TABLE I. Primers Used to Identify Polymorphisms Within the *ACE2* Gene

Region	Primer name	Primer sequence (5′–3′)	Product size
5′ flanking region	ACE2-pro-1-sense	TAA TTC AGT CAG TGC TTG C	676 bp
	ACE2-pro-1-anti	AAT AGT GGA GGC ATA GAT AAA	
5′ flanking region	ACE2-pro-2-sense	TTT GTG AGC TGC TTT ATT TT	618 bp
	ACE2-pro-2-anti	TGC CAG AGT GTA TGT ATG AG	
New alternate exon	ACE2-new-sense	TTA TTG CAA TGT CAC CTG A	470 bp
	ACE2-new-anti	TTA TGA CTA CTC TCC ACT CCA	
5′ flanking region	ACE2-pro-3-sense	TTT GAA TAG GTA AGT GAA GG	669 bp
	ACE2-pro-3-anti	TAG AAC TAG GGA TCA TGA AGA	
5′ flanking region	ACE2-pro-4-sense	TGA ATT CCA TAA AGA CAA GG	653 bp
	ACE2-pro-4-anti	AAA CTT GTC CAA AAA TGT CTT	
Exon 1	ACE2-ex1-sense	ATC TTT AAC AGC TTT CTA GGA	644 bp
	ACE2-ex1-anti	AAC ATC CAA TCT CAC AAC TC	
Exon 2	ACE2-ex2-sense	AAC TCA TCT ATG TCA CAG CAC	636 bp
	ACE2-ex2-anti	AAA TTA TAT GGA CAC CTT ACC	
Exon 3	ACE2-ex3-sense	ACT TCT TTG GGT TTT GGT AG	627 bp
	ACE2-ex3-anti	ACA TCA GGT CAT AAA GTG GTT	
Exon 4	ACE2-ex4-sense	TCA TTT CAG TGG TTT ATT TTC	521 bp
	ACE2-ex4-anti	CTT TTC TTT TTC CCC AGT A	
Exon 5	ACE2-ex5-sense	CTT GTA TGG TTC TTG TGC TT	535 bp
	ACE2-ex5-anti	GGG CTG TCC TAT TAT TCT CTA	
Exon 6	ACE2-ex6-sense	ACC TGT GTT CTC CCA AGT A	568 bp
	ACE2-ex6-anti	CTT TAT CAT TTG AAT TGC AG	
Exon 7	ACE2-ex7-sense	TCA CCA AGT TAA GTA CAC GAA	562 bp
	ACE2-ex7-anti	TAC ACC TGC AAT TCA AGT TAT	
Exon 8	ACE2-ex8-1-sense	TTG CAG TGA GAA CAT TTG AAA	560 bp
	ACE2-ex8-1-anti	CCT CTG TTG TCT CCC ATT T	
Exon 8	ACE2-ex8-2-sense	GCT GTG CAG TAG ATC TCA AA	643 bp
	ACE2-ex8-2-anti	CAG ATT GTC CAC AGG TTC A	
Exon 9	ACE2-ex9-sense	CTA TGA GCA AGA GAA CAG G	577 bp
	ACE2-ex9-anti	TCA CCA GTA GTA ATT TCC AGT	
Exon 10	ACE2-ex10-sense	AGG GAG GAA ACT GAA ACT AAT	587 bp
	ACE2-ex10-anti	GGT ATC CAA ATG GAG ACT AAA	
Exon 11	ACE2-ex11-sense	GTG CAC ACC TAT AAA CCA AG	615 bp
	ACE2-ex11-anti	TGA GCA TGT TTA GGG TAG AC	
Exon 12	ACE2-ex12-sense	GTG AAA GGG CTA TTA ATC TGT	612 bp
	ACE2-ex12-anti	GAG AGG GCT GTA GTT ATG A	
Exon 13	ACE2-ex13-sense	CAG GAA CCT AGA CCA TAC AA	636 bp
	ACE2-ex13-anti	GTT GCT TTC ACT ATG TCT CA	
Exon 14	ACE2-ex14-sense	GTA CAA ATT AGG TCA TGG C	550 bp
	ACE2-ex14-anti	GAC GAG AGT CAA TTG AAA G	
Exon 15	ACE2-ex15-sense	ATT ATT GGG TTT CAT CTC G	637 bp
	ACE2-ex15-anti	TAT AGG TCA ATG AAG GCA G	
Exon 16	ACE2-ex16-sense	CAG AAC AAA TAG TGC CAA A	610 bp
	ACE2-ex16-anti	CAT AGT GGT AAC TTG CTT GAT	
Exon 17	ACE2-ex17-sense	GCT CTG TCA CCT AGG CTA G	633 bp
	ACE2-ex17-anti	CTA GGA AGA TGA ACT GCT GAT	
Exon 18	ACE2-ex18-1-sense	TTA AGA TGA ATC CTA GCA GTG	655 bp
	ACE2-ex18-1-anti	CAT TTA GAT TAT CCC TGA ACA	
Exon 18	ACE2-ex18-2-sense	TCT GGA TTT GAC TTC TGT TC	623 bp
	ACE2-ex18-2-anti	AAC ACT GTG AGC AAA TAC AAA	
Exon 18	ACE2-ex18-3-sense	GAA CAG GTA GAG GAC ATT G	531 bp
	ACE2-ex18-3-anti	GGG TAG TGA CTG TGA GAA ATA	

