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Functional role of ICAM-3 polymorphism in genetic susceptibility to SARS infection

Introduction

Genetic polymorphisms are associated with vulnerability to a variety of human infections. Association between susceptibility to severe acute respiratory syndrome (SARS) and major histocompatibility complex (MHC) class I has been reported.¹ Clinical outcome of SARS patients vary; only a small proportion needed intubation or intensive care. Moreover, immunopathological damage rather than uncontrolled viral replication may correlate with clinical progression of the disease beyond the first week.

Dendritic cells (DCs) are crucial in the defence against pathogens. The dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN) is a mannose binding lectin expressed on the surface of DCs. The ICAM-3 is the natural ligand for DC-SIGN. Expressed constitutively on T-cells, ICAM-3 is a potent signalling molecule and major ligand in the initiation of T-cell-mediated immune response.² The interaction of adhesion molecules between antigen-presenting cells and T-cells is critical for activating antigen-specific T-cells. The binding between ICAM-3 and its ligands provides transient engagement of naive T-cells with DCs, which then allow the T-cells to sample large numbers of MHC molecules for the presence of specific peptides.³ This initial cell-to-cell engagement step is critical for induction of T-cell responses, which play a central role in the immuno-regulation of infectious diseases. We hypothesised that polymorphisms of ICAM-3 may also influence susceptibility to SARS infection.

Aims

1. To determine whether ICAM-3 Asp143Gly polymorphism associates with susceptibility of SARS infection and its relation to clinicopathological outcome of SARS patients.
2. To investigate the role of ICAM-3 polymorphism influencing DC-SIGN-ICAM-3 interaction and immune response in SARS infection using functional studies.

Methods

This study was conducted from January 2006 to December 2007. A case-control genetic association study was performed to examine the contribution of ICAM-3 Asp143Gly polymorphism to SARS infection and/or any association with the clinicopathological outcome of SARS patients. A total of 817 SARS patients confirmed by serology and/or real-time polymerase chain reaction were recruited from follow-up outpatient clinics of the Pamela Youde Nethersole, Princess Margaret, United Christian, Queen Mary, Alice Ho Miu Ling Nethersole, and Prince of Wales hospitals. The controls included (1) 260 patients from the general outpatient clinics seen at least 2 months after the SARS outbreak, with no clinical history, signs or symptoms of inflammation/infection; (2) 307 health care workers who had worked in SARS wards but were disease-free and sero-negative for SARS; (3) 309 household contacts of SARS patients that remained unaffected and sero-negative for SARS. To prevent genotype and allele frequency distribution bias, family members of the same household who were genetically related were taken into consideration in the statistical analysis of genotypes. All control subjects were Hong Kong Chinese.

Key Messages

1. Severe acute respiratory syndrome (SARS) patients who are homozygous for intercellular adhesion molecule-3 (ICAM-3) Gly143 showed significant association with higher lactate dehydrogenase levels and lower total white blood cell counts on admission.
2. In vitro functional studies demonstrated low level binding of ICAM-3 to DC-SIGN and a wide variation in T-cell response of the wild-type ICAM-3 genotype.

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Main outcome measures

The clinical data of the SARS patients were retrospectively obtained from Hospital Authority, with the permission of all attending clinicians. Data collected include: age, sex, length of hospital stay, treatment in intensive care unit, and whether patients received assisted ventilation, steroid treatment, pulse steroids or intravenous immunoglobulin, and final outcome of patients (survival and death). Results of haematological and biochemical laboratory investigations on admission included the haemoglobin level, absolute lymphocyte count, platelet count, white blood cell (WBC) count, and biochemical indices (serum/plasma alanine aminotransferase, albumin, globulin, creatinine kinase, lactate dehydrogenase [LDH], urea, sodium, potassium and serum creatinine).

Study instruments

Genotyping of the ICAM-3 polymorphism

Genotyping of the initial SARS patients and health care workers was performed using Sequenom MassARRAY (Sequenom Inc, San Diego, CA, USA). Genotyping of the additional SARS patients, household contacts and outpatient controls for the ICAM-3 Gly143 SNP was performed by Allelic Discrimination TaqMan Assay (Applied Biosystems Inc, Foster City, CA, USA). Appropriate controls and replicates were included for quality control.

