

## Results

Demographic information is shown in Table 1. The mean age was not different between SARS cases and contacts ( $p = 0.1781$ ). Although females appeared more frequently in SARS cases than contacts, the male/female ratio was not statistically different between the two groups ( $p = 0.0869$ ). Sixteen individuals out of 103 contacts were revealed to have anti-SARS-CoV antibodies. When we classified these individuals into an infected group together with SARS patients, age and gender showed no significant difference between the infected and non-infected groups ( $p = 0.2139$ ;  $0.2065$ ). SARS cases were classified by the requirement for oxygen therapy. Age and gender did not differ between these subgroups either ( $p = 0.4198$ ;  $0.7411$ ).

We analyzed SNPs of IFN-inducible genes showing association with HCV infection as described above [20–22], and compared their genotypes and allele frequencies between 44 SARS cases and 103 controls with contact history to SARS patients (Table 2).

We observed a higher frequency of the G-allele positive genotypes (GA and GG) of non-synonymous SNP in exon 3 of *OAS-1* gene in SARS patients (odds ratio 2.68; 95% CI; 1.17–6.15;  $p = 0.0178$ ). Allele frequency of the G-allele in exon 3 was significantly higher in SARS patients ( $p = 0.0090$ ). Allele frequency of the G-allele in exon 6 of *OAS-1* was also found more frequently in SARS patients than in the controls that showed marginal significance ( $p = 0.0542$ ).

The genotype and allele frequencies of *OAS-1* polymorphisms were compared among 60 SARS-CoV infected individuals and 87 uninfected individuals (Table 3). It was shown that frequencies of the G-alleles in exon 3 and exon 6 were significantly higher in infected individuals than in uninfected ( $p = 0.0156$  and  $p = 0.0176$ , respectively). These two polymorphisms in the *OAS-1* gene were in strong linkage disequilibrium ( $|D'| = 0.931$ ,  $r^2 = 0.530$ ). Genotype and allele frequencies in 50 controls with no contact history with SARS patients are also shown in Table 3. In controls of subjects having no contact history, allele frequencies of G-allele in exons 3 and 6 were lower than those of the infected group, but higher than those of the uninfected group.

The SNPs in *MxA* gene and *PKR* gene were not associated with the development of SARS (Table 2). However, on comparison of the *MxA* genotype between hypoxemic SARS patients requiring oxygen therapy

Table 2

Genotype and allele frequencies in SARS cases and controls with contact history

	SARS cases (n = 44)	Controls (n = 103)	p value
<i>OAS-1</i> exon 6			
Genotype			
AA	18 (40.9%)	60 (58.3%)	0.0537
AG	21 (47.7%)	36 (35.0%)	
GG	5 (11.4%)	7 (6.7%)	
Allele			
A	0.65	0.76	0.0542
G	0.35	0.24	
<i>OAS-1</i> exon 3			
Genotype			
AA	9 (20.5%)	42 (40.8%)	0.0178
AG	24 (54.5%)	48 (46.6%)	
GG	11 (25.0%)	13 (12.6%)	
Allele			
A	0.48	0.64	0.0090
G	0.52	0.36	
<i>MxA</i> -88			
Genotype			
GG	23 (52.3%)	43 (41.7%)	0.2400
GT	16 (36.4%)	52 (50.5%)	
TT	5 (11.3%)	8 (7.8%)	
Allele			
G	0.70	0.67	0.5597
T	0.30	0.33	
<i>PKR</i> -168			
Genotype			
CC	18 (41.9%)	49 (47.6%)	0.5278
CT	22 (51.2%)	46 (44.7%)	
TT	3 (7.0%)	8 (7.8%)	
Allele			
C	0.67	0.70	0.6780
T	0.33	0.30	

and non-hypoxemic SARS patients who did not, GG genotype was found more frequently in patients of the former category (odds ratio 3.75; 95% CI 1.08–10.7;  $p = 0.0346$ ). It was also shown that the G-allele was more frequent in the former group ( $p = 0.0195$ ) (Table 4). The other SNPs did not show any significant  $p$  values between these two groups (data not shown).

## Discussion

Our study showed that the polymorphisms in the IFN-inducible *OAS-1* gene might affect susceptibility to SARS-CoV infection or the development of SARS.

Table 1  
Characteristics of SARS cases and healthy contacts

Characteristics	Contacts			SARS cases		
	(n = 103)	Anti-SARS-CoV Ab (-) (n = 87)	Anti-SARS-CoV Ab (+) (n = 16)	(n = 44)	Non-hypoxemic group (n = 22)	Hypoxemic group (n = 22)
Age (year), mean [range]	36.5 [15–69]	36.6 [15–69]	36.6 [25–50]	39.3 [17–76]	37.7 [17–61]	41.0 [23–76]
Male/female (n)	46/57	39/48	7/9	13/31	6/16	7/15

Table 3  
Genotype and allele frequencies of *OAS-1* polymorphisms in SARS infected, uninfected, and controls without contact history

<i>OAS-1</i>	SARS infected (n = 60)	Uninfected (n = 87)	p value	Controls without contact (n = 50)
<b>Exon 6</b>				
Genotype				
AA	25 (41.7%)	53 (60.9%)	0.0215	27 (54.0%)
AG	28 (46.7%)	29 (33.3%)		17 (34.0%)
GG	7 (11.7%)	5 (5.7%)		6 (12.0%)
Allele				
A	0.65	0.76	0.0176	0.71
G	0.35	0.24		0.29
<b>Exon 3</b>				
Genotype				
AA	14 (23.3%)	37 (42.5%)	0.0163	17 (34.0%)
AG	33 (55.0%)	39 (44.8%)		26 (52.0%)
GG	13 (21.7%)	11 (12.6%)		7 (14%)
Allele				
A	0.51	0.65	0.0156	0.60
G	0.49	0.35		0.40

Table 4  
Genotype and allele frequencies of *MxA* -88 G/T polymorphism in the subgroups of SARS cases

	SARS cases (n = 44)		p value
	Non-hypoxemic group (n = 22)	Hypoxemic group (n = 22)	
Genotype			
GG	8 (36.4%)	15 (68.2%)	0.0346
GT	10 (45.4%)	6 (27.3%)	
TT	4 (18.2%)	1 (4.5%)	
Allele			
G	0.59	0.82	0.0195
T	0.41	0.18	

In the presence of double-stranded RNA (dsRNA), *OAS-1* catalyzes the 2',5'-oligomers of adenosine in order to permit the binding and activation of a latent ribonuclease, RNase L, which cleaves cellular and viral RNAs [11,25]. *OAS-1* gene has two major transcripts that are generated by alternative splicing at the last two exons [23]. E16 (NM\_002534) is a short transcript with 5 exons and is translated to p40 isoform. E18 (NM\_016816) is a long transcript with 6 exons and is translated to p46 isoform. Another transcript 9-2 is generated using a different splice acceptor site that comes from E18 at exon 6 and is translated to 9-2 protein [26]. The 9-2 protein has a unique property due to the Bcl-2 homology domain 3 present in its unique carboxyl-terminal region. This is also distinctive in causing cellular apoptosis by binding to the anti-apoptotic proteins of the Bcl-2 family [26]. Therefore, *OAS-1* has dual functions representing the synthesis of 2',5'-oligomers of adenosine and the promotion of cellular apoptosis.

Knapp et al. [22] described how the GG genotype in exon 6 of *OAS-1* gene was more frequent in persistent

HCV infection than in self-limiting infection. In our study, the G-allele was more frequently observed in SARS-CoV infected individuals than in the uninfected group. In both these studies, the G-allele was susceptible to virus infection. The A/G polymorphism in exon 6 is located downstream of the stop codon for E18 transcript meaning therefore that it is included in the 3'-untranslated region. However, it is located upstream of the stop codon for 9-2, and the A/G SNP results in amino acid substitution Arg397Gly of 9-2 protein, which is located near the Bcl-2 homology domain (amino acid positions 372–393). It will be an interesting aspect if this phenomenon occurs with any functional importance. We also analyzed the A/G polymorphism in exon 3 of *OAS-1* gene and found that there was strong linkage disequilibrium between the two SNPs. The A/G polymorphism in exon 3 causes amino acid substitution Ser162Gly in three isoforms, which is located near the dsRNA binding domain (amino acid positions 104–158) of *OAS-1* [27]. We are unable at this point to determine which SNP is directly related to susceptibility to SARS or SARS-CoV infection. One can also consider that the other unidentified polymorphism of strong linkage disequilibrium with these SNPs may serve as the basis for any functional difference. Judging from the results obtained in this study, polymorphisms in *OAS-1* gene are likely to be involved in SARS-CoV infection or the development of SARS, at least in part, bearing in mind the fact that *OAS-1* might have antiviral potential against SARS-CoV.

SARS-CoV is usually cultured in Vero E6 cell line [13–17,19], which cannot produce IFNs because it lacks *IFN* genes [28,29]. Recently, Cinatl et al. [30] infected permissive Caco-2 cells with SARS-CoV and analyzed the effects of SARS-CoV on cellular gene expression by high-density oligonucleotide arrays. They found that SARS-CoV infection of Caco-2 cells up-regulated IFN-inducible *OAS-2*, *OASL*, and *MxA* but not *PKR* genes. *OAS-2* and *OASL* are members of the human *OAS* gene family [25]. The role of *OAS-1* as an inhibitor of SARS-CoV replication should be clarified to examine the hypothesis that Caco-2 cells permitted considerable infection with SARS-CoV because they did not induce *OAS-1*.

