

US Khoo 邱瑋璇
 KY Chan 陳遠光
 VS Chan 陳秀芳
 JCY Ching 程子忻
 L Yam 任燕珍
 CM Chu 朱頌明
 ST Lai 黎錫滔
 TY Wong 黃天祐
 P Tam 譚廣亨
 SP Yip 葉社平
 GM Leung 梁卓偉
 CL Lin 林成龍
 JSM Peiris 裴偉士

Role of polymorphisms of the inflammatory response genes and DC-SIGNR in genetic susceptibility to SARS and other infections

Introduction

The SARS outbreak had a powerful impact on both the health care system and the economy of Hong Kong. A proportion of unprotected people exposed to the virus did not develop the disease, however. It has been shown that genetic polymorphism is associated with vulnerability to diseases, including infectious diseases. DC-SIGNR (also known as L-SIGN) serves as a receptor for many viral and bacterial pathogens, including SARS coronavirus (SARS-CoV).¹ Heterozygous expression of polymorphic neck-region variants of L-SIGN might alter ligand-binding affinity² and thus influence binding affinity and susceptibility to infection. The inflammatory response cytokine genes play a role in SARS infection and single nucleotide polymorphisms (SNPs) of these genes may contribute to determining susceptibility to and/or the clinical outcome of infection with SARS.

Aims and objectives

1. To determine whether L-SIGN neck-region tandem repeats are associated with susceptibility to SARS infection.
2. To determine the ligand-binding capacity of L-SIGN homo/hetero-zygotes with SARS-CoV and HIV1 envelop-protein (gp120).
3. To investigate the contribution of SNPs of the cytokine genes to SARS infection susceptibility, and their relation to clinicopathological outcomes.

Methods

This study was conducted from December 2004 to November 2006.

Study design

Case-control genetic association studies were performed to examine the contribution of (1) the L-SIGN neck-region tandem repeat to SARS-CoV infection and (2) the cytokine gene SNPs to the risk of contracting SARS and/or any association with clinicopathological outcomes. In-vitro binding experiments were performed to investigate the role of homo/hetero-zygotic L-SIGN in viral binding capacity for SARS-CoV and HIV1-gp120 protein.

Sample size

- (1) A total of 285 SARS patients with infections confirmed by serology and/or reverse transcription-polymerase chain reaction for SARS were recruited. The controls included 380 healthy blood donors randomly recruited before the SARS outbreak, 290 patients from general out-patient clinics with no clinical history, signs or symptoms of inflammation/infection at least 2 months after the SARS outbreak; and 172 health care workers (HCW) who had worked in SARS wards but remained disease-free and were seronegative for SARS. Known genetic relatives were excluded from study.
- (2) 309 household contact controls who had lived in households with SARS patients but remained unaffected by the disease and were found to be seronegative at the end of the outbreak were also recruited. To prevent

Key Messages

1. A genetic risk-association study involving more than 1200 subjects showed individuals homozygous for L-SIGN tandem repeats are less susceptible to SARS infection.
2. This was supported by in vitro binding studies that demonstrated homozygous L-SIGN, compared to heterozygous, had higher binding capacity for SARS coronavirus (SARS-CoV), with higher proteasome-dependent viral degradation. In contrast, homozygous L-SIGN demonstrated lower binding capacity for HIV1-gp120.
3. Genetic-association studies for single nucleotide polymorphisms of the inflammatory response genes, namely TNF- α , INF- α , INF- β , INF- γ , IL1- α , IL1- β , IL-4, IL-6 and iNOS, failed to show a significant association with SARS clinical outcomes or susceptibility.