Production of wild-type and polymorphic variant ICAM-3 protein

The coding sequences of ICAM-3 containing Asp143 and Gly143 allele were separately cloned and then stably transfected into 293 cell lines. The stable transfectants thus expressed a fusion protein containing the ICAM-3 of known genotype. The secreted soluble protein was purified and concentrated for in vitro binding studies.

Culturing monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat samples obtained from the Hong Kong Red Cross Blood Transfusion Service. CD14⁺ monocytes were isolated from PBMCs, from which DCs were induced, using MACS separation (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). These CD14⁺ cells were cultured in the presence of 50 ng/ml GM-CSF and 10 ng/ml IL-4. The cells were harvested at the 6th day and analysed by flow cytometry.

ICAM-3 binding assay

To investigate the binding affinity of soluble wild-type ICAM-3 (Asp143) with DC-SIGN transfectants, a stable DC-SIGN transfectant was used whereas parental 293 cell line was used as negative control. These cells were treated with (1) recombinant human ICAM-3 protein (R&D Systems Inc, MN, USA, Cat No 715-IC), (2) purified ICAM-3 protein containing Asp143, or (3) purified ICAM-3 protein containing Gly143. Cells not treated with ICAM-3 protein were used as reference. After incubation at 4°C for 40 min, the treated cells were washed twice to remove unbound ICAM-3 proteins, and then stained by FITC-labelled anti-ICAM-3 antibody for analysis by BD FACSCalibur flow cytometer (BD Biosciences, San

Jose, CA, USA). Cells not treated with ICAM-3 but stained by anti-ICAM-3 antibody were used as reference. To investigate the binding affinity of soluble wild-type ICAM-3 with DC-SIGN expressed on monocyte-derived DCs, similar procedures were performed, except that male human serum of AB blood type was included in the binding buffer to reduce non-specific binding by blocking the Fc receptors known to be present on DCs.

T-cell response

Convalescent SARS patients with the homozygous wild-type, heterozygous, and homozygous variant genotypes were identified. Those who were HLA-A2 positive were re-contacted to donate further blood samples for study. Isolated PBMCs were used for T-cell response analysis using ELISPOT assay.

Statistical analysis

For the risk association analysis, genotype distributions of the patient and control groups were assessed by χ^2 test. Odds ratio (OR) and 95% confidence intervals (CI) were used to measure the strength of association. Genotyping results were checked for Hardy-Weinberg equilibrium. As a significant proportion of the household contacts recruited were genetically related with each other, logistic regression with the cluster and robust methods (STATA program, College Station, TX, USA) was used to factor in genetic relations. For in vitro studies, statistical significance was calculated using Student's *t*-test. For analysis for association with clinical outcome, the χ^2 test was used to test for possible association with nominal clinical outcome measures. For analysis of numerical variables, each parameter was first analysed by Student's *t* test. Parameters that were significantly different were further studied using the χ^2 test.

RESULTS

Genetic association study

The demographics of the SARS patients, control groups, and their clinical features are summarised in Table 1. The genotype and allele frequencies of ICAM-3 Asp143Gly SNP of the 817 SARS patients, 260 outpatient controls, and 309 household contacts were in Hardy-Weinberg equilibrium. As the genotype distribution of the health care workers was not in Hardy-Weinberg equilibrium, this group was excluded from risk association analysis. No significant difference in genotype or allele frequency distribution was found when comparing these 2 control groups with SARS patients (data not shown). This lack of risk association was confirmed even with blood relationship taken into account, either by excluding patients who were blood relatives or by using logistic regression model analysis. Among clinical outcome measures, Student's *t* test showed significant association for LDH levels on admission ($P=0.036$) and for WBC counts ($P=0.036$) when comparing homozygous wild-type Asp versus homozygous Gly (Fig). The LDH levels and WBC counts were divided into high- and low-level groups, and the χ^2 test performed. For LDH levels, the overall genotype yielded a *P* value of 0.015; homozygous Gly versus

Table 1a. Demographics of severe acute respiratory syndrome (SARS) patients and controls

Demographics	SARS patients (n=817)		Controls		
			Health care workers (n=307)	Household contacts (n=309)	Out-patients (n=260)
Mean±SD, median (range) age (years)	40±14, 38 (5-88)		35±10, 33 (21-60)	42±14, 43 (18-80)	50±20, 47 (4-95)
Male:female	2:3		2:6.6	2:2.4	2:4
No. (%) of female	505 (61.8)		235 (76.5)	168 (54.4)	173 (66.5)