As regards the G/T polymorphism at position -88 in promoter region of *MxA* gene, GG genotype and G-allele were found to be more frequent in patients with an enhanced clinical progression, requiring oxygen therapy, although the number of cases was rather small. GG genotype was found more frequently in non-responders of IFN treatment in hepatitis C, and a luciferase reporter assay revealed that the *MxA* promoter sequence of G haplotype had lower promoter activity than that of T haplotype [31]. Recently, Arcas et al. [32] reported that GG genotype expressed lower amount of *MxA* mRNA than GT or TT genotype in IFN-treated peripheral

blood mononuclear cells in vitro. Spiegel et al. [15] reported that SARS-CoV replication was not affected in Vero E6 cells that were stably expressing MxA. They concluded that antiviral effect of IFN against SARS-CoV was not mediated by MxA. In our study, -88 SNP in *MxA* promoter was not related to disease susceptibility. Taking these observations together, MxA may not have a strong inhibitory effect on replication of SARS-CoV, but lower MxA expression may play a role in the worsening of SARS clinical progression.

If SARS re-emerges, IFN could be a promising candidate to treat SARS patients [12–19]. In the present study, the SNPs in *OAS-1* were associated with SARS-CoV infection or development of SARS, and the SNP in *MxA* was associated with the progression of SARS. It could be interesting to consider that they may also be related to the response of SARS patients to IFNs, and that SARS patients with AA genotype of the A/G SNP in exon 3 of *OAS-1* may respond to IFN treatment more effectively than those with AG or GG genotypes. During the course of our study, age was not a risk factor contributing to any worsening of SARS, probably because the majority of the patients consisted of relatively young medical staff members [6].

In conclusion, we showed that the polymorphisms in *OAS-1* gene were associated with SARS-CoV infection or development of SARS and that the polymorphism in *MxA* gene was also associated with hypoxemic status in SARS cases in Vietnam. These findings may lead to an understanding of IFN-induced antiviral response to SARS infection.

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## Short Communication

# Trial Surveillance of Cases with Acute Respiratory Symptoms at IMCJ Hospital

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**SUMMARY:** We have developed a surveillance system that can detect a severe acute respiratory syndrome (SARS) outbreak in a hospital as quickly as possible using the "SARS alert" strategy proposed by the World Health Organization (WHO). Our research examined hospital staff and in-patients during the winter of 2003/2004. We defined patients with a fever of over 38°C and respiratory symptoms as "cases with acute respiratory symptoms." During the study period, 215 such cases (78% in-patients; 22% hospital staff members) were reported. A rapid diagnostic test for influenza was performed on 131 individuals, with 52 having positive results. There were no cases fulfilling the definition of SARS provided by the WHO in their SARS alert. The present surveillance system will be of use in the early detection of a SARS epidemic in a hospital as well as in early detection of similar illnesses accompanied by acute respiratory symptoms, such as influenza.

Severe acute respiratory syndrome (SARS) haunted the world from November 2002 to July 2003. According to the World Health Organization (WHO), over 8,000 infected patients and nearly 800 deaths were reported in 26 regions during this period. An extremely large problem in the case of SARS is the number of health care workers (HCWs) infected; at 1,706 persons, the figure accounted for 21% of all reported cases (1; [http://www.who.int/csr/sars/country/table2004\\_04\\_21/en/](http://www.who.int/csr/sars/country/table2004_04_21/en/)). Because of this problem, the WHO has proposed a new surveillance strategy known as the "SARS alert" (2; <http://www.who.int/csr/sars/postoutbreak/en/>). If a SARS alert occurs, the WHO recommends that strict infection control procedures be adopted immediately. However, the introduction of this policy requires daily surveillance in accordance with the definition of a SARS alert. Additionally, this surveillance targets not only in-patients but also hospital personnel. To date, the WHO has not yet indicated any specific methods for the application of SARS alert surveillance to hospital personnel.

Therefore, we attempted to create a new surveillance system to detect clinical SARS cases as defined by the SARS alert in both patients and HCWs. To facilitate the detection of SARS as well as other respiratory infectious diseases such as influenza, the present surveillance focused on cases with "acute respiratory symptoms".

These definitions used for this surveillance were a fever of over 38°C and one or more symptoms of respiratory tract illness (RTI), including both upper RTI (rhinorrhea or sore throat) and lower RTI (coughing, sputum, shortness of breath, decreased SpO<sub>2</sub>, or radiographic evidence of lung infiltrates consistent with pneumonia or respiratory distress syndrome [RDS]).

The subjects were all in-patients, nurses, doctors, technicians, pharmacists or other medical staff at the International Medical Center of Japan (IMCJ) hospital, Tokyo, Japan. The

study period was from December 2003 to March 2004. If a patient or HCW with acute respiratory symptoms was identified, the head of each section filled in a surveillance report and submitted it to an infection control team (ICT). The results of the surveillance were analyzed and released weekly to hospital staff by hospital intranet.

During the study period, 215 cases with acute respiratory symptoms were reported. Their median age was 39.0 years of age (range: 5 mos - 99 years of age), and the male:female ratio was 1:1.05. Wards in which numerous cases were reported were the pediatric ward (36 cases), the respiratory ward (20 cases) and the private room ward (18 cases). The identified cases included 168 in-patients (78%), 26 nurses (12%), 15 doctors (7%), 4 technicians (2%) and 2 pharmacists (1%). A rapid test for influenza (Espline®; Fujirebio, Inc., Tokyo, Japan) (3) was performed in 131 cases (61%), and 40% of tested individuals were found to be positive. Trends in the reported cases are shown in Figure 1. There was a peak in the number of reported cases from the 3rd week of January to the 2nd week of February, coinciding with a peak in influenza cases at the IMCJ hospital. Additionally, these peaks coincided with a peak in the nation wide spread of influenza in Japan (4; <http://idsc.nih.go.jp/idwr/kanja/weeklygraph/01flu-e.html>).

During the surveillance period, one cluster of cases with acute respiratory symptoms was found in our hospital. The episode was observed in the respiratory ward and included 11 cases with acute respiratory symptoms; 4 of which tested positive on the rapid diagnostic test for influenza. This finding caused the ICT to quickly introduce appropriate infection control measures such as cohort isolation, prophylactic use of oseltamivir, and limitations on the admission of new patients. With this intervention, the cluster was quickly controlled.

During the study period, no actual SARS alert cases that met the WHO definition were observed.

SARS is characterized by its high transmissibility to HCWs and becomes widespread via nosocomial infection (5,6). Therefore, both in-patients and HCWs with symptoms must be constantly monitored in order to detect a SARS outbreak

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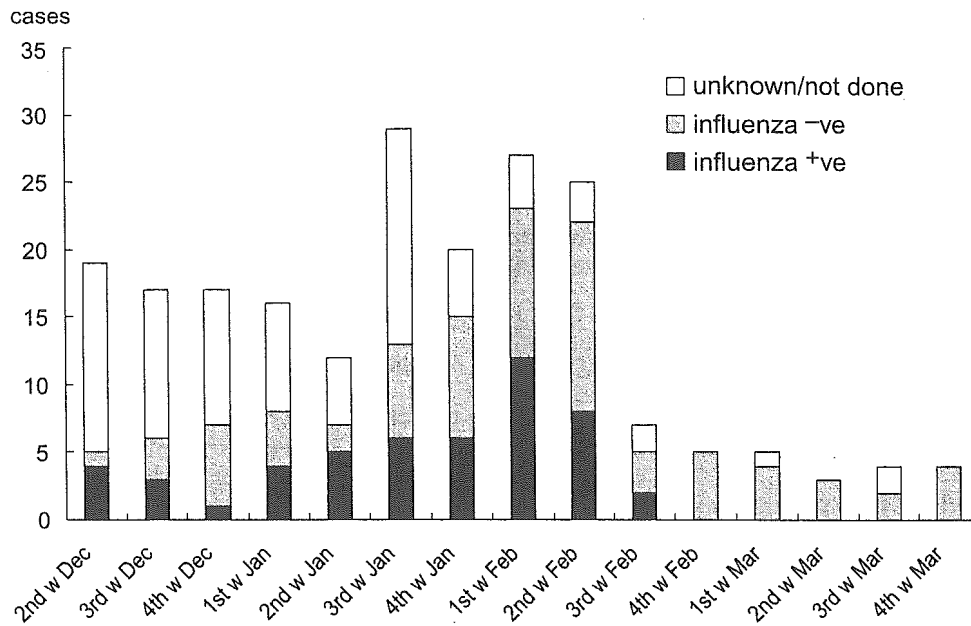


Fig. 1. Trend graph of reported case with acute respiratory symptoms between the 2nd week of December 2003 and the 4th week of March 2004.

in a hospital in the early stages. The SARS alert strategy proposed by the WHO is an operational definition used to ensure that appropriate infection control and public health measures are implemented until SARS has been ruled out as a cause of pneumonia or RDS.