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Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China:

Department of Pathology

US Khoo, KY Chan, JCY Ching

Department of Surgery

VS Chan, P Tam, CL Lin

Department of Community Medicine

GM Leung

Department of Microbiology

JSM Peiris

Pamela Youde Nethersole Eastern Hospital, Hong Kong SAR, China

L Yam

United Christian Hospital, Hong Kong SAR, China

CM Chu

Princess Margaret Hospital, Hong Kong SAR, China

ST Lai

Infection Control Branch, Centre for Health Protection, Hong Kong SAR, China

TY Wong

Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hong Kong SAR, China

SP Yip

RFCID project number: 02040202

Principal applicant and corresponding author:

Dr Ui-soon Khoo

Department of Pathology, Queen Mary Hospital,

The University of Hong Kong, Pokfulam, Hong Kong SAR, China

Tel: (852) 2855 4410

Fax: (852) 2872 5197

E-mail: uskhoo@pathology.hku.hk

genotype and allele frequency distribution bias, the number of members of the same household who were genetically related was taken into consideration in the statistical analysis of genotypes. All control subjects were ethnically Chinese Hong Kong residents.

Study instruments

(1) L-SIGN study

Genotyping of the L-SIGN neck-region tandem repeats

L-SIGN neck-region tandem repeats were genotyped by amplifying exon-4 of L-SIGN using sequence specific primers.³ Amplified products were separated and detected by gel electrophoresis. Some representative cases were confirmed by Southern blot analysis and by direct sequencing.

Statistical analysis

Genetic association for heterozygote and homozygote genotype comparison was assessed by the χ^2 test, two-sided, and odds ratio (OR) and 95% confidence intervals (CIs) were used to measure the strength of the association in the genetic risk-association study. The Mantel-Haenszel test for stratified analysis was also performed. Genotyping results were checked for Hardy-Weinburg Equilibrium. For all in-vitro studies, statistical significance was calculated using Student's *t* test.

Plasmids and cell lines

The 5 neck-region repeat (N5) of L-SIGN expressing plasmid was genetically engineered using DNA amplified from a N5-allele carrier, whereas the 7 neck-region repeats (N7) expressing plasmid was obtained from the AIDS Research Program, National Institutes of Health, US. The N7-L-SIGN plasmid, N5-L-SIGN plasmid or a mixture of N7+N5 plasmids (ratio 1:1) were transfected into CHO cells. L-SIGN expression was verified by immunostaining with L-SIGN specific antibody. Stable clones of CHO cells expressing N5 or N7/5-L-SIGN variants were also established using antibiotic selection.

Measurement of SARS-CoV ORF-1b for viral copy number

The total viral ribonucleic acid (RNA) copy number was determined using a quantitative reverse transcription-polymerase chain reaction amplifying the SARS-CoV ORF-1b sequence. The total copy number was calculated against the calibration standard curve.

Homol/hetero-zygote neck-region repeat of L-SIGN binding with SARS-CoV and HIV1-gp120

N5-, N7-, and N7/N5-L-SIGN transient transfectant and mock transfected CHO cells were pulsed with SARS-CoV. After incubation with the virus for 1, 24 and 48 hours, the cells were subjected to RNA extraction for ORF-1b viral copy number measurement. For proteasome inhibition experiment, N7-L-SIGN transfected or mock-transfected CHO cell pulsed with the viruses were cultured in the presence of proteasome inhibitors before ORF-1b copy number measurement.

N5-L-SIGN, N7/N5-L-SIGN stable CHO transfectants, and the parental CHO cells were used to test HIV1-gp120 binding. The percentage of cells binding with HIV1-gp120 was detected by flow cytometry. N7-, N5-, and N7/N5-L-SIGN transfectants were each tested three times in triplicate.

(2) Genetic association study of inflammatory response genes

SNPs selection

SNPs of nine inflammatory response cytokine genes were selected from the dbSNP database based on the following criteria:

- All information was based on Han Chinese genotype data obtained from the HapMap International Database (<http://www.hapmap.org>), available from the dbSNP of the National Center for Biotechnology Information.
- SNPs in the promoter region (1.8 kb), exons (including 5' untranslated regions, coding exons, and 3' untranslated regions), and 3' flanking regions (400 bp)
- SNPs with minor allele frequency (>10%) in the Han Chinese population. This gave the study at least 80% power to detect a 2-fold risk-associated genotype in 285 cases.

A total of 17 SNPs were selected from eight cytokine genes. An appropriate SNP for TNF α could not be identified.