Subgrouping of Subjects Based on the Status of Anti-SARS-CoV Antibody

Basic characteristics and sub-grouping of subjects are shown in Table II. The 44 SARS cases, 103 contacts, and 50 non-contacts were analyzed in the present study. Based on anti-SARS-CoV antibody titer in serum, the contacts were further divided into two subgroups, antibody-positive contacts, and antibody-negative contacts (data not shown).

Identification of Polymorphisms Within *ACE2* Gene

All exons including the new exon, exon-intron boundaries and the corresponding 5′-flanking region of *ACE2* were tested

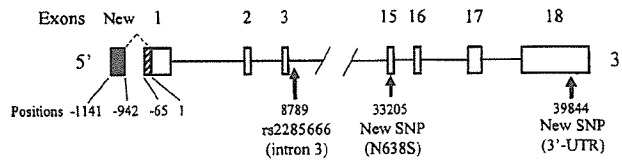

Fig. 1. A schematic diagram of the *ACE2* gene structure and the positions of SNPs. The known exons are depicted as open boxes. A solid box and a striped box indicate the new exon and the new extended region of the exon 1, respectively. The arrows represent locations of the SNPs analyzed in a case-control study. The broken line depicts an alternative-splicing site.

TABLE II. Demographic Findings of Subjects and Subgroups

Groups	SARS cases (n = 44)	Contacts (n = 103)	Anti-SARS-CoV antibody		Non-contacts (n = 50)
			Positive (n = 16)	Negative (n = 87)	
Age (years), mean [range]	39.3 [17–76]	36.5 [15–68]	36.0 [25–50]	36.6 [15–68]	—[a]
Male/female	13/31	46/57	7/9	39/48	17/33

[a]Data not available.

to identify variations of *ACE2* among SARS cases and contacts. As shown in Table III, 19 single nucleotide polymorphisms (SNPs) were identified. Six of them have already registered on dbSNP database, and 13 SNPs including one non-synonymous substitution, from asparagine to serine at 638 (N638S) in the exon 15 (position 33205) were identified. All SNPs but one in intron 3 (rs2285666, position 8789) and another in exon 18 (position 39844) were found to be considerably rare among both SARS cases and contacts tested. In subsequent analysis, we therefore chose polymorphisms, and analyzed possible non-synonymous substitution, excluding rare non-coding variants among SARS patients and contacts.

Genotype and Allele Frequency of Three SNPs

Two SNPs in intron 3 and exon 18 with minor allele frequencies higher than 0.05 and a newly identified non-synonymous SNP, N638S in exon 15 were analyzed in all samples (Table IV). Relative positions of these SNPs are shown in Figure 1. Genotyping results by direct sequencing method were confirmed by RFLP or SSCP methods. Because *ACE2* is located to the X chromosome in humans, samples from both males and females were analyzed, respectively. Two possible non-synonymous SNPs that are shown in the dbSNP database (rs4646116 and rs11798104) were not found in our samples this time. When the antibody-negative contacts group was compared with antibody-positive group including SARS cases in either males or females, no difference was observed between the two groups both in regards to genotype and allele frequencies. Comparison between antibody-positive contacts and SARS cases, and comparison between contacts and non-contacts did not show any significant differences in genotype and allele frequencies of the tested polymorphisms.