This policy defines SARS cases clinically as cases with a fever of over 38°C, with one or more symptoms of lower RTI (coughing, difficulty breathing, or shortness of breath), with radiographic evidence of lung infiltrates consistent with pneumonia or RDS, and with no alternative diagnosis that can fully explain the illness. SARS alert situation is defined as one or both of the following:

- i) two or more HCWs in the same health care unit fulfilling the clinical case definition of SARS and whose onset of illness occurs within the same 10-day period; and
- ii) hospital-acquired illness in three or more persons (HCWs and/or other hospital staff and/or patients and/or visitors) in the same health care unit fulfilling the clinical case definition of SARS and whose onset of illness occurs within the same 10-day period.

Because the threat of infection involves not only SARS but also other emerging respiratory virus infections (i.e., new types of influenza), we attempted to create a system that can also detect acute respiratory infections such as influenza in a hospital. Because the early clinical features of SARS and influenza are quite similar, some confusion in clinical settings is expected. Hence, a "syndromic surveillance" system, that is, a system that detects acute respiratory symptoms without regard to the pathogenic virus, must be developed. Therefore, we partially modified the WHO's SARS alert strategy and introduced a new method of surveillance for the early detection of SARS and influenza.

Our criteria for the definition of disease differed from that of the WHO in that it included upper RTI and (ii) it did not require pneumonia findings in chest X-rays. We felt that adding these changes would allow the detection of influenza outbreaks in a hospital as well.

An epidemic of cases with acute respiratory symptoms during the aforementioned period was effectively monitored

during surveillance at IMCJ hospital. An outbreak of influenza at the hospital was also detected by the present surveillance system. Information provided by surveillance was effectively used for infection control. Fortunately, there were no cases that met the definition of SARS provided by the WHO in their SARS alert. Hospital staff should be informed as soon as possible about the spread of infectious diseases in the hospital. We used hospital intranet for this purpose, and information was quickly conveyed to the appropriate divisions of the hospital.

The present surveillance strategy will be of use in the early detection of a SARS epidemic in a hospital as well as in the early detection of similar illnesses accompanied by acute respiratory symptoms such as human influenza and new types of influenza. Further study is needed to improve the sensitivity and specificity of this surveillance.

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## Assessment of synthetic peptides of severe acute respiratory syndrome coronavirus recognized by long-lasting immunity

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**Key words:**

IgG; long-lasting immunity; SARS; structural protein; synthetic peptides

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**Abstract:** In order to determine highly immunogenic severe acute respiratory syndrome coronavirus (SARS-CoV) epitope peptides capable of inducing long-lasting immunity, we first screened immunoglobulin-G (IgG) antibodies reactive to 197 different overlapping 15-mers from the SARS-CoV proteins in the sera of three infected patients. Forty-two peptides among them were reactive to the sera from all three patients. Consequently, we tested for the reactivity of these 42 peptides to patients' sera ( $n = 45$ ) at 6-month post-infection. The significantly higher levels of IgG antibodies specific to three (S791, M207 and N161) of 42 peptides were detectable in the post-infection sera from 23 (51%), 27 (60%) and 19 (42%) of 45 patients, respectively. These three peptides, recognized by their long-lasting immunity, may provide a better understanding of the immunogenicity of SARS-CoV.

A novel coronavirus (SARS-CoV) was discovered in association with the cases of life-threatening severe acute respiratory syndrome (SARS) that occurred in March of 2003 (1, 2). The genome of the SARS-CoV is 29,727 nucleotides in length and has 11 open reading frames, and its genome organization is similar to that of other coronaviruses (3). Since March of 2003, studies to determine immunogenic epitopes have been performed at a fast pace, within a short period of time, because of the urgent need to develop both therapeutic and diagnostic modalities for the SARS-CoV (4–8). The results of these studies indicate that both the spike (S) and nucleocapsid (N) proteins of the SARS-CoV contain immunogenic regions. However, further studies are needed in order to identify the hot spots, for which diagnostic and therapeutic tools can be developed. In order to determine highly immunogenic regions, an investigation was performed of SARS-CoV epitope peptides capable of inducing long-lasting immunity, and the three candidate peptides have been reported in this study.

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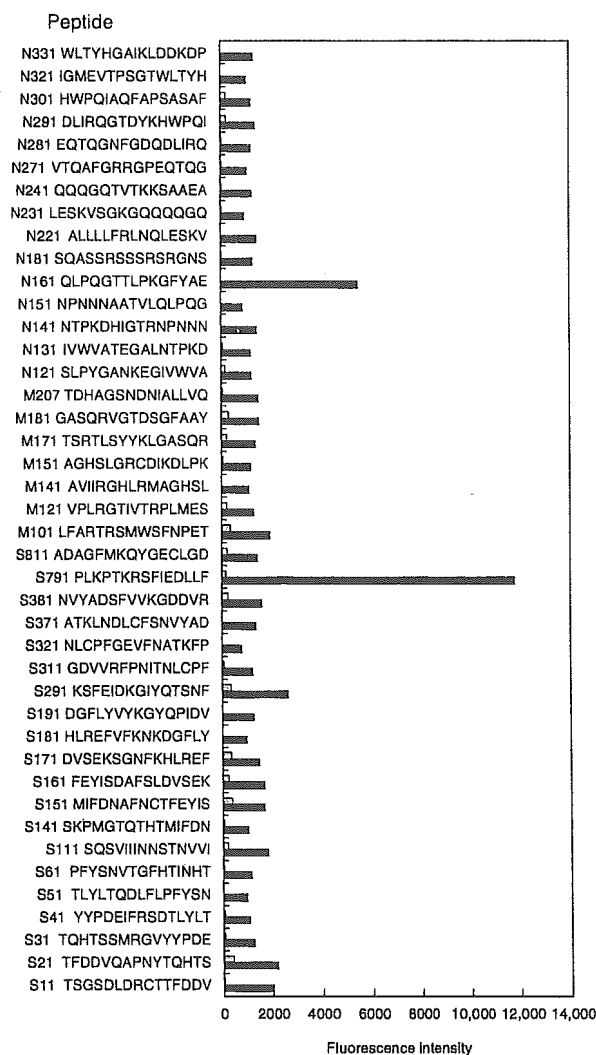
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## Forty-two peptides recognized by sera from SARS patients

