-SIGN in PBMC derived DCs with the heterozygous -336AG genotype was not significantly different from that with -336AA genotype ($P=0.767$, t -test) [Fig 3]. However, the number of cases was small and did not allow conclusions to be made. Furthermore, the effect of genotype on surface expression of DC-SIGN may only be significant with the homozygous -336GG genotype, which we were unable to study owing to the rarity of this genotype.

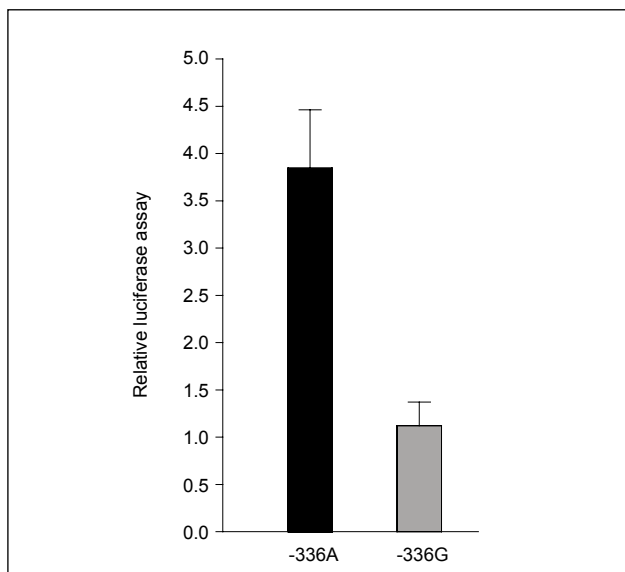


Fig 2. Luciferase assay results of the DC-SIGN -336A (pGL3-basic/DC-SIGN-336A) and -336G (pGL3-basic/DC-SIGN-336G) promoter constructs in HeLa cells

Results are the means from three independent triplicate experiments. The luciferase activity readings of -336A construct is normalised against that of -336G construct which is assigned an arbitrary relative value of 1. Luciferase levels for -336G are significantly lower ($P=0.011$, Student's t test with unequal variance). Error bars denote standard deviations

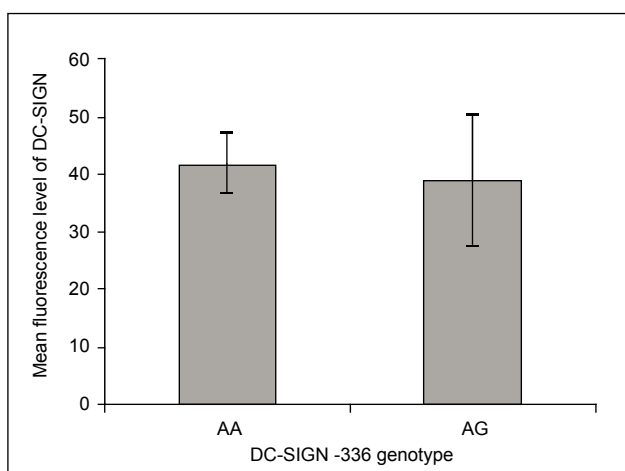


Fig 3. Comparison of mean fluorescence level of DC-SIGN of dendritic cells having -336AA or -336AG genotype

Difference in DC-SIGN level is not significant ($P=0.767$, Student's t test). Error bars denote standard deviations

Discussion

The G allele of the DC-SIGN -336A/G SNP has been shown to play a protective role against parenteral HIV-1 infection in European-American populations,³ against dengue in Thailand,¹ and confer a low risk of tuberculosis in African populations.² Although the -336A/G SNP was not associated with susceptibility to SARS infection, the G allele of this SNP was associated with lower LDH levels in SARS patients being admitted to hospital apart from being independently associated with good prognosis for the disease.⁴ The association remained significant after adjustment for patient age and gender in both overall cases and an unrelated patient subset.

Increase in serum LDH activity was postulated to be a result of massive tissue destruction during the acute phase of SARS-CoV infection. A high LDH level on admission was an independent prognostic indicator for severity of SARS infection and could help clinicians predict adverse clinical outcomes.⁴ A high LDH level reflected tissue necrosis related to immune hyperactivity in SARS. A cytokine and chemokine storm occurs with a significant elevation of T-helper cell cytokine IFN- γ , inflammatory cytokines and Th1 IFN- γ -induced protein-10 during the early phase of SARS-CoV infection. The respiratory tracts of affected individuals who died during the first 10 days of illness showed diffuse alveolar damage with a mixed alveolar infiltrate, lung oedema and hyaline membrane formation indicating lung injury during progression of the disease.

Our EMSA and promoter activity results showed lower transcription activity of the -336G allele of DC-SIGN promoter compare to the -336A allele, suggesting that -336A/G has a functional role of in transcriptional regulation of DC-SIGN. Sp1 is a possible transcription factor binding site spanning the -336A/G SNP. Our findings are in concordance with those reported by Sakuntabhai et al¹ and suggest that individuals with the G allele might be less immune-responsive to SARS-CoV. Lower G-allele promoter activity may result in lower levels of DC-SIGN protein expressed, resulting in a lower T-cell response and reduced cytokine and chemokine secretion infiltrating the alveoli. Thus, the G allele of DC-SIGN -336A/G might

protect the lung from injury during the progression of SARS infection.

Interestingly, the frequency of the minor allele -336G identified varied in different ethnic groups; Asian populations have the lowest frequency. The frequency of the minor allele -336G in Chinese (8.7%) was similar to that in Thais (8%),¹ but was significantly lower than in European Caucasians, Africans, and American-Africans (>20%).^{1,2} This genetic heterogeneity between different populations suggests that the DC-SIGN SNP might be associated with genetic predisposition to disease. Moreover, the significant association of the DC-SIGN SNP to various infectious diseases suggests that the genetic difference may be caused by natural selection of individuals who survived lethal disease/pathogen challenges.

Combined genotype analysis of ICAM3 +443T>C and DC-SIGN -336A>G showed that overall combined genotypes were significantly associated with disease severity. We previously reported that ICAM3 +443C carriers were associated with high LDH levels on admission. This study found that homozygous -336AA subjects were associated with high LDH levels on admission compared to -336G carriers. The combined +443CC/-336AA genotype was in agreement with this high LDH level association. Patients with this +443CC/-336AA genotype had a 4.3-fold greater risk than standardised LDH levels on admission. Nonetheless, multivariate logistic regression analysis was unable to demonstrate any synergistic effect between the two SNPs.

In summary, a SNP of DC-SIGN -336A/G is associated with LDH levels on admission, which is an independent prognostic indicator for the severity of SARS. This functional SNP affects the promoter activity of DC-SIGN and may alter gene expression and hence host immune

response.

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