DISCUSSION

During the worldwide outbreak of SARS in 2003, a subset (about 20%–30%) of SARS patients required mechanical ventilation, having developed pneumonia. The fatality rate was 11%, although the majority of patients recovered without unfavorable outcome [Peiris et al., 2003b]. As a natural consequence, asymptomatic individuals produce antibodies against SARS-CoV in their sera [Ip et al., 2004; Woo et al., 2004]. In one of the studies, it was shown that 2.3% of contacts who did not develop clinical SARS had serum antibody titer over the threshold [Ip et al., 2004], and this implies the presence of asymptomatic individuals.

We hypothesized that the functional polymorphism of ACE2, which is considered as being a virus receptor of SARS-CoV, might influence the clinical history of SARS-CoV infection at least in part. This is because, a variation of the co-receptor to HIV, CCR5-Δ32 where allele frequency is approximately 10% in the European population [Martinson et al., 1997], has been well known to resist HIV infection and alter its clinical course [Dean et al., 1996; Liu et al., 1996; Samson et al., 1996].

TABLE III. SNPs Within the *ACE2* Gene

Region	Position[a]	dbSNP rs# cluster ID	Change of nucleotide (major/minor allele)	Change of amino acid (major/minor allele)	No. of individuals who had the minor allele	
					SARS cases	Contacts
5′ flanking region	−751	NEW[b]	C/T	—	1	1
5′ flanking region	−671	NEW	G/A	—	1	1
5′ flanking region	−634	NEW	C/G	—	1	0
Intron 3	***8789***	***rs2285666***	***A/G***	—	***15***	***32[c]***
Intron 6	13286	rs4646140	G/A	—	0	1
Intron 9	25082	NEW	G/A	—	0	1
Intron 10	25424	NEW	G/A	—	0	1
Intron 10	27418	rs4646165	G/A	—	0	1
Intron 12	28946	rs2301693	C/T	—	0	2
Intron 12	29018	rs2301692	A/G	—	0	2
Intron 14	30816	NEW	A/G	—	1	1
Intron 14	30867	rs4646174	C/G	—	0	2
Intron 14	33121	NEW	G/C	—	1	0
Exon 15	33205	NEW	A/G	N/S	0	1
Intron 16	36655	NEW	G/A	—	0	1
Intron17	38926	NEW	C/T	—	0	1
Exon 18 (3′-UTR)	39663	NEW	C/G	—	0	1
Exon 18 (3′-UTR)	39705	NEW	A/G	—	0	1
Exon 18 (3′-UTR)	***39844***	***NEW***	***G/A***	—	***3***	***4[c]***
					No. of samples tested = 20	No. of samples tested = 57

[a]Position numbers indicate distance from 5′ end of the original exon 1.
[b]Newly identified SNPs are shown as NEW.
[c]Minor allele frequencies of the SNPs shown in bold and italic were higher than 0.05.

TABLE IV. Genotype and Allele Distribution of Three Single Nucleotide Polymorphisms (SNPs)

					Contacts		
				SARS cases	Antibody (+)	Antibody (−)	Non-contacts
Intron 3 (rs2285666)							
	Male	Genotype/allele[a] no. (frequency)	A	5 (0.38)	4 (0.57)	21 (0.54)	5 (0.31)
			G	8 (0.62)	3 (0.43)	18 (0.46)	11 (0.69)
			Total no.	13	7	39	16
	Female	Genotype no. (frequency)	A/A	12 (0.39)	4 (0.44)	15 (0.31)	11 (0.33)
			A/G	16 (0.51)	3 (0.33)	24 (0.50)	17 (0.52)
			G/G	3 (0.10)	2 (0.22)	9 (0.19)	5 (0.15)
			Total no.	31	9	48	33
		Allele no. (frequency)	A	40 (0.65)	11 (0.61)	54 (0.56)	39 (0.59)
			G	22 (0.35)	7 (0.39)	42 (0.44)	27 (0.41)
Exon 15 (N638S)							
	Male	Genotype/allele no. (frequency)	A	13 (1.00)	7 (1.00)	39 (1.00)	17 (1.00)
			G	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
			Total no.	13	7	39	17
	Female	Genotype no. (frequency)	A/A	31 (1.00)	8 (0.89)	47 (0.98)	33 (1.00)
			A/G	0 (0.00)	1 (0.11)	1 (0.02)	0 (0.00)
			G/G	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
			Total no.	31	9	48	33
		Allele no. (frequency)	A	62 (1.00)	17 (0.94)	95 (0.99)	66 (1.00)
			G	0 (0.00)	1 (0.06)	1 (0.01)	0 (0.00)
Exon 18 (3′-UTR)							
	Male	Genotype/allele no. (frequency)	G	12 (0.92)	7 (1.00)	37 (0.95)	17 (1.00)
			A	1 (0.08)	0 (0.00)	2 (0.05)	0 (0.00)
			Total no.	13	7	39	17
	Female	Genotype no. (frequency)	G/G	27 (0.87)	8 (0.89)	46 (0.96)	29 (0.88)
			A/G	4 (0.13)	1 (0.11)	2 (0.04)	4 (0.12)
			A/A	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
			Total no.	31	9	48	33
		Allele no. (frequency)	G	58 (0.94)	17 (0.94)	94 (0.98)	62 (0.94)
			A	4 (0.06)	1 (0.06)	2 (0.02)	4 (0.06)