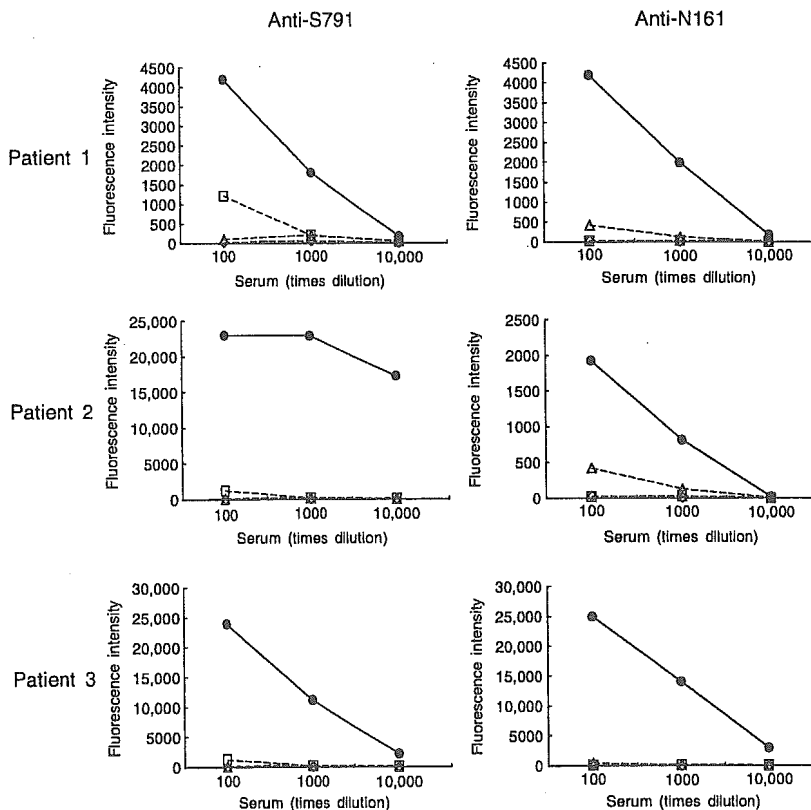


**Fig. 1. Screening of peptides.** A total of 197 different 15-mer peptides (>70%) possessing 5-amino acid overlap sequences based on the full genomic sequences of the severe acute respiratory syndrome coronavirus (SARS-CoV), including 125 spike (S), 43 nucleocapsid (N), 22 membrane (M) and seven envelope (E) proteins (3), were purchased from American Peptide Company, Inc. (Vista, CA). Each peptide was dissolved in dimethylsulfoxide (DMSO) and was then stored at  $-20^{\circ}\text{C}$  until use. These peptides were tested for their reactivity to the sera of early stages of three Taiwanese SARS-CoV-infected patients by using flowmetry analysis with Luminex<sup>TM</sup> (Luminex Corp., Austin, TX) (9). The sera were collected from Jen-Ai Municipal Hospital, SaAn District, Taipei, Taiwan. The patients' sera showed significantly higher levels of immunoglobulin-G (IgG) ( $P < 0.05$ ) activities reactive to 42 of 197 peptides tested, including 20 spike (S)-, seven membrane (M)- and 15 nucleocapsid (N)-derived peptides, when the means of the scores of fluorescence intensity (FI) from the sera (1000-fold dilution) of the three patients (closed bar) were compared to those of the three healthy donors (HD) (open bar). The peptides were coupled to colour-coded beads, according to the modified manufacturer's instructions (Luminex Corp.). In brief, 100  $\mu\text{l}$  of colour-coded beads were mixed with 100  $\mu\text{l}$  of peptide (1 mg/ml in 0.1M morpholinohanesulfonic acid (MES) buffer, pH4.5). The peptide-loaded beads were then incubated with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide 2-(N-Morpholino)ethanesulfonic acid (EDC) (1 mg ml<sup>-1</sup>) at room temperature for 30 min in darkness, and the beads were washed with Tween-20 phosphate-buffered saline (PBS). The beads were treated with 2-aminoethanol for 10 min at room temperature in darkness, washed twice and then re-suspended with 1 ml of 0.05% Block Ace (Snow Brand Milk Products Co., Ltd, Hokkaido, Japan) in Tween-20 PBS. Two microlitres of serum at dilutions of 100–10,000 times was incubated with 25  $\mu\text{l}$  of the peptide-coupled colour-coded beads for 2 h at room temperature on a plate shaker in a 96-well filter plate (MultiScreen<sup>TM</sup>-BV, Millipore Co., Bedford, MA). After incubation, the plate was washed by using a vacuum manifold apparatus and was incubated with 100  $\mu\text{l}$  of biotinylated goat anti-human IgG (gamma-chain-specific; Vector Laboratory Inc., Burlingame, CA) for 1 h at room temperature on a plate shaker. The plate was then washed, and 100  $\mu\text{l}$  of streptavidin-PE (Molecular Probes, Eugene, OR) was added into wells, followed by incubation for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100  $\mu\text{l}$  of Tween-20 PBS into each well, and the plate was placed for 3 min on a plate shaker. Fifty microlitres of sample was analysed by using the Luminex<sup>TM</sup> system with the help of the method reported previously (13, 9, 15).

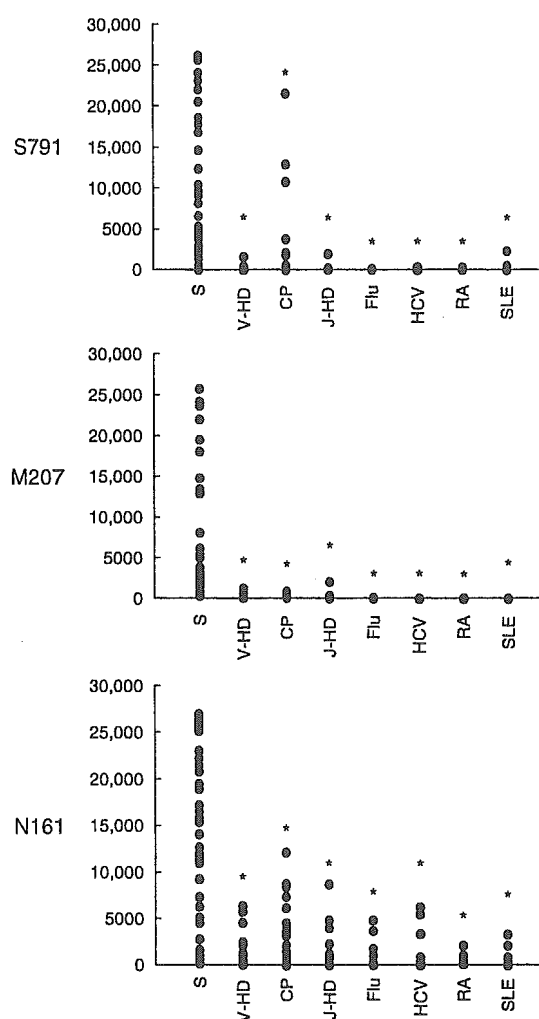
We first measured the levels of immunoglobulin-G (IgG) antibodies reactive to each of the 197 peptides in the sera of the Taiwanese SARS-CoV-infected patients ( $n=3$ ) and the Japanese healthy donors (HD) ( $n=3$ ) as negative controls by means of the flowmetry analysis with Luminex™ (Luminex Corp., Austin, TX), a new method that has the great advantage of allowing users to measure a large number of serum samples against a large number of peptide antigens at relatively low cost, time and labour intensity, as recently reported by us (9). The patients' sera showed significantly higher levels of anti-peptide activities ( $P < 0.05$ ) against 42 of 197 peptides tested, including 20 spike (S), seven membrane (M) and 15 nucleocapsid (N)-derived peptides (Fig. 1). The scores, for instance, for the fluorescence intensity (FI) of the anti-SARS-CoV spike protein at positions 791–805 (termed anti-S791) were highest among the peptides tested, and were 1813, 22,964 and 11,240 in the sera of the three patients, whereas those of the controls were 207, 58 and 210, respectively. The scores for the FI of the anti-SARS-CoV nucleocapsid protein at positions 161–175 (termed anti-N161) were 697, 815 and 14,084, whereas those of the controls were 0, 11 and 129, respectively.

The results of the dose-dependent curves were obtained in all of the 42 peptides for all three cases. The representative results of the anti-S791 and anti-N161 antibodies have been presented in Fig. 2.

The 42 peptides shown in Fig. 1 were tested for their reactivity to the post-infection (6th month) sera from patients with the Vietnamese SARS-CoV infection ( $n=45$ ). As negative controls, sera of both Vietnamese HD ( $n=50$ ) and the contact persons ( $n=230$ ), who were free from illness but worked in the same hospitals, were simultaneously tested at a serum dilution of 1:100. Both the mean and the median of the FI of sera from Vietnamese HD, contact persons and the patients reactive to each of the 42 peptides have been presented in Table 1. The levels of anti-S791, anti-M207 and anti-N161 activities in the sera of the SARS-CoV patients were significantly ( $P < 0.005$ ) higher than those of both Vietnamese HD and the contact persons as evaluated by means of both Student's *t*-test and the Mann-Whitney test (Table 1). In contrast, there were no significant differences in the reactivity against any of 42 peptides between the HD and the contact persons.



**Fig. 2. Dose dependency.** The dose dependency of the anti-peptide activities was observed in all of the 42 peptides at three different serum dilutions (100-, 1,000- and 10,000-fold). Representative results of the dose-dependent curves of the anti-S791 and anti-N161 activities have been shown in this figure. The levels of immunoglobulin-G (IgG) have been presented as closed circles (patients), and as an open circle, open square and open triangle (three healthy donors), respectively.



**Fig. 3. Anti-peptide immunoglobulin-G (IgG) at post-infection.** The 42 peptides shown in Fig. 1 were tested for their reactivity to sera from post-infection (6th month) patients with the Vietnamese SARS-CoV infection ( $n=45$ ). As negative controls, the sera of both Vietnamese healthy donors (V-HD) ( $n=50$ ) and the contact persons (CP), who were free from illness of SARS-CoV infection but worked in the same hospitals, were tested. Sera from Japanese patients with hepatitis-C virus (HCV), influenza (Flu), rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), along with Japanese HD (J-HD), were also tested at the same times at a serum dilution of 1:100. Representative results of the fluorescence intensity (FI) scores at a serum dilution of 100:1 of anti-S791, anti-M207 and anti-N161 activities have been shown in this figure. The significance of the differences ( $*P < 0.05$ ) in Luminex reactivities of SARS patients against all of other negative control groups, including V-HD, CP, J-HD, Flu, HCV, RA and SLE, was observed by means of Mann-Whitney test. Plasma from 78 Vietnamese post-infection (6th month,  $n=45$ ) SARS-CoV patients was collected at Hanoi French Hospital and Bach Mai Hospital in Vietnam. All cases met a modified World Health Organization (WHO) case definition of SARS (16). This study was approved by both the Japanese and Vietnamese Institutional Review Board. Written informed consent was obtained from each of the participants. Sera from Japanese patients with Flu ( $n=12$ ), HCV ( $n=12$ ), RA ( $n=15$ ) and SLE ( $n=10$ ) and Japanese healthy donors (J-HD,  $n=27$ ) were provided by Kurume University Hospital and Kurume Medical Center after informed consent was obtained. SARS-CoV, severe acute respiratory syndrome coronavirus.)

Each of the FI scores at a serum dilution of 1:100 of anti-S791, anti-M207 and anti-N161 activities has been plotted in Fig. 3. The cut-off value of the FI scores for anti-S791 peptide activity at a serum dilution of 1:100 was set at 970 (mean: 199 plus 2 SD,  $386 \times 2$  of 50 HD). Under these circumstances, significant levels ( $>970$  at a serum dilution of 1:100) of anti-S791 activity were detected in the sera from 23 of 45 patients (51%), 18 of 230 contact persons (7.8%) and four of 50 HD (8%). When the cut-off value for anti-M207 activity was set at 896 (mean: 356 plus 2 SD,  $270 \times 2$  of 50 HD), significant levels ( $>896$ ) of anti-M207 activity were detected in the sera from 27 of 45 patients (60%), 10 of 230 contact persons (4.3%) and three of 50 HD (6%). Similarly, when the cut-off value for anti-N161 activity was set at 2705 (mean: 525 plus 2 SD,  $1090 \times 2$  of 50 HD), significant levels ( $>2705$ ) of anti-N161 activity were detected in the sera from 19 of 45 patients (42%), 21 of 230 contact persons (9.1%) and two of 50 HD (4%) (Fig. 3).

