Table 2. Resistance to rifampicin and mutations in the *rpoB* gene of *S. aureus* in tuberculosis wards

Hospital[a]	Specimen or reference strain	No. of isolates	Rifampicin MIC (μg/ml)	PFGE genotype[b]	*rpoB* gene		Type no. assigned
					Nucleotide changes[c]	Amino acid changes[d]	
T	Sputum	1	48	A14	TCT→CCT	Ser464→Pro	1
T	Sputum	1	>256	A2(M1)	CAA→CTA	Gln468→Leu	2
T	Sputum	1	>256	F6	GAC→AAC	Asp471→Asn[e]	3
T	Sputum	1	>256	F2	GCT→GAT, ATT→CTT	Ala477→Asp, Ile527→Leu[e]	4
T	Sputum	2	>256	F4	GCT→GAT, ATT→CTT	Ala477→Asp, Ile527→Leu[e]	5
C	Gastric juices	1	>256	M2(A18)	GCT→GAT	Ala477→Asp	6
C	Sputum	12	>256	A2(M1)	GCT→GAT	Ala477→Asp	7
C	Sputum	1	>256	AO	GCT→GAT	Ala477→Asp	8
C	Sputum	1	>256	M7	GCT→GAT	Ala477→Asp	9
T	Sputum	1	>256	G2	GCT→GAT	Ala477→Asp	10
T	Sputum	1	>256	J1	GTT→TTT, TCA→TTA	Val453→Phe[e], Ser486→Leu	11
T	Arterial blood	1	>256	J1	TCA→TTA	Ser486→Leu	12
	Sputum	9					
	Nasal cavity	1					
T	Nasal cavity	1	>256	J2	TCA→TTA	Ser486→Leu	13
T	Sputum	1	>256	J4	TCA→TTA	Ser486→Leu	14
T	Thorax drain	1	>256	R1	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	15
	Sputum	1					
T	Sputum	3	>256	J7(R2)	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	16
	Arterial blood	1					
	Urine	1					
T	Sputum	1	>256	J8	GCA→ACA, CAT→GAT	Ala473→Thr, His481→Asp	17
T	Sputum	1	>256	A1	CAT→TAT	His481→Asp	18
T	Sputum	1	>256	A2(M1)	CAT→TAT	His481→Asp	19
T	Urine	1	>256	S	CAT→GAT	His481→Asp	20
T	Sputum	1	>256	A22	CAT→TAT	His481→Asp	21
T	Sputum	1	0.015	AU1	No change	No change	22
C	Sputum	2	0.005	A3	No change	No change	23
	ATCC29213		0.005	–	No change	No change	
	N315		0.004	–	–	–	

MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis
[a]T, Tokyo hospital; C, Chubu district hospital
[b]Data from references 8–13
[c]Base changes are underlined
[d]The numbering of the amino acids is based on that of *S. aureus* N315 (GenBank accession no. NC-002745)
[e]Novel mutation

J1, and had mutation Ser486→Leu; and 5 isolates from Tokyo (type 16) were resistant to RFP (MIC, >256μg/ml), showed PFGE pattern J7(R2), and had two mutations of Ala 473→Thr and His 481→Asp, indicating that there was clonal expansion of these RFP-resistant MRSA strains in tuberculosis wards in both hospitals. Sixteen isolates of types 1–4, 6, 8–11, 13, 14, and 17–21 were resistant to PFP, but showed different genotypes (PFGE patterns and *rpoB* mutations), indicating that individual strains of RFP-resistant MRSA existed in tuberculosis patients. Collectively, these results suggest that there were two types of transmission mode of MRSA isolates: some were transmitted within tuberculosis wards and the others were brought from outside the wards.

In conclusion, MRSAs obtained from tuberculosis wards in two hospitals in Japan had resistance to RFP and mutation(s) in the particular regions of *rpoB*. It is difficult to conclude that RFP-resistant MRSA isolates were emerging in the wards during RFP therapy. Nevertheless, the present results strongly suggest an emergence of such MRSAs in tuberculosis wards in Japan. It is necessary to monitor PFP resistance in both tuberculosis and other wards.

The DNA sequences of part of the *rpoB* of MRSA reported here were registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers: AB195713, AB195714, and AB195715.