[a]Genotype distribution is the same as allele distribution in male.

Using the PCR-based cloning procedure, we identified for the first time an alternative exon upstream of the original exon 1 of *ACE2* that is expressed in various organs, including the lung and trachea, primary-cultured bronchial epithelial cells, and the small intestine. These are considered to be important replication sites of SARS-CoV [Haagmans et al., 2004]. Both 5′- and 3′-ends of the intron between the new alternative exon and the original exon 1 followed the GT/AG rule of Breathnach and Chambon [1981]. Although the organ specificity of the transcripts was not confirmed in this study due to the limitation of non-quantitative PCR amplification, implication of the new exon was definitely shown in the lung and small intestine. Also, we found the extended region of the original exon 1, 65 bp on the 5′ side. Neither the new alternative exon nor the new extended region of exon 1 gave rise to a new coding region and they were considered as 5′-untranslated region.

It was recently reported that genetic variations of ACE2 did not affect SARS susceptibility or outcome in Hong Kong [Chiu et al., 2004]. In that study, five intronic SNPs (rs2106809, rs2285666, rs4646142, rs714205, and rs2074192) were chosen and analyzed in a case-control manner, based on the previously known exon-intron structure and SNPs already registered in the database. By contrast, we attempted to analyze not only previously known SNPs but also variations newly identified among actual SARS patients and contacts. Based on the information from the exon-intron structure of *ACE2* cloned by ourselves, we searched for nucleotide sequences in all the exons including the new alternative exon and the corresponding 5′-flanking region, which are thought to contain promoters of the new exon and the original exon 1. We found one novel non-synonymous substitution N638S and 18 non-coding SNPs including two relatively common SNPs with minor allele frequency higher than 5%. We selected these SNPs and analyzed them furthermore in a case-control manner, because, while they are rare occurrence, non-synonymous substitution may directly modulate the function of the protein, and because relatively common SNPs can often be used as markers to ascertain a causative variation. Of 19 SNPs found in this study, 13 were new polymorphisms, 3 of which were located in 3′-UTR. Two possible non-synonymous SNPs in dbSNP database were not found in the population tested. Judging from the results so far obtained in this case-control study, there was no statistical evidence that ACE2 polymorphisms affect SARS infection or alter its clinical course. However, type II error was not negligible because of a relatively small size of samples tested.

Taking also into consideration, the results from a previous study of *ACE2* polymorphisms by others [Chiu et al., 2004], it is unlikely that the genetic defect of ACE2 is involved in the disease resistance that has been shown in CCR5-Δ32 in HIV-1 infection cases. Nevertheless, this newly identified alternative 5′-untranslated exon expressed in the lung, and also newly recognized polymorphisms in this study might be of great help concerning investigations into the regulation of *ACE2* gene expression and the possible significance of the variations in further more in-depth studies.

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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.

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 μl, containing 1.5 mM $MgCl_2$, 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 (AmpriTaq Gold DNA polymerase, Applied Biosystems).