Genotyping of cytokine gene SNPs

Multiplex genotyping was performed using Sequenom. Primer design was successful for 12 out of 17 SNPs which could be multiplexed into two multiplex reactions: INF- α , INF- α , INF- β , INF- γ , INF- γ , IL1- α , IL1- α , IL1- β , IL-4, IL-6, iNOS, iNOS. In the genotyping assay, proper controls and replicates were included for quality control.

Statistical analysis

Genetic association was analysed using a two-sided χ^2 test. A clustering logistic regression was used to adjust for genetic relations of HHC. The χ^2 test was used for ordinal variables (ie intensive care unit, use of steroid, use of pulse steroid, use of intravenous immunoglobulin, death, and ventilation) whilst ANOVA and Student's *t* tests were used to analyse continuous clinical parameters (ie alanine-aminotransferase, albumin, creatinine-kinase, haemoglobin, lymphocyte, platelet, white blood cell, globulin, lactate-dehydrogenase, total length of stay, total length of stay in intensive care unit, days from admission to start of steroid treatment, days from the start of steroids to the start of pulse steroids). The issue of multiple testing of 12 SNPs, was dealt with by using Bonferroni's correction procedure to adjust for it.

Results

(1) L-SIGN study

Risk-association analysis of L-SIGN neck-region tandem repeat

As at least one fifth of SARS patients in Hong Kong were

Table 1. Statistical analysis of L-SIGN homo/hetero-zygosity and comparison of the allele and genotype frequencies**

(a)

L-SIGN neck region	All SARS samples	Random controls
Heterozygotes†	153 (53.7%)	171 (45.0%)
Homozygotes	132 (46.3%)	209 (55.0%)

(b)

L-SIGN neck region	Non-HCW population		HCW population	
	Community SARS	Out-patient controls	HCW SARS	HCW controls
Heterozygotes†	115 (52.8%)	127 (43.8%)	38 (56.7%)	71 (41.3%)
Homozygotes	103 (47.2%)	163 (56.2%)	29 (43.3%)	101 (58.7%)

* The frequencies of homozygotes and heterozygotes are compared between (a) all SARS samples (health care workers [HCW] SARS + community SARS) and random controls, and (b) non-HCW population and HCW population for stratified analysis

† The heterozygotes are used as the reference group in comparisons in (a) and (b). All SARS samples vs random controls: $\chi^2(df=1)=4.91$, $P=0.027$, $OR=0.706$ (95% CI=0.519-0.961). Community SARS vs out-patient controls: $\chi^2(df=1)=4.01$, $P=0.045$, $OR=0.698$ (95% CI=0.490-0.993). HCW SARS vs HCW controls: $\chi^2(df=1)=4.63$, $P=0.031$, $OR=0.536$ (95% CI=0.303-0.950). In (b), the Mantel-Haenszel test is performed in SARS vs controls stratified by non-HCW/HCW populations. Test of heterogeneity of ORs: $\chi^2(df=1)=0.590$, $P=0.442$. Test of overall association: $\chi^2(df=1)=7.977$, $P=0.005$, overall OR estimate=0.649 (95% CI, 0.481-0.876)

Table 2. Summary of the L-SIGN genotypes in study groups*3

L-SIGN neck region†	HCW SARS		Community SARS		HCW controls		Out-patient controls‡		Random controls‡	
	No.	%	No.	%	No.	%	No.	%	No.	%
5/5	1	1.5	8	3.7	19	11.0	14	4.8	10	2.6
5/9	2	3.0	7	3.2	3	1.7	8	2.8	15	3.9
6/5	0	0.0	3	1.4	7	4.1	2	0.7	4	1.0
6/6	0	0.0	1	0.5	0	0.0	0	0.0	2	0.5
6/9	1	1.5	3	1.4	1	0.6	3	1.0	4	1.0
7/5	16	23.9	49	22.5	25	14.5	50	17.2	63	16.6
7/6	7	10.4	12	5.5	6	3.5	25	8.6	25	6.6
7/7	27	40.3	86	39.4	76	44.2	145	50.0	191	50.3
7/8	0	0.0	0	0.0	1	0.6	0	0.0	0	0.0
7/9	12	17.9	41	18.8	28	16.3	39	13.4	60	15.8
9/9	1	1.5	8	3.7	6	3.5	4	1.4	6	1.6
Total	67		218		172		290		380	