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Original Article

"Syndromic Surveillance within a Hospital" for the Early Detection of a Nosocomial Outbreak of Acute Respiratory Infection

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SUMMARY: We have performed intra-hospital syndromic surveillance to rapidly detect nosocomial acute respiratory infection outbreaks in both inpatients and health care workers in a hospital. Syndromic surveillance allows the rapid detection of sudden outbreaks, including infections caused by unknown pathogens. This approach depends on the identification of specific "symptoms" as signs of a possible outbreak, with no need for specific diagnoses. Moreover, syndromic surveillance is quick, easy, and inexpensive. Nosocomial infection surveillance is usually performed on inpatients only. However, during the outbreaks of SARS and seasonal influenza, for example, many hospital personnel were infected. In cases of this kind, in order to quickly detect the prevalence of such infections, a surveillance system that includes hospital personnel is essential. This surveillance is promising as a strategy to prepare for re-outbreaks of SARS and the emergence of novel influenza pandemics.

INTRODUCTION

Severe acute respiratory syndrome (SARS) emerged from 2002 to 2003. According to the World Health Organization (WHO), over 8,000 infected patients were reported during this period. A notable problem of SARS is the number of health care workers infected: at 1,706 persons, such cases accounted for 21% of all reported cases (1).

For the early detection of hospital outbreaks of acute respiratory infections (ARIs) that develop with a short incubation period and can spread by airway droplet transmission, such as SARS (2) and influenza (3,4), the surveillance method should be simple and rapid, and hospital personnel should be included in the surveillance.

We therefore applied the strategy of syndromic surveillance - a method based on surveillance for symptoms only, which does not depend on conclusive diagnoses or laboratory results (5). The system applied not only to inpatients but also hospital personnel.

Our objective was to assess and validate the usefulness of syndromic surveillance that includes hospital personnel to detect the outbreak of ARIs in our hospital.

MATERIALS AND METHODS

In Japan, ARIs are most prevalent in winter (from November to March), and this study was therefore performed during winter months. Basically, the first season of the period of study was from 2003 to 2004, the second season was from 2004 to 2005, and the third season was from 2005 to 2006.

Subjects showing both a fever of >38°C and respiratory symptoms were rated as having symptoms of ARIs and were included in the surveillance. Respiratory symptoms included upper airway symptoms (nasal discharge and sore throat), lower airway symptoms (cough, sputum, dyspnea, and reduced SpO2), or chest X-ray evidence of pneumonia. For each reported case with respiratory symptoms, the Infection Control Team (ICT) recommended a rapid test for influenza.

Subjects consisted of all patients hospitalized at the International Medical Center of Japan (IMCJ) hospital during the above-mentioned periods, in addition to the nurses, nursing assistants, physicians, laboratory technicians, pharmacists, administrative personnel, and students. If a case consistent meeting the definition of an ARI was identified, the head of each section immediately filled out a surveillance sheet and submitted it to an ICT. The ICT visited each ward every day, and had an interview with the head nurse, asking whether there were cases showing the target syndrome, and collected report papers. Report papers submitted on holidays were collected by the ICT on the next day, and each week after the collection, the results of the reports were documented on the hospital intranet for the hospital personnel. The IMCJ is a general hospital that is located in Tokyo. It has 925 beds and 28 health-care units. It also has 155 physicians, 585 nurses, 100 laboratory technicians and pharmacists, and 80 administrative personnel.

This study was approved by institutional review boards and the infection control committee of the IMCJ.

RESULTS

The numbers of cases that showed acute respiratory symptoms in each season are shown in Figure 1.

During the 106-day period from December 17, 2003 to March 31, 2004 (first season), 215 cases were reported. Rapid tests for influenza were performed on 109 individuals (51%), of whom 49 were rated as positive (the positivity rate was 23% of the total reported cases, and 45% of the rapid-tested cases). All positive cases were type A influenza. The surveillance period of the 1st season was comparatively short, because surveillance was performed as a provisional trial in this season. Reported cases included 168 inpatients (78%),