Nucleotide sequences of primers were as follows: ACE1: 5′-CATC CTTTCTCCCATTTCTC-3′, ACE2: 5′-TGGGATTACAGGCGTGA TACAG-3′, and ACE3: 5′-ATTTCAGAGCTGGAATAAAATT-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 χ^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
Underlying diseases		
Diabetes mellitus	0	0
Hypertension	2	1
Heart disease	0	0
Lung disease		
COPD	3	2
Bronchial asthma	0	2
Tuberculosis	1	1
Pulmonary embolism	0	1
Contact status		
Direct	37	63
Indirect	7	40
Occupation		
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 (χ^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 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; χ^2 value $= 6.22$, df $= 1$, $p = 0.013$).

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

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 2
Characteristics of subgroups of SARS cases

Characteristics	SARS cases ($n = 44$)			
	Non-hypoxemic group	Hypoxemic group		
	($n = 22$)	($n = 22$)	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 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]
Genotype frequency							
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%
Allele frequency							
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 ($n = 22$)
Genotype frequency		
DD	1 (4.5%)	5 (22.7%)
DI	7 (31.8%)	10 (45.5%)
II	14 (63.6%)	7 (31.8%)
Allele frequency		
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
Univariate analysis		
ACE (No. of D allele)	3.04 (1.15–8.02)	0.025
Multivariate analysis		
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 of 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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Brief communication

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

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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.

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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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Tissue Antigens 2004: **64:** 600–607

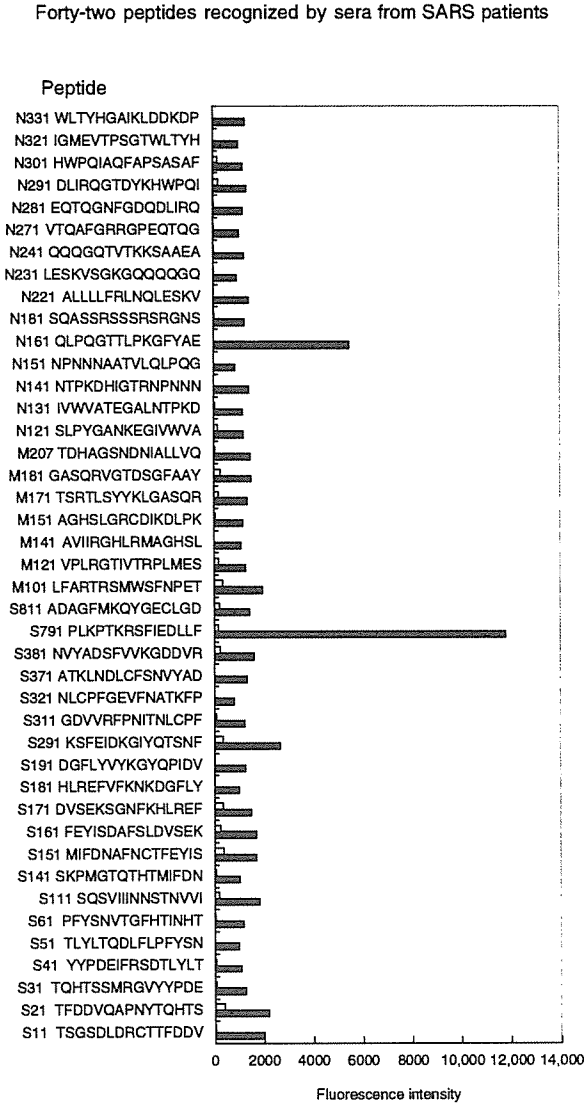

Fig. 1. **Screening of peptides.** A total of 197 different 15-mer peptides (>70%) possessing 5-amino acid overlap sequences based on the full genomic sequences of the severe acute respiratory syndrome coronavirus (SARS-CoV), including 125 spike (S), 43 nucleocapsid (N), 22 membrane (M) and seven envelope (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°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™ (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 µl of colour-coded beads were mixed with 100 µl of peptide (1 mg/ml in 0.1 M morpholinoehanesulfonic acid (MES) buffer, pH 4.5). The peptide-loaded beads were then incubated with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide 2-(N-Morpholino)ethanesulfonic acid (EDC) (1 mg ml^{-1}) 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 µl of the peptide-coupled colour-coded beads for 2 h at room temperature on a plate shaker in a 96-well filter plate (MultiScreen™-BV, Millipore Co., Bedford, MA). After incubation, the plate was washed by using a vacuum manifold apparatus and was incubated with 100 µl 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 µl 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 µl 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™ 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^{TM} (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.