* HCW SARS were health care workers infected by SARS in the course of duty; community SARS were SARS patients recruited from the community; HCW controls were unaffected HCW who had cared for SARS patients and were proven seronegative for SARS; Out-patient controls were individuals randomly recruited from out-patient clinics who had neither inflammatory nor infectious conditions; Random controls were healthy blood donors randomly recruited before the SARS outbreak

† Numbers of the L-SIGN neck-region tandem repeats. Hardy-Weinberg Equilibrium for HCW SARS, community SARS, HCW controls, out-patient controls and random controls give P values of 0.893, 0.432, <0.0001, 0.054, and 0.412, respectively

‡ Neither the genotype frequencies nor the homo/hetero-zygosity frequencies differed significantly ($P=0.737$ and $P=0.755$, respectively) between out-patient controls and random controls.

HCW, we sub-classified our SARS patients into: (1) 67 HCW infected during the course of duty ("HCW SARS"), and (2) the remaining 218 recruited from the community ("community SARS").³

When all SARS patients (HCW SARS + community SARS) were compared with random controls, homozygous individuals had a significantly lower risk association for SARS infection with an $OR=0.706$ (95% CI, 0.519-0.961; $P=0.027$, Table 1a), compared with heterozygotes. Analysis was performed separately in the non-HCW population and the HCW population. In the non-HCW population, community SARS versus out-patient controls gave an $OR=0.698$ (95% CI, 0.490-0.993; $P=0.045$, Table 1b). In the HCW population, HCW SARS versus HCW controls demonstrated an OR of 0.536 for homozygotes (95% CI, 0.303-0.950; $P=0.031$; Table 1b). The stratified analysis between these two populations did not reveal significant differences ($P=0.442$, Table 1b), allowing us to perform overall association tests, yielding an overall $OR=0.649$ (95%

CI, 0.481-0.876; $P=0.005$, Table 1b), further confirming that homozygosity conferred a reduced risk. As the out-patient controls and the random controls did not differ from each other in their individual genotype frequencies or homo/hetero-zygote frequencies ($P=0.737$ and 0.755 respectively, Table 2), these two controls were grouped together as "combined controls". Analysis of all SARS patients versus combined controls again gave an $OR=0.691$ for homozygotes (95% CI, 0.523-0.913; $P=0.009$).

Binding assay of homo/hetero-zygote L-SIGN to SARS-CoV and HIV1-gp120

Homozygous L-SIGN demonstrated higher SARS-CoV binding capacity than heterozygous L-SIGN (Fig a).³ After binding to L-SIGN, there was little or no viral dissociation, and viruses underwent degradation over time, with the homozygous L-SIGN more efficient than the heterozygous (Fig b).³ L-SIGN-mediated viral degradation could be partially inhibited by proteasome inhibitors (Fig c).³ In contrast, the homozygote L-SIGN transfectants showed less binding