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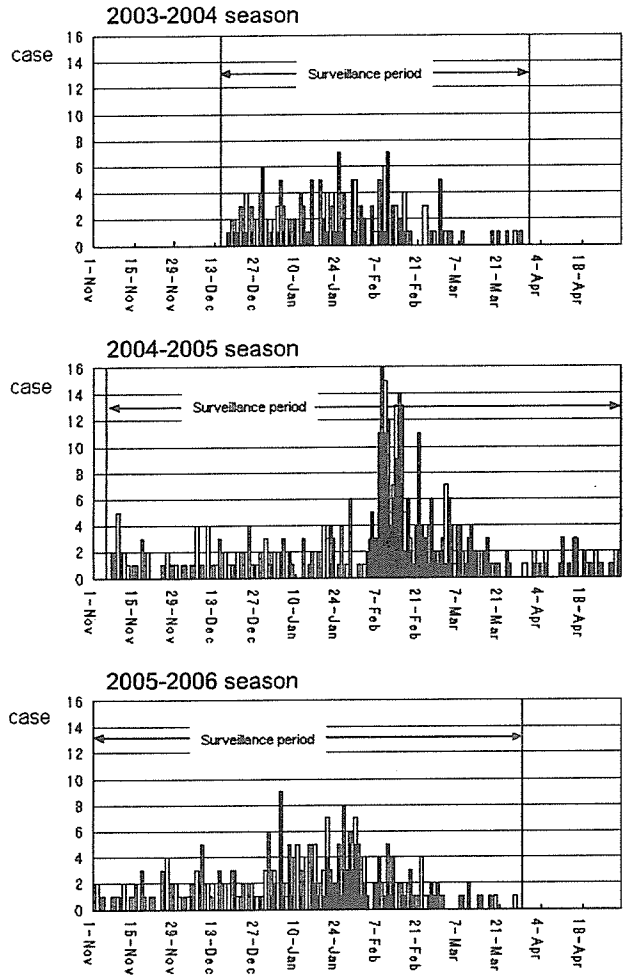

Fig. 1. Number of cases that showed acute respiratory symptoms in the hospital. Closed bar, rapid test influenza positive.

25 nurses (12%), 14 physicians (7%), and 4 technologists (2%). The outcome of this provisional trial has already been reported (6).

During the 175-day period from November 7, 2004 to April 30, 2005 (the second season), 382 cases were reported. An obvious outbreak of acute respiratory symptom cases was observed in early February. Rapid tests for influenza were performed on 261 individuals (68%), of whom 169 were rated as positive (the positivity rate was 44% of the total reported cases, and 65% of the rapid-tested cases). This peak consisted of an influenza outbreak at our hospital. Influenza was rated as type B in 130 cases (77%) and type A in the remaining cases. Reported cases included 268 inpatients (70%), 68 nurses (18%), 29 physicians (8%), 8 technologists (2%), and 8 administrative personnel (2%). Cases reported during this period were classified into inpatients and hospital personnel, and their courses are shown in Figure 2. Reports from inpatients constituted the majority of reports during the non-epidemic period, while reports from hospital personnel accounted for about 50% of reports during the influenza epidemic period.

During the 151-day period from November 1, 2005 to March 31, 2006 (the third season), 270 cases were reported. Rapid tests for influenza were performed on 204 individuals (76%), of whom 89 were rated as positive (the positivity rate was 33% of the total reported cases, and 44% of the

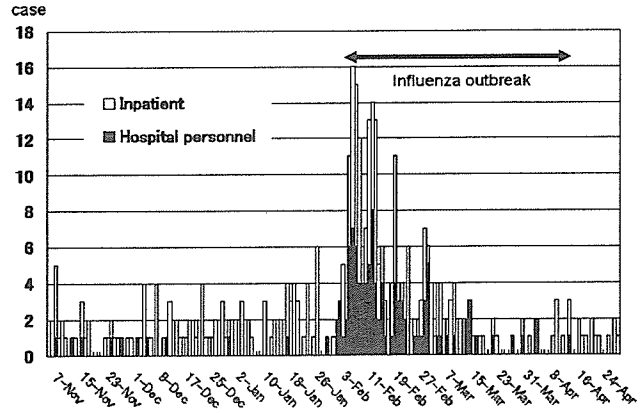

Fig. 2. Number of cases with respiratory symptoms defined by patient background. November 7, 2004 - April 30, 2005.

rapid-tested cases). Eighty-eight cases (99%) were type A influenza. Reported cases included 215 inpatients (80%), 35 nurses (13%), 8 physicians (3%), and 6 technologists (2%).

When cases showing respiratory symptoms or many cases of influenza were observed, the ICT immediately performed an intervention and took measures such as the isolation of patients, strengthening of anti-infection measures, and recommendation of rest to hospital personnel with infection.