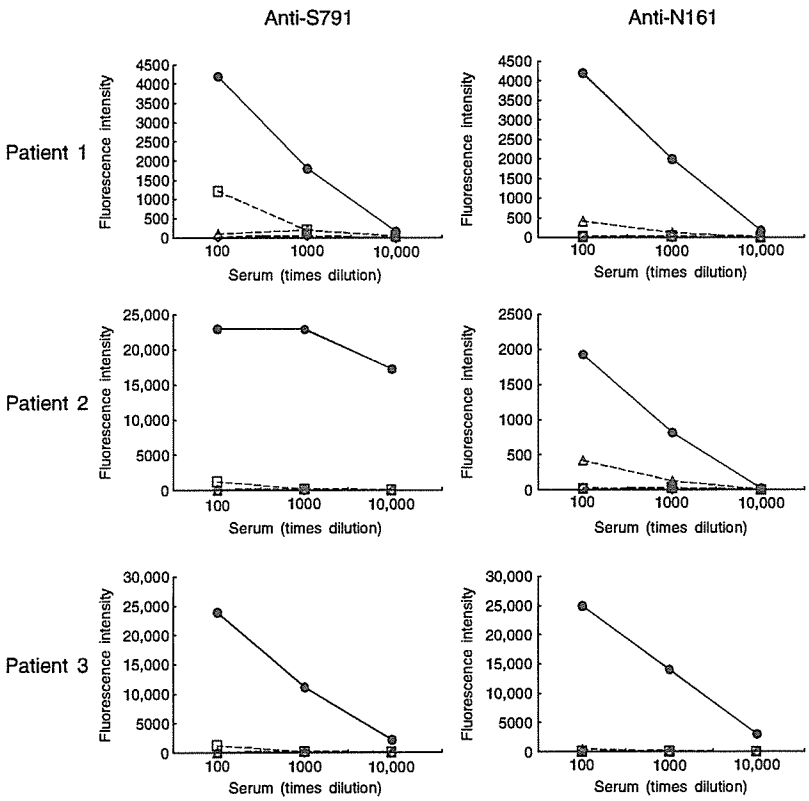

***Fig. 2.* Dose dependency.** The dose dependency of the anti-peptide activities was observed in all of the 42 peptides at three different serum dilutions (100-, 1,000- and 10,000-fold). Representative results of the dose-dependent curves of the anti-S791 and anti-N161 activities have been shown in 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.