The levels of anti-M181 activity in the sera of the SARS-CoV patients were also significantly higher than those of Vietnamese HD and contact persons as evaluated with the help of both Student's *t*-test and Mann-Whitney test (Table 1). However, the positive cases showing FI scores of greater than the mean plus 2SD were only six of 45 patients (13%). In contrast to these four peptides, significant levels of IgG reactive to the remaining 36 peptides were either scarcely or not detected in the patients (Table 1).

Wang et al. (4) reported four different epitope peptides recognized by the sera of SARS-CoV patients. One of them, the N66 (nucleocapsid protein at positions 161–182) peptide, was 7-amino acids longer at the C-terminal than the N161 (at positions 161–175) peptide reported in this study. These findings suggest that this region of nucleocapsid proteins is one of the most highly immunogenic epitopes of the SARS-CoV when peptides are used. However, the S791 peptide shown to be the other candidate of immunogenic peptides in the present study was not tested by Wang et al. (4), because they selected peptides with relatively high hydrophilicity. They also did not detect the M207 peptide as an immunogenic epitope, although they tested the M206 peptide, which is one N-terminal amino acid longer than the M207 peptide.

In order to determine the cross-reactivity of the patients' sera to the other infectious diseases or auto-immune diseases, sera from Japanese patients with hepatitis-C virus ( $n=12$ ), influenza virus ( $n=12$ ), rheumatoid arthritis ( $n=15$ ) and systemic lupus erythematosus (SLE) ( $n=10$ ) and the Japanese HD ( $n=27$ ) were also tested at the same times at a serum dilution of 1:100. However, anti-S791, anti-M207 or anti-N161 activity was not detectable in the

sera of any groups tested, including SLE patients, although the cross-reactivity between the SARS-CoV and SLE was suggested in the study by using an Enzyme-linked immunosorbent assay (ELISA) kit coated by non-purified antigen (10). These results indicate that anti-S791, anti-M207 and anti-N161 activities were largely restricted to the SARS-CoV infection, although sera of Vietnamese patients shall be provided as controls to confirm this issue.

Both the Luminex™ and ELISA were employed for the measurement of anti-peptide antibodies in order to ensure the reliability of the former new method. As expected, both the assays could detect anti-SARS peptide activity reactive to each of the three peptides with relatively higher sensitivity in the Luminex assay for the measurement of anti-M207 antibody. Representative results of the three cases have been presented in Fig. 4.

**Reactivity of the synthesized 15-mer peptides with sera from post-infection (6th month) SARS patients with the help of flowmetric analysis by using Luminex™**

Name of peptide	Peptide sequence	FI of HD <sup>a</sup> (mean ± SD) (n = 50)	FI of CP <sup>b</sup> (mean ± SD) (n = 230)	FI of S <sup>c</sup> (mean ± SD) (n = 45)	Percentage of positive S samples	FI of HD <sup>a</sup> median <sup>d</sup> (75, 25)	median (75, 25)	median (75, 25)
S11	TSGSDLDRCTTFDDV	767 ± 1505	735 ± 1496	469 ± 631	0	217 (107, 520)	200 (109, 581)	210 (131, 474)
S21	TFDDVQAPNYTQHTS	92 ± 396	113 ± 356	117 ± 382	4	18 (13, 38)	24 (12, 56)	18 (9, 30)
S31	TQHTSSMRGVYYPDE	423 ± 766	573 ± 962	493 ± 847	7	231 (158, 376)	257 (164, 478)	207 (162, 288)
S41	YYPDEIFRSDTLYLT	32 ± 103	44 ± 130	43 ± 79	4	8 (0, 27)	15 (0, 32)	23 (7, 37)
S51	TLYLTQDLFLPFYSN	14 ± 64	7 ± 28	7 ± 21	0	0 (0, 0)	0 (0, 0)	0 (0, 0) 0 (0, 3)
S61	PFYSNVTGFHTINHT	17 ± 46	13 ± 45	13 ± 29	2	0 (0, 12)	0 (0, 9)	0 (0, 19)
S111	SQSVIIINNSTNVVI	48 ± 158	34 ± 75	53 ± 159	4	13 (5, 26)	17 (8, 30)	17 (12, 28)
S141	SKPMGTQHTMIFDN	265 ± 630	290 ± 599	320 ± 686	4	119 (52, 269)	106 (51, 224)	121 (67, 208)
S151	MIFDNAFNCTFEYIS	353 ± 651	806 ± 3057	423 ± 623	7	180 (95, 344)	247 (141, 512)	239 (151, 387)
S161	FEYISDAFSLDVSEK	128 ± 423	168 ± 371	143 ± 279	4	46 (29, 85)	63 (37, 112)	60 (36, 87)
S171	DVSEKSGNFKHLREF	327 ± 610	528 ± 1865	418 ± 697	4	178 (122, 366)	198 (126, 371)	213 (164, 359)
S181	HLREFVFNKDGFLY	20 ± 54	12 ± 39	10 ± 13	0	0 (0, 15)	0 (0, 7)	4 (0, 16)
S191	DGFLYVYKGYQPIDV	22 ± 65	14 ± 32	39 ± 113	7	0 (0, 13)	0 (0, 11)	0 (0, 35)
S291	KSFEIDKGIYQTSNF	440 ± 1088	604 ± 1321	564 ± 1054	4	165 (109, 331)	207 (121, 428)	229 (149, 371)
S311	GDVVRFPNITNLCPF	171 ± 338	180 ± 345	180 ± 315	2	73 (46, 135)	82 (52, 165)	125 (65, 191)
S321	NLCPFGEVFNATKFP	627 ± 1008	835 ± 2051	508 ± 631	2	322 (175, 596)	341 (202, 664)	333 (212, 481)
S371	ATKLNLCFSNVYAD	495 ± 1056	875 ± 2035	719 ± 1405	7	231 (157, 474)	289 (189, 607)	237 (179, 521)
S381	NVYADSFVVKGDVDR	376 ± 1047	488 ± 1047	498 ± 1037	7	130 (72, 317)	158 (84, 339)	124 (83, 244)
S791	PLKPTKRSFIEDLLF	199 ± 386	489 ± 2027	3374 ± 5981 <sup>e,h</sup>	51	39 (6, 214)	37 (9, 124)	1057 (189, 3160) <sup>f,g,h</sup>
S811	ADAGFMKQYGECLGD	268 ± 711	399 ± 970	356 ± 775	4	90 (66, 160)	105 (66, 216)	108 (88, 232)
M101	LFARTRSMWSFNPET	86 ± 224	152 ± 367	151 ± 358	4	42 (18, 76)	48 (18, 101)	51 (32, 117)
M121	VPLRGITVTRPLMES	69 ± 338	61 ± 207	109 ± 330	4	0 (0, 11)	0 (0, 14)	2 (0, 39)
M141	AVIIRGHRLMAGHSL	17 ± 58	12 ± 32	8 ± 13	0	0 (0, 10)	0 (0, 9)	0 (0, 15)
M151	AGHSLGRCDIKDLPK	781 ± 726	951 ± 1623	1714 ± 3558	16	586 (324, 1035)	562 (296, 951)	627 (368, 1314)
M171	TSRTLSTYYKLGASQR	77 ± 372	86 ± 230	114 ± 329	4	8 (0, 27)	14 (0, 51)	18 (7, 39)
M181	GASQRVGTDSGFAAY	904 ± 1655	1470 ± 2614	2596 ± 3007 <sup>e</sup>	20	499 (296, 869)	588 (369, 1300)	1306 (706, 3237) <sup>e,h</sup>
M207	TDHAGSNDNIALLVQ	356 ± 270	339 ± 254	2867 ± 4928 <sup>e,h</sup>	60	271 (177, 438)	279 (178, 451)	1150 (612, 2224) <sup>e,h</sup>
N121	SLPYGANKEGIVWA	61 ± 375	101 ± 809	71 ± 268	2	0 (0, 0)	0 (0, 0)	0 (0, 9)
N131	IVWVATEGALNTPKD	169 ± 576	208 ± 576	194 ± 513	7	50 (26, 116)	46 (20, 104)	41 (22, 90)
N141	NTPKDHIGTRNPNNN	276 ± 397	283 ± 393	254 ± 294	2	162 (79, 290)	157 (71, 341)	136 (65, 338)
N151	NPNNNAATVLQLPQG	71 ± 288	189 ± 976	119 ± 411	7	7 (0, 47)	12 (0, 44)	12 (0, 36)
N161	QLPQGTTLPKGFYAE	525 ± 1090	962 ± 2664	6079 ± 7604 <sup>e,h</sup>	42	136 (61, 376)	201 (82, 678)	1250 (111, 12099) <sup>e,h</sup>