Table 1b. Characteristics of severe acute respiratory syndrome (SARS) patients

Characteristics	No. (%) of SARS patients
Treated in intensive care unit (ICU)	136 (16.6)
Received assisted ventilation	76 (9.3)
Received steroid treatment	795 (97.3)
Received pulse steroid/intravenous immunoglobulin	517 (63.3)
Mean±SD, median (range) length of hospital stay (days)	28.2±17.9, 23 (4-235)
Death	12 (1.5)
Mean±SD, Median (range) of length of ICU stay (days)	2.7±10.8, 0 (0-139)

homozygous Asp (P=0.007, OR=4.31, 95% CI=1.37-13.56). Allelic association was observed (P=0.0093, OR=1.75, 95% CI=1.14-2.67, Table 2a). Association for WBC counts was also demonstrated for homozygous Gly versus homozygous Asp (P=0.022, OR=0.30, 95% CI=0.10-0.89, Table 2b). Notably, the homozygous Gly genotype associated with higher LDH levels and lower WBC counts. No significant association was found for nominal clinical outcome measures.

Functional studies

Using DC-SIGN transfectants, binding experiments showed that ICAM-3 protein did bind to DC-SIGN but at a low level; at most binding was 15%. Similar binding was also shown in the soluble ICAM-3 produced and purified in our laboratory, expressing either wild-type Asp143 genotype or variant ICAM-3 Gly143 genotype (data not shown). Binding experiments using DC-SIGN expressed on DCs did not demonstrate ICAM-3 binding to DC-SIGN. Blood

samples from a total of 15 convalescent SARS-infected individuals with different ICAM-3 genotypes and HLA-A2 positive were retrieved. T-cells harvested from these samples were incubated overnight with IL-2 before stimulation by SARS peptide and measured for IFN-γ. Results showed a wide range of response for the wild-type genotype, but with no significant difference in T-cell responses between the wild-type and variant genotypes (data not shown).

Discussion

In this large genetic association study for SARS susceptibility, no significant risk association was found for SARS infection for the ICAM-3 Asp143Gly SNP. Nonetheless, genotype analysis of our 817 SARS patients showed significant association of higher LDH levels and lower WBC counts in SARS patients on admission with the homozygous Gly143 genotype of ICAM-3, which supports the role of ICAM-3 in the immunopathogenesis of SARS. These findings are in keeping with the role of ICAM-3 in T-cell activation and the immune response. As the SARS patients were from six different hospitals throughout Hong Kong, there may have been confounding factors, such as differences in management preferences pertaining to length of hospital stay, level of intensive care, and decision to initiate assisted ventilation or administer steroids. This may have contributed to non-identification of significant associations with other clinical parameters. Laboratory parameters on the other hand were more standardised.

Although the LDH level is a relatively non-specific reflection of tissue destruction, the association with higher

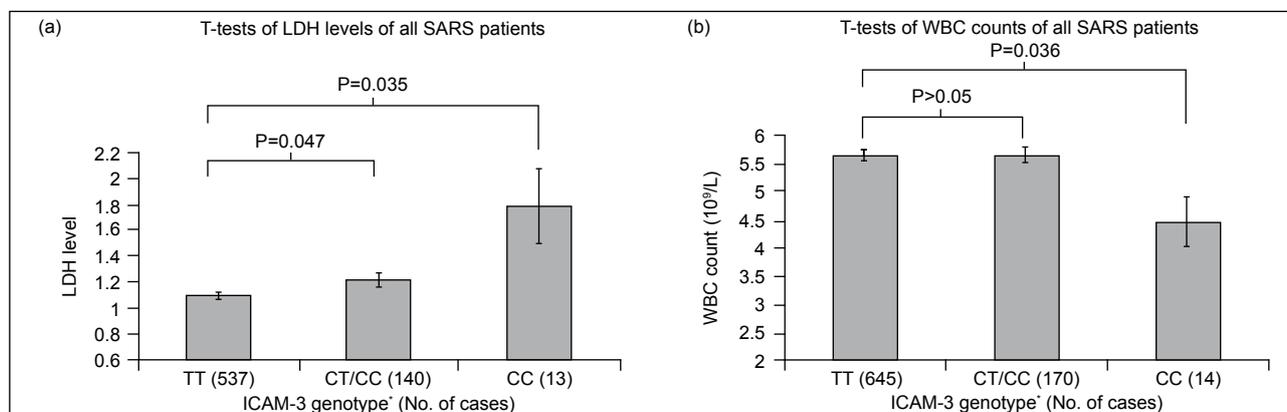


Fig. Genotype frequencies of intercellular adhesion molecule-3 (ICAM-3) of all severe acute respiratory syndrome (SARS) patients: (a) normalised lactate dehydrogenase (LDH) levels on admission (as ratio to the upper limit of the normal reference range) (b) white blood cell (WBC) counts on admission (copyright 2007 Journal of Infectious Diseases)