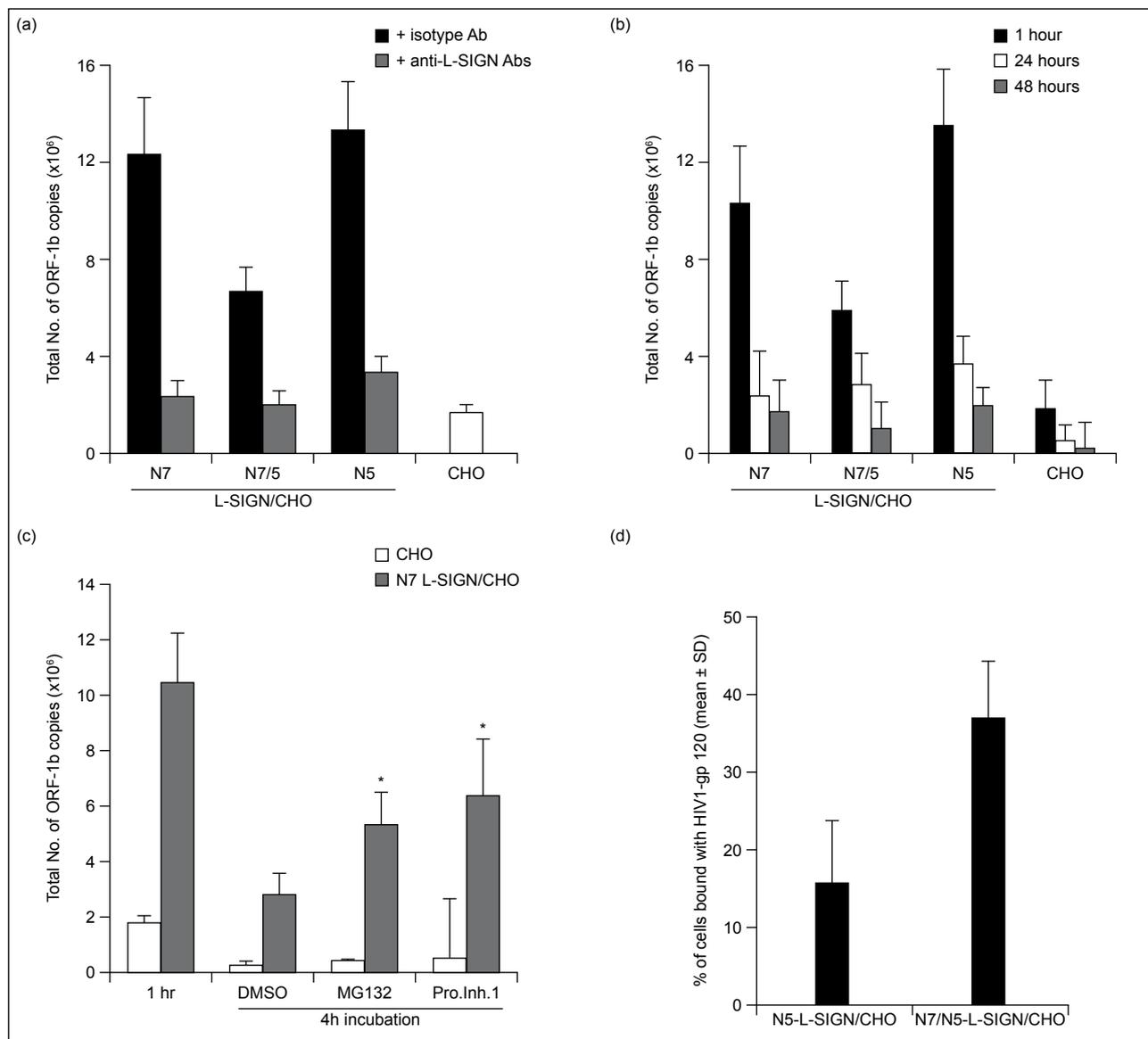


Fig. SARS-CoV infection and HIV1-gp120 binding on the N7-, N5-, and N7/N5-L-SIGN/CHO transfectants*