continued

Name of peptide	Peptide sequence	FI of HD <sup>a</sup> (mean ± SD) (n = 50)	FI of CP <sup>b</sup> (mean ± SD) (n = 230)	FI of S <sup>c</sup> (mean ± SD) (n = 45)	Percentage of positive S samples	FI of HD <sup>a</sup> median <sup>d</sup> (75, 25)	median (75, 25)	median (75, 25)
N181	SQASSRSSSRSRGNS	661 ± 2588	437 ± 1240	388 ± 794	0	111 (63, 207)	87 (44, 231)	79 (49, 245)
N221	ALLLFRNLQLESKV	181 ± 490	226 ± 505	220 ± 557	4	79 (36, 128)	80 (30, 171)	75 (40, 137)
N231	LESKVSGKQQQQGQ	314 ± 408	522 ± 1857	312 ± 430	4	150 (109, 431)	209 (100, 370)	181 (99, 362)
N241	QQQGQTVTKKSAAEA	263 ± 594	428 ± 1851	283 ± 496	4	156 (57, 262)	148 (79, 253)	100 (66, 278)
N271	VTQAFGRRGPEQTQG	329 ± 926	681 ± 2088	421 ± 731	4	141 (95, 254)	185 (107, 385)	140 (113, 288)
N281	EQTQGNFGDQLIRQ	126 ± 344	340 ± 1687	269 ± 375	9	72 (34, 110)	71 (35, 147)	118 (69, 238) <sup>e-h</sup>
N291	DLIRQGTDYKHWPQI	390 ± 865	760 ± 2302	493 ± 949	4	188 (138, 323)	243 (148, 434)	235 (170, 332)
N301	HWPQIAQFAPSASAF	176 ± 397	249 ± 946	155 ± 292	4	34 (17, 146)	40 (18, 97)	56 (37, 131) <sup>h</sup>
N321	IGMEVTPSGTWLTYH	419 ± 2038	307 ± 1782	202 ± 465	0	29 (11, 95)	27 (10, 73)	55 (32, 121) <sup>e-h</sup>
N331	WLTYHGAIKLDKDP	200 ± 330	225 ± 334	216 ± 292	4	142 (12, 228)	111 (50, 245)	113 (74, 214)

<sup>a</sup>IgG level was determined by using Luminex as described in the legend of Fig. 1. A total of 50, 230 and 45 sera were collected from <sup>a</sup>Vietnamese healthy donors (V-HD), <sup>b</sup>contact persons (CP) and <sup>c</sup>SARS patients (S), respectively, for the experiments.

<sup>d</sup>median (25, 75); the FI at 25 and 75 percentiles were shown.

The significance of the differences (*P*) in Luminex reactivities between HD and patients was analysed by means of <sup>e</sup>Student's *t*-test and <sup>f</sup>Mann-Whitney test.

<sup>g</sup>S vs HD.

<sup>h</sup>S vs CP, *P* < 0.005 (statistical analysis).

SARS, severe acute respiratory syndrome.

FI, fluorescence intensity.

Table 1

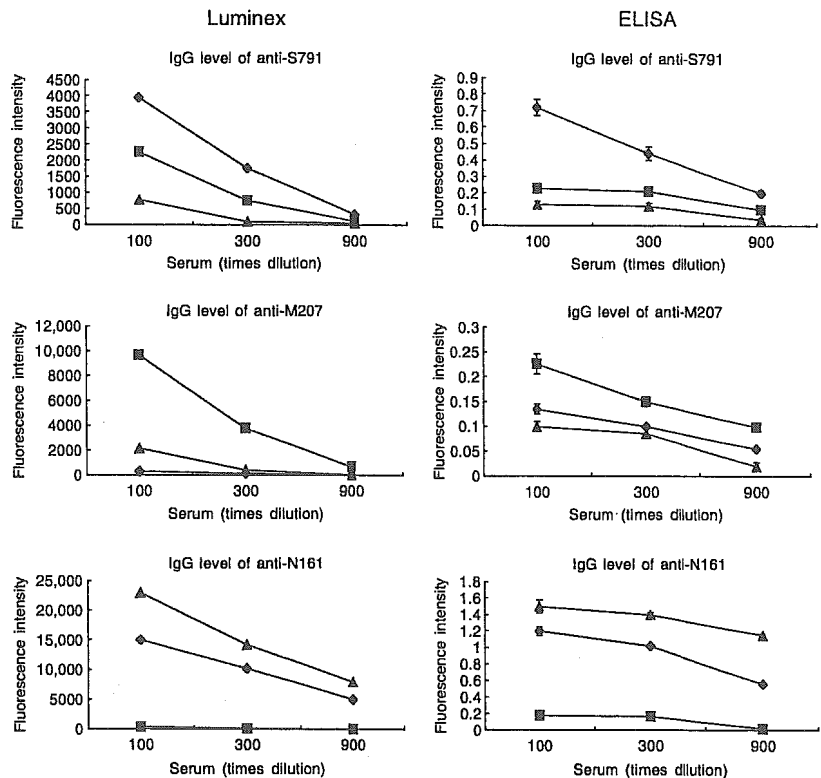
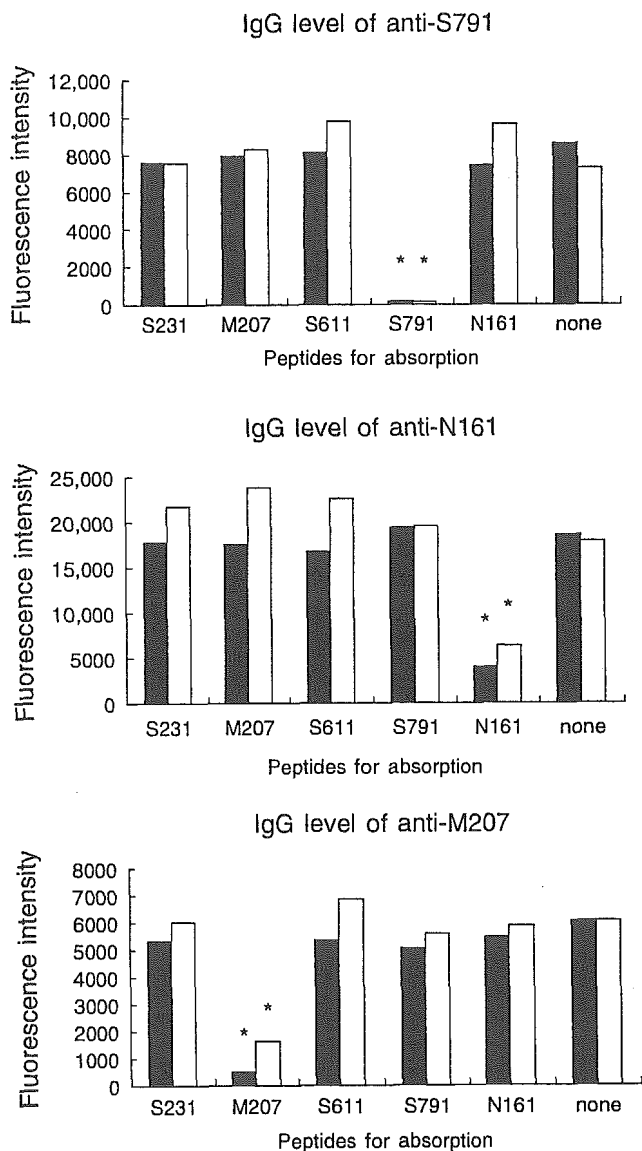


Fig. 4. Assayed by conventional ELISA methods. For the preparation of the peptide immobilized ELISA plate for the antibody absorption test, peptides were diluted in 0.1M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (DSS) (Pierce, Rockford, IL), as reported previously (17). ELISA plates were coated overnight at 4°C with the target peptides at a dose of 200 µg/well. The wells were rinsed three times with 0.05% Tween-20 PBS (PBST). The plates were blocked overnight at 4°C with Block Ace. The representative results have been shown in this figure.



**Fig. 5. Absorption test.** The immunoglobulin-G (IgG) activity to each of the S791, M207 and N161 peptides was absorbed by using a triplicate assay with an immobilized corresponding peptide and each of the five different irrelevant peptides. The method for the preparation of immobilized peptides was the same as the method used for ELISA plate preparation, as described in the legend of Fig. 4. The results of the absorption test were analysed by means of a two-tailed Student's *t*-test. All tests of significance were two-sided. In order to test the specificity of anti-peptide IgG in the serum samples, 100 µl/well of serum samples (1:100 dilution with 0.05% PBST) was absorbed with the immobilized peptide (200 µg/well: closed bar or 40 µg/well: open bar, as final concentrations) in wells kept for 2 h at room temperature. The absorption was repeated three times, and then the level of peptide-specific IgG in the resultant supernatant was measured. PBST, Tween-20 PBS.

The specificity of anti-peptide activities was then confirmed by means of the absorption test with two different concentrations of peptides (200 and 40 µg/ml) for immobilization. As expected, anti-S791, anti-M207 or anti-N161 activities were significantly reduced by absorption with the corresponding peptide, but not with any of the irrelevant peptides tested in Fig.5. The same results were obtained at the two different doses of peptides for plate immobilization, suggesting that the 200 µg/ml of peptide, a concentration usually used for immobilization, was excessive.