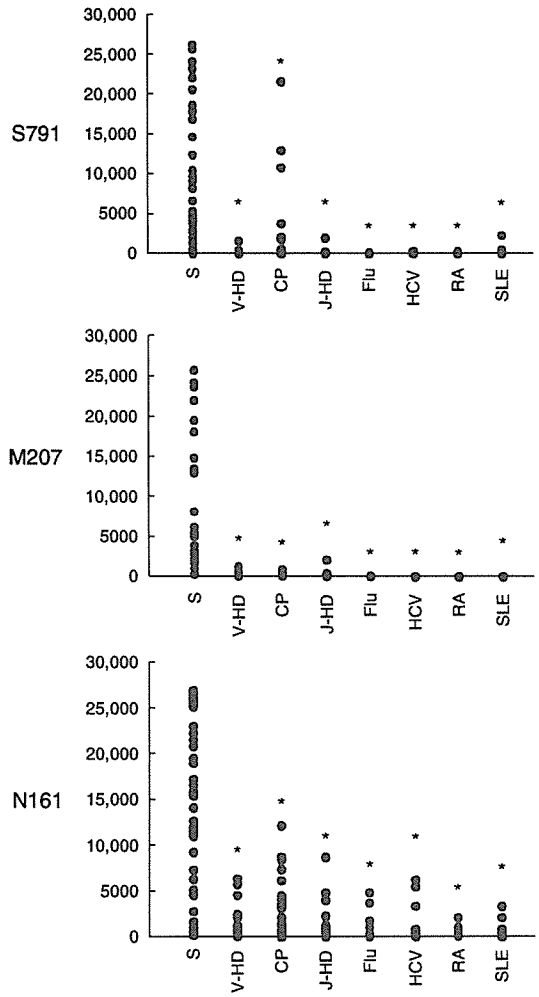

***Fig. 3.* Anti-peptide immunoglobulin-G (IgG) at post-infection.** The 42 peptides shown in Fig. 1 were tested for their reactivity to sera from post-infection (6th month) patients with the Vietnamese SARS-CoV infection ($n = 45$). As negative controls, the sera of both Vietnamese healthy donors (V-HD) ($n = 50$) 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 × 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 × 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 × 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[a] (mean ± SD) (n = 50)	FI of CP[b] (mean ± SD) (n = 230)	FI of S[c] (mean ± SD) (n = 45)	Percentage of positive S samples	FI of HD[a] median[d] (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	SKPMGTQTHTMIFDN	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	HLREFVFKNKDGFLY	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	ATKLNDLCFSNVYAD	495 ± 1056	875 ± 2035	719 ± 1405	7	231 (157, 474)	289 (189, 607)	237 (179, 521)
S381	NVYADSFVVKGDDVR	376 ± 1047	488 ± 1047	498 ± 1037	7	130 (72, 317)	158 (84, 339)	124 (83, 244)
S791	PLKPTKRSFIEDLLF	199 ± 386	489 ± 2027	3374 ± 5981[e,g,h]	51	39 (6, 214)	37 (9, 124)	1057 (189, 3160)[f,g,h]
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	VPLRGTIVTRPLMES	69 ± 338	61 ± 207	109 ± 330	4	0 (0, 11)	0 (0, 14)	2 (0, 39)
M141	AVIIRGHLRMAGHSL	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	TSRTLSYYKLGASQR	77 ± 372	86 ± 230	114 ± 329	4	8 (0, 27]	14 (0, 51)	18 (7, 39)
M181	GASQRVGTDSGFAAY	904 ± 1655	1470 ± 2614	2596 ± 3007[g]	20	499 (296, 869)	588 (369, 1300)	1306 (706, 3237)[g,h]
M207	TDHAGSNDNIALLVQ	356 ± 270	339 ± 254	2867 ± 4928[g,h]	60	271 (177, 438)	279 (178, 451)	1150 (612, 2224)[g,h]
N121	SLPYGANKEGIVWVA	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[g,h]	42	136 (61, 376)	201 (82, 678)	1250 (111, 12099)[g,h]

continued

Name of peptide	Peptide sequence	FI of HD[a] (mean ± SD) (n=50)	FI of CP[b] (mean ± SD) (n=230)	FI of S[c] (mean ± SD) (n=45)	Percentage of positive S samples	FI of HD[a] median[d] (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	ALLLLFRLNQLESKV	181 ± 490	226 ± 505	220 ± 557	4	79 (36, 128)	80 (30, 171)	75 (40, 137)
N231	LESKVSGKGQQQQGQ	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	EQTQGNFGDQDLIRQ	126 ± 344	340 ± 1687	269 ± 375	9	72 (34, 110)	71 (35, 147)	118 (69, 238)[g,h]
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)[h]
N321	IGMEVTPSGTWLTYH	419 ± 2038	307 ± 1782	202 ± 465	0	29 (11, 95)	27 (10, 73)	55 (32, 121)[g,h]
N331	WLTYHGAIKLDDKDP	200 ± 330	225 ± 334	216 ± 292	4	142 (12, 228)	111 (50, 245)	113 (74, 214)

[a]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 [a]Vietnamese healthy donors (V-HD), [b]contact persons (CP) and [c]SARS patients (S), respectively, for the experiments.
[d]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 [e]Student's t-test and [f]Mann–Whitney test.
[g]S *vs* HD.
[h]S *vs* CP, $P < 0.005$ (statistical analysis).
SARS, severe acute respiratory syndrome.
FI, fluorescence intensity.