* (a) Viral ORF-1b copy number of N7, N5, and N7/5-L-SIGN transfected CHO cells (L-SIGN/CHO), and mock transfectants (CHO), pre-treated with anti-L-SIGN antibodies (clone 120526 and 120612, 10 µg/mL each) or isotype control antibody before being pulsed with 1 pfu/cell SARS-CoV and incubated for 1h at 4°C and washed.³ (b) Total viral ORF-1b copy number (cell lysates plus supernatants) of N7, N5 and N7/5 L-SIGN transfected CHO cells, and mock transfectants, pulsed with 1 pfu/cell SARS CoV for 1h, washed and subsequently incubated at 37°C for 24 and 48 hours.³ (c) Total viral ORF-1b copy number of N7 L-SIGN transfected CHO cells, and mock transfectants, pulsed with 1 pfu/cell SARS CoV for 1h at 37°C, washed and subsequently incubated for 4 hours in the presence or absence of 10 µM MG132 or proteasome inhibitor I (Pro. Inh. 1). P<0.05 in comparison to cells treated with DMSO solvent control. Data are expressed as mean ± SD from triplicate, and are representative of three experiments.³ (d) L-SIGN transfectants and HIV1-gp120 binding experiments. Percentage of N5 and N7/5-L-SIGN transfectants that bound to HIV1-gp120. Transfectants were incubated in 0.5 µg HIV1-gp120 proteins (recombinant HIV1BaL-gp120 protein, an R5-topic HIV1-gp120, NIH AIDS Reagent Program, US) for 45 minutes at 4°C and washed, before incubation in 5 µg/mL anti-human HIV1-gp120 (clone 2G12, NIH AIDS Reagents Program) for 40 minutes at 4°C. HIV1-gp120 binding was detected by FITC-conjugated goat anti-human IgG antibody and analysed by BD FACSCalibur flow cytometer. N7/N5-L-SIGN transfectants showed to have significant higher binding affinity to HIV1-gp120 than N5-L-SIGN transfectants (P=0.0022, *t*-test). Data are expressed as mean ± SD from triplicate, and are representative of three experiments

to HIV1-gp120 than heterozygote transfectants (Fig d).

(2) Cytokine genes study

Genotype analysis found no significant association between SARS patients and HHC, nor between SARS patients and HCW. An analysis of clinicopathological parameters also

found no statistically significant association after multiple-testing adjustment.

Discussion

For our genetic-association study for L-SIGN tandem

repeats, direct comparison of SARS-infected individuals with their controls, together with the stratified analysis, showed that L-SIGN homozygotes were significantly less susceptible to SARS infection.³ This finding is supported by our observation that homozygous L-SIGN had a higher SARS-CoV binding capacity than heterozygous L-SIGN. We also showed that after binding to L-SIGN, viruses underwent degradation over time, with the homozygous more efficient than the heterozygous.³ It has been reported that SARS-CoV sub-genomic RNA was increased 24 hours after infection of L-SIGN expressing cells.¹ The increase was transient, however, and SARS-CoV antigen was detected by immunofluorescence staining in <1% of the infected L-SIGN expressing cells, which argues against efficient and productive viral replication mediated by L-SIGN.¹ Furthermore, L-SIGN-mediated viral degradation is in part proteasome-dependent because it can be partially inhibited by proteasome inhibitors.

For HIV1-gp120 binding to homo/hetero-zygote L-SIGN transfectants, in contrast to our SARS-CoV findings, homozygote L-SIGN transfectants showed less binding to HIV1-gp120 (Fig d). The reasons for such differences are yet to be investigated. One possibility may be related to differences between the binding epitope of L-SIGN for HIV1-gp120 and that for SARS-CoV. Unlike SARS-CoV, where homozygous L-SIGN plays a protective role, in HIV-1 infection a heterozygote advantage has been reported,⁴ with homozygous N7-repeat carriers associated with a higher infection risk (17.5% high-risk HIV1-seronegative vs 28.5% HIV1-seropositive individuals, $P=0.0015$), supportive of the findings of our HIV1-gp120 binding experiments. Whether the HIV1 virus is internalised and degraded on binding with L-SIGN as demonstrated for SARS-CoV remains to be investigated.

Our genetic association study for cytokine gene SNPs showed no significant association with SARS infection susceptibility or with any clinicopathological parameters. Although there is a reported susceptibility to SARS

associated with the +874A/T SNP⁵ being located deep within intron 1 of IFN- γ , its functional significance is unclear. Moreover, it is unlikely to be in linkage-disequilibrium with the promoter or 3' flanking IFN- γ SNPs that we studied, which demonstrated no significant association with risk or clinical outcome. Although a significant association with SARS infection has been reported for several other genes, with the exception of the mannose-binding lectin gene, the study sample sizes were all small and need to be repeated using large sample sizes.

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