Kinetic studies showed that anti-nucleocapsid protein antibodies could be detected in <20%, 70–80% and >90% of probable SARS patients 1–7 days, 8–14 days and 15–61 days after the illness began, respectively (11). However, there was no information on sera obtained 6 months after the onset of the disease, at least in the literatures we read. Viral RNA may persist for some time in patients, who have humoral responses to it, whereas some patients may lack an antibody response to the SARS-CoV after the onset of illness (12, 13). Prolonged shedding of viral RNA in respiratory secretions (11 days after the onset of illness), plasma (up to 9 days) and stool specimens (25 days) has been documented (13). Serum antibody levels do not correlate with protection, although local antibody is believed to play an important role in protection (14). Rather, the upsurge of the IgG antibody to the SARS-CoV and its correlation with the progression of SARS were observed (13). Therefore, a comprehensive investigation of the timing and intensity of humoral responses and their association with clinical manifestations of the disease is needed to better understand Fig.5. the pathogenesis of the SARS-CoV and to develop appropriate treatment modalities. In order to achieve the necessary level of understanding, we will perform kinetic studies for humoral responses to the three peptides reported in this study in the near future.

Gao et al. (6) reported that an adenovirus-based vaccine could induce strong SARS-CoV-specific immune responses in the monkey, and this research holds promise for the development of a protective vaccine against the SARS causal agent. However, in order to develop this type of protective vaccine, many hurdles in terms of the safety, efficacy, cost benefits and durability must be overcome by performing basic and clinical studies. One of the key basic studies for vaccine development could be to identify immunogenic regions capable of inducing long-lasting immunity, and thus the results shown in this study may provide new information that will help us determine suitable vaccine candidates.

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## ACE1 polymorphism and progression of SARS

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### Abstract

We have hypothesized that genetic predisposition influences the progression of SARS. Angiotensin converting enzyme (ACE1) insertion/deletion (I/D) polymorphism was previously reported to show association with the adult respiratory distress syndrome, which is also thought to play a key role in damaging the lung tissues in SARS cases. This time, the polymorphism was genotyped in 44 Vietnamese SARS cases, with 103 healthy controls who had had a contact with the SARS patients and 50 controls without any contact history. SARS cases were divided into either non-hypoxemic or hypoxemic groups. Despite the small sample size, the frequency of the D allele was significantly higher in the hypoxemic group than in the non-hypoxemic group ( $p = 0.013$ ), whereas there was no significant difference between the SARS cases and controls, irrespective of a contact history. ACE1 might be one of the candidate genes that influence the progression of pneumonia in SARS.

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**Keywords:** Angiotensin converting enzyme; Severe acute respiratory syndrome; Association study; Hypoxemia; Polymorphism

The severe acute respiratory syndrome (SARS) spread worldwide as an emergent pneumonia [1]. Typical clinical features of SARS are high fever, myalgia, and other symptoms caused by systemic inflammation and the subsequent atypical pneumonia [2–4]. Approximately 40% of SARS cases developed hypoxemia [5] and further advanced cases were presented as acute respiratory distress syndrome (ARDS). Pathological analysis

of the lung at autopsy in cases of SARS revealed diffuse alveolar damage, characterized by desquamation of pneumocytes, inflammatory infiltrates, edema, and hyaline-membrane formation [6–8]. Such pathological feature of the lung in SARS cases was consistent with that in ARDS.

The early reports showed that higher age, diabetes mellitus, and heart disease are risk factors for the prognosis of SARS [9,10], whereas there has been so far little discussion on contribution of genetic factors for the development or progression of SARS [11,12]. To date, there are only a few reports showing genetic involvement

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in ARDS as well [13,14]. In the present study, we focused on the angiotensin converting enzyme (ACE) deletion (D) allele that was once reported to be associated with poor outcome in ARDS [13].

ACE is a metallopeptidase that converts angiotensin I (AT-I) to angiotensin II (AT-II), acting as a vasoconstrictor, and it also degrades bradykinin, acting as a vasodilator. ACE is a prototype of angiotensin converting enzyme, which is distinct from its human homologue ACE2 also known as a SARS receptor [15]. It is well known that ACE is rich in microvascular endothelium and is likely involved in angiopathy of the lung. The human ACE gene on chromosome 17q23 consists of 26 exons, and has insertion (I) or deletion (D) of a 287-bp Alu repeat sequence in intron 16 [16]. Although the function of this polymorphism remains unclear, the genotype or allele frequency of this polymorphism has been often reported to be associated with pathological status including the development of myocardial infarction and deterioration of the renal function in diabetes mellitus in humans [17]. In the present study, we explored the possibility of this polymorphism, based on the assumption that it might be associated with progression of SARS in Vietnamese cases.

Although Vietnam experienced the outbreak of SARS in February 2003, they successfully controlled SARS and Vietnam was removed from the list of the affected areas just nine weeks later. Consequently, in Vietnam, there were 62 probable cases of SARS; 87% was nosocomial infection in a single hospital and the community-acquired infection was only 13% [1].

## Materials and methods

**Study population.** This study was reviewed and approved by Ethics Committees in Ministry of Health of Vietnam as well as International Medical Center of Japan. Out of 62 cases fulfilling the World Health Organization case definition of probable SARS [18], five cases died, three were not Vietnamese, such that they were excluded from this study. In the remaining 54, written informed consent was obtained from 44 individuals, who were enrolled in this study as cases. Furthermore, 103 staff members who came in contact with SARS patients in the hospital A without development of SARS, and 50 individuals reflecting general Vietnamese population who had no contact history participated in this study. Peripheral blood sample of all the subjects was taken, blood cells were separated from plasma by centrifuge method, and genomic DNA was subsequently extracted by a method described elsewhere [19].

**Genotyping of ACE I/D polymorphism.** ACE I/D genotype was determined by three-primer polymerase chain reaction amplification method that was described previously [20]. Briefly, primers ACE1 and ACE3 were chosen outside the insertion sequence and another primer ACE2 was placed on the insertion sequence. Reactions were performed with 25 pmol of each primer ACE1 and ACE3, and with 7.5 pmol of primer ACE2 in a final volume of 25  $\mu$ l, containing 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.001% (W/V) of gelatin, 0.2 mM of each dNTP, and 1 U Taq polymerase (AmpliQ Gold DNA polymerase, Applied Biosystems).

Nucleotide sequences of primers were as follows: ACE1: 5'-CATCCTTTCTCCCATTTCTC-3', ACE2: 5'-TGGGATTACAGGCGTGA TACAG-3', and ACE3: 5'-ATTTAGAGCTGGAATAAAATT-3'.

After the initial denaturation at 94 °C for 10 min, the reaction mixture was subjected to 30 cycles of 94 °C for 1 min and 55 °C for 1 min. This method yields amplification products of 84 bp for the D allele and 65 bp for the I allele, and products were electrophoresed and visualized on 4% agarose gels with ethidium bromide.

**Clinical profiles and backgrounds of the subjects.** Clinical profiles and backgrounds of all subjects were extracted from medical records and questionnaires taken by trained interviewers. Information about age, sex, degree of contacts with SARS patients, and requirement of supplementary oxygen was obtained. Chest radiographs of SARS cases on the acute phase were also available. Entire lung field on the chest X-ray films was divided into six zones, which were right upper, middle, and lower zones and left upper, middle, and lower zones. The number of affected zones on the film where the lung is most severely inflamed during the clinical course was counted in each case. Two different chest physicians judged the number of involved zones in the entire lung field independently.

**Statistical analysis.** Disease associations were assessed by the  $\chi^2$  test. Inter-observer variability for the number of involved zones in the lung was assessed with the Spearman rank correlation coefficient. Logistic regression analysis was performed to identify predictors of hypoxemia or progression of SARS. The *p* values less than 0.05 were considered significant in all the tests and data analysis was carried out using the SAS system for Windows version 8.2 (SAS Institute, Cary, NC).

## Results

### Demographics

Demographic information of 44 SARS cases and 103 healthy contacts in this study is shown in Table 1. Of the 44 cases, 13 were male and 31 were female. Their mean age was 39.3 years old. Two cases had hypertension but they had neither diabetes mellitus nor heart disease. Of the 103 healthy contacts, 46 were male and 57 were female. Their mean age was 36.5 years old and this was comparable with that of SARS patients. One case had hypertension.

Individuals who faced SARS patients directly were defined as "direct contacts." Health care workers working in the hospital A during the outbreak but did not come in direct contact with SARS patient were described as "indirect contacts."

### Validation of parameters for progression of SARS

Based on the requirement of supplementary oxygen when pneumonia deteriorated, cases were separated into two groups. Oxygen was supplied to maintain a partial pressure of arterial oxygen of higher than 60 mm Hg, or arterial oxygen saturation of more than 90% at room air on the basis of Provisional guidelines issued by Ministry of Health in Vietnam. Half of the cases did not require supplementary oxygen and were defined as "non-hypoxemic" group and the other half required supplementary oxygen and constituted "hypoxemic"

Table 1  
Characteristics of SARS cases and healthy contacts

Characteristics	SARS cases (n = 44)	Contacts (n = 103)
Age (years), mean [range]	39.3 [17–76]	36.5 [15–68]
Male/female	13/31	46/57
<i>Underlying diseases</i>		
Diabetes mellitus	0	0
Hypertension	2	1
Heart disease	0	0
<i>Lung disease</i>		
COPD	3	2
Bronchial asthma	0	2
Tuberculosis	1	1
Pulmonary embolism	0	1
<i>Contact status</i>		
Direct	37	63
Indirect	7	40
<i>Occupation</i>		
Nurse	16	24
Doctor	5	13
Co-medical staff	8	22
Non-medical staff	1	44
Visitor	2	—
Inpatient	2	—
Family or maid of patient	5	—
Others	5	—

group (Table 2). Five cases of the hypoxemic group further received mechanical ventilation because they could not keep spontaneous breathing.