Table 1

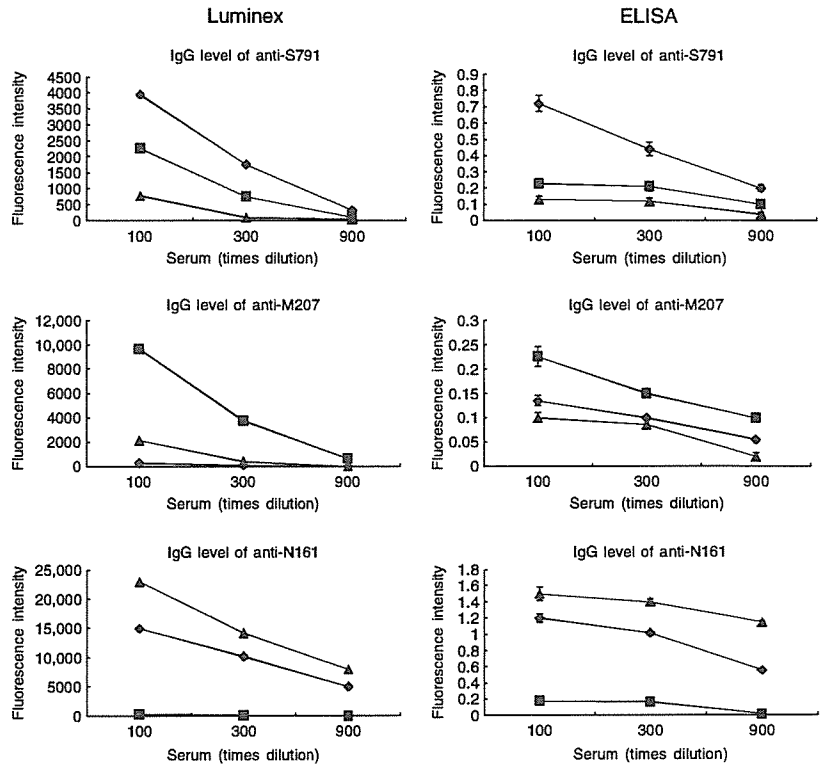

***Fig. 4.* Assayed by conventional ELISA methods.** For the preparation of the peptide immobilized ELISA plate for the antibody absorption test, peptides were diluted in 0.1 M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (DSS) (Pierce, Rockford, IL), as reported previously (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.

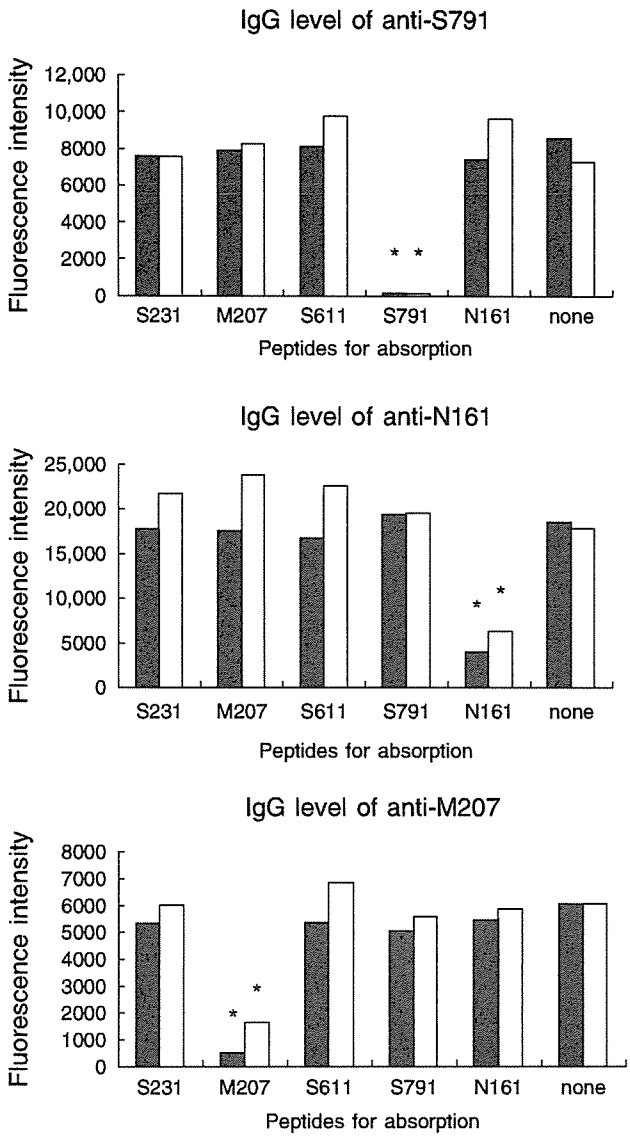

Fig. 5. **Absorption test.** The immunoglobulin-G (IgG) activity to each of the S791, M207 and N161 peptides was absorbed by using a triplicate assay with an immobilized corresponding peptide and each of the five different irrelevant peptides. The method for the preparation of immobilized peptides was 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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