* TT denotes homozygous Asp143, CT heterozygous Gly/Asp, and CC homozygous Gly143

Table 2a. Genotype and allele analysis of the intercellular adhesion molecule-3 (ICAM-3) Asp143Gly, with lactate dehydrogenase (LDH) levels on admission of all severe acute respiratory syndrome (SARS) patients

ICAM-3	No. (%) of SARS patients		Odds ratio (95% CI)	P value [†]
	Lower LDH levels [*]	Higher LDH levels [*]		
Genotype	n=582	n=95		
TT (Asp)	469 (80.6)	68 (71.6)	Reference	0.015
CT (GlyAsp)	105 (18)	22 (23.2)	-	0.168
CC (Gly)	8 (1.4)	5 (5.2)	4.31 (1.37-13.56)	0.007
C-carrier (Gly-carrier) [CT and CC]	113 (19.4)	27 (28.4)	1.65 (1.009-2.69)	0.044
Allele	n=1164	n=190		
T (Asp)	1043 (89.6)	158 (83.2)	Reference	-
C (Gly)	121 (10.4)	32 (16.8)	1.75 (1.14-2.67)	0.009

^{*} The cutoff value of normalised LDH levels on admission is 1.6

[†] χ^2 test for overall genotype

Table 2b. Genotype and allele analysis of the intercellular adhesion molecule-3 (ICAM-3) Asp143Gly, with white blood cell (WBC) counts of all severe acute respiratory syndrome (SARS) patients

ICAM-3	No. (%) of SARS patients		Odds ratio (95% CI)	P value [†]
	Lower WBC counts [*]	Higher WBC counts [*]		
Genotype	n=246	n=569		
TT (Asp)	186 (75.6)	459 (80.7)	Reference	0.047
CT (GlyAsp)	52 (21.1)	104 (18.3)	-	0.270
CC (Gly)	8 (3.3)	6 (1.0)	0.30 (0.10-0.89)	0.022
C-carrier (Gly-carrier) [CT and CC]	60 (24.4)	110 (19.3)	-	0.102
Allele	n=492	n=1138		
T (Asp)	424 (86.2)	1022 (89.8)	Reference	-
C (Gly)	68 (13.8)	116 (10.2)	0.71 (0.51-0.98)	0.034

^{*} The cutoff value of WBC counts on admission is 4.5

[†] χ^2 test for overall genotype

LDH levels and lower WBC counts in SARS patients suggests immune response-associated leukocyte destruction. Indeed high peak LDH levels have been reported to be independent predictors of adverse outcome.⁴ Thus SARS patients who are homozygous for Gly 143 genotype of the ICAM-3 Asp143Gly SNP have a four-fold chance of higher LDH levels on admission and a poorer prognosis. This may have implications for other infectious diseases in which viral-induced cell death and/or immune responses contribute significantly to outcome. Thus, by knowing the genotype of the patient, we might be able to predict clinical outcome and offer suitable treatment in advance.

Using DC-SIGN transfectants, in vitro ICAM-3 binding experiments demonstrated low levels of soluble ICAM-3 binding to DC-SIGN, in keeping with previous reports suggesting binding between ICAM-3 with DC-SIGN might be transient, allowing T-cells to sample large numbers of MHC molecules for the presence of specific peptides. Our method of detection was thus not sensitive enough to demonstrate significant differences in binding affinity between the two different genotypes. Although binding levels may be low, in vivo they may be sufficient for transient interactions between DCs and T-cells.

A wide variation in T-cell response suggested that at least for wild-type ICAM-3, other factors also affect the response. The DCs might transfer SARS coronavirus to other cells through DC-SIGN, with interactions between

ICAM-3 and DC-SIGN contributing to trans-infection from DCs to T cells. Thus the higher LDH levels and lower WBC counts associated with homozygous Gly143 genotype could also be attributed in part by viral-induced cell death.

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