Serial chest X-rays were taken in all cases during the acute stage. We compared the number of involved lung zones with requirement of supplementary oxygen (Table 3). Assessment of the number of involved lung zone correlated well between two medical observers (Spearman rank correlation coefficient,  $r = 0.879$ ,  $p < 0.0001$ ). The number of involved lung zones was significantly associated with requirement of supplementary oxygen ( $\chi^2$  value = 31.5;  $df = 3$ ;  $p < 0.0001$ ). In 13 out of 22 cases with hypoxia, more than three zones of the lung were involved on chest X-rays, whereas in 14 out of 22 cases without hypoxia, only one zone was involved.

#### Genotypic and allele frequencies of ACE I/D polymorphism

Genotypic distribution and allele frequency of the ACE I/D polymorphism in SARS cases and controls

Table 2  
Characteristics of subgroups of SARS cases

Characteristics	SARS cases (n = 44)			
	Non-hypoxemic group (n = 22)	Hypoxemic group (n = 22)	Without mechanical ventilation (n = 17)	With mechanical ventilation (n = 5)
Age (year), mean	37.7	41.0	36.8	55.4
Male/female (n)	6/16	7/15	5/12	2/3
Direct/indirect contact (n)	19/3	18/4	16/1	2/3

Table 3  
The number of involved lung zones on chest radiographs in the subgroups of SARS cases

Number of involved zones	Non-hypoxemic group (n = 22)	Hypoxemic group (n = 22)
One	14	1
Two	6	6
Three	2	2
More than three	0	13

with or without contact history to SARS patients were compared (Table 4). Genotypic and allele frequencies of ACE I/D polymorphism were not different among those groups. Roughly, one-third of the alleles were D in the Vietnamese population, which were comparable with the previous data from Asians including Japanese and Chinese, but quite different from those in British population, where a half of them were D allele.

As shown in Table 5, frequency of the D allele of the hypoxemic group was significantly higher than that of the non-hypoxemic group (9 of 44 alleles versus 20 of 44 alleles;  $\chi^2$  value = 6.22,  $df = 1$ ,  $p = 0.013$ ).

#### Logistic regression analysis on progression of SARS

In a univariate logistic regression analysis, the number of ACE D allele in SARS cases was a significant risk factor for hypoxemia of SARS cases (odds ratio 3.04; 95% CI 1.15–8.02;  $p = 0.025$ ) (Table 6). Even when age, gender, and contact status were added to the ACE allele to carry out multivariate logistic regression analysis, contribution of the D allele as a risk factor for hypoxemia was still robust (Table 6). Age did not contribute to this parameter. However, the mean age of 5 hypoxemic cases that received mechanical ventilation was 55.6 and obviously higher than 36.6 of hypoxemic cases that did not require mechanical ventilation (Table 2). Comparison of parameters between survivors and non-survivors was not possible, because no DNA samples were available from non-survivors.

#### Discussion

In Vietnam, 87% of SARS cases were medical staff members, in-patients with another disease or visitors to the hospital in question [1]. Although a standard

Table 4  
Genotypic and allele frequencies of ACE I/D polymorphisms in SARS cases and controls

	SARS cases (n = 44)	Controls		References			
		With contact (n = 103)	Without contact (n = 50)	Japanese (n = 341) [21]	Chinese (n = 102) [22]	Korean (n = 135) [23]	British (n = 1903) [13]
<i>Genotype frequency</i>							
DD	6 (13.6%)	12 (11.7%)	3 (6.0%)	13.1%	16.7%	24.4%	26.1%
DI	17 (38.6%)	44 (42.7%)	25 (50.0%)	40.5%	47.0%	43.0%	49.8%
II	21 (47.8%)	47 (45.6%)	22 (44.0%)	46.3%	36.3%	32.6%	24.1%
<i>Allele frequency</i>							
D	0.33	0.33	0.31	0.33	0.4	0.46	0.51
I	0.67	0.67	0.69	0.67	0.6	0.54	0.49

Table 5  
Genotypic and allele frequencies of ACE I/D polymorphism in the subgroups of SARS cases

	SARS cases (n = 44)	
	Non-hypoxemic group (n = 22)	Hypoxemic group (n = 22)
<i>Genotype frequency</i>		
DD	1 (4.5%)	5 (22.7%)
DI	7 (31.8%)	10 (45.5%)
II	14 (63.6%)	7 (31.8%)
<i>Allele frequency</i>		
D	0.2	0.45
I	0.8	0.55

Table 6  
Logistic regression analysis on hypoxemia of SARS

Variable	Odds ratio (95% CI)	p value
<i>Univariate analysis</i>		
ACE (No. of D allele)	3.04 (1.15–8.02)	0.025
<i>Multivariate analysis</i>		
ACE (No. of D allele)	3.04 (1.12–8.25)	0.029
Age (>60 years)	2.12 (0.19–24.1)	0.546
Female	1.07 (0.25–4.52)	0.928
Direct contact	0.53 (0.09–3.12)	0.484

precaution was taken, this nosocomial infection was neither modified by isolation of patients nor protected by measures such as wearing N95 masks against the new pathogen, because the cause of this atypical pneumonia was unknown in the early phase of the outbreak [24]. All the infection spread originally from one case from Hong Kong within 2 months. It implies that natural course of nosocomial infection of SARS was observed in Vietnam. This was an unfortunate event but was a major advantage for us to assess genetic risk factors, minimizing other confounding factors. Moreover, the study population was limited to be Vietnamese ethnicity for genetic analysis. Questionnaires taken by trained interviewers and chest radiographs of all cases in this study, and clinical records of most of the cases were available to evaluate clinical status in detail. As a result, 44 out of 54 accessible cases of ethnic Vietnamese were enrolled and this achieved high coverage

of this study. Although the number of cases is not enough to reach a definite conclusion in a case-control design, we were able to extract an attractive candidate gene in this study.

Herein, 50% of the cases required supplementary oxygen. This was comparable with a report by others [5]. Oxygen was given on the basis of local guidelines, when hypoxemia was detected at room air. There was a close correlation between the number of involved zones of chest radiography and status of supplementary oxygen; therefore, we considered that progression of SARS in the lung is reasonably evaluated by the requirement of supplementary oxygen.

In the SARS cases, the frequency of D allele in hypoxemic group was higher than that of non-hypoxemic group with statistical significance, despite the small sample size. This indicates that ACE gene could be one of the candidates which influence the progression of SARS, although another gene that is located close to the gene may be responsible for the disease. The frequency of D allele of SARS cases was not different from that of the healthy contacts, and the D allele did not appear to influence development of SARS itself. There was also no difference in the frequency of D allele between healthy contacts and non-contacts. In other words, contact history did not affect the genotype distribution.

Most severe cases of SARS show diffuse alveolar damage. This pathological status is characterized by increased permeability of alveolar–capillary barrier, which consists of two separate barriers, microvascular endothelium and alveolar epithelium [25]. When these barriers are damaged, the permeability of alveolar–capillary barrier increases and protein-rich edema fluids flow into alveolar spaces from blood vessel [25]. A previous report indicated that AT-I increased vascular permeability in Rat [26]. Another report showed that AT-II induces apoptosis of human alveolar epithelial cells [27]. The degree of epithelial injury seems to influence the outcome of ARDS [25]. The serum ACE levels of individuals who have genotype DD are almost twice as high as that of genotype II [16]. It is conceivable that D allele might influence the activity of renin–angiotensin system via ele-

vation of serum or local ACE level, and then this may lead to damage of endothelium or epithelium of the lung.

In this study, neither higher age nor underlying disease was found to be risk factors for hypoxemia in SARS. One of the reasons might be that SARS cases in Vietnam were mostly nosocomial infection among relatively young medical staff members who did not have underlying diseases. This situation may be different from ones in other countries. However, as described above, the number of cases that had specific underlying diseases, which may influence the prognosis of SARS, was too few to conclude the role of underlying disease as a risk factor in our cases.

Although no case of SARS had cardiovascular disorders or diabetes mellitus in Vietnam, it is interesting that heart disease and diabetes mellitus have been reported to be risk factors in the prognosis of SARS cases in other countries where incidence rates of these life-style related diseases are rather high [9,10]. The ACE insertion/deletion polymorphism has also been reported to be a risk factor of the diseases mentioned above [17] and this might be associated with systemic angiopathy and influence progression of SARS in the lung.

The reason why we used hypoxia as a parameter of disease progression and did not use other parameters such as requirement of mechanical ventilation or outcome of death was that there were only 5 cases which required mechanical ventilation in our samples tested and no samples were obtained from the fatal cases. Autopsies were not performed in Vietnamese SARS patients for religious reasons. It would promote further studies to clarify genetic predisposition of infection, development or progression of SARS in the near future in other countries where a larger number of cases were reported.

In conclusion, we showed that ACE insertion/deletion polymorphism was statistically associated with hypoxemic status in SARS cases of Vietnam. Genetic predisposition may be one of the risk factors for the progression of SARS.

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