DISCUSSION

Usual nosocomial infection surveillance can be divided into two types: "hospital-wide surveillance" and "targeted surveillance" (7). Hospital-wide surveillance involves all patients managed at a given hospital. Its advantage lies in the fact that detection of the outbreak of nosocomial infection is easier with this type of surveillance. Its disadvantages are the amount of labor needed, low efficiency, and difficulty in comparing the results with those from surveillance at other hospitals. The second type, or targeted surveillance, has the advantage of being generally effective and allowing easy comparison with the results of other hospitals. Targeted surveillance focuses on surgical-site, bloodstream, and urinary tract infection, and ventilator-associated pneumonia. However, targeted surveillance is not suitable for the early detection of diseases such as influenza and SARS, which tend to show sudden major outbreaks. Therefore, we need a much easier method of "hospital-wide surveillance" to detect these types of infection outbreaks. In the present study, we focused on the strategy of "syndromic surveillance" (5). Syndromic surveillance allows the rapid detection of sudden outbreaks, including infection caused by unknown pathogens. This approach depends on the identification of specific "symptoms" as signs of a possible outbreak, with no need for specific diagnoses. In recent years, this method has been used for the early detection of bioterrorism. Moreover, syndromic surveillance is quick, easy, and inexpensive.

The surveillance allowed the precise detection of outbreaks of influenza within our hospital during the survey periods. In particular, during the second season of the study, sudden outbreaks of influenza within our hospital were clearly documented. The peak of the outbreak in the hospital coincided with the peak of influenza prevalence in the whole of Japan reported by the National Institute of Infectious Diseases, Japan (8). However, further investigation is needed to fully elucidate the sensitivity and specificity.

Nosocomial infection surveillance is usually performed on inpatients only. However, during the outbreaks of SARS in 2003, many hospital personnel were infected, as previously mentioned (1,2). In cases of this kind, in order to quickly detect the prevalence of such infections, a surveillance system that includes hospital personnel is essential. The WHO has proposed a strategy called "SARS Alert" in preparation for a recurrence of the disease (9). This is a judgment standard stipulating that "if two or more health care workers have clinical evidence of SARS in the same health-care unit and with onset of illness in the same 10-day period, a recurrence of SARS must be suspected". In order to detect cases that are consistent with the SARS Alert, a symptomatic surveillance that includes hospital personnel must be performed. Apart from SARS, there are many other ARIs that may involve hospital personnel, such as influenza, respiratory syncytial (RS) virus, and others. Even in the case of a major outbreak of a novel influenza virus, which is widely feared to be possible in the near future, a method of syndromic surveillance that includes hospital personnel will be effective. We were able to obtain clear information about infection among hospital personnel through the surveillance. For example, a significantly large number of personnel cases were reported during the influenza season (Figure 2). Highly infectious diseases such as influenza also induce outbreaks involving hospital personnel. Hospital personnel with respiratory symptoms immediately put on masks (10), and those who were found to have influenza were instructed to undergo treatment at home. As a result of these measures, it was possible both to control nosocomial infection and, simultaneously, implement treatment among hospital personnel who had fallen ill.

Finally, the problems associated with this method require some discussion. This surveillance adopted a method by which hospital personnel who detected a symptomatic case filled in a case report form and submitted it to the ICT. With this method, the cooperation of hospital personnel is indispensable. To gain adequate cooperation of personnel, it is necessary to provide information and an explanation about the planned surveillance to hospital staff. Since this method depends on "reporting" from the place of clinical practice, there is a possibility that the number of reported cases decreases with a decrease in the sense of impending crisis in physicians and nurses, which was suggested by the definite decrease in the number of reported cases after an influenza epidemic compared with the number before the epidemic in all 3 seasons (Figure 1). Ideally, this surveillance would be performed throughout the year. However, surveillance only during high-risk periods may be more practical. In addition, a prerequisite condition is the effective functioning of the teams responsible for initiating the anti-infection measures, such as the ICT.

We have proposed syndromic surveillance as a method for the early detection of outbreaks of ARIs in hospitals. This method is simple and quick, and can be performed by any hospital. It may be applicable to developing countries as well. This surveillance method is promising as a strategy to prepare for re-outbreaks of SARS and the emergence of novel influenza pandemics.

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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/). 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_2, 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/01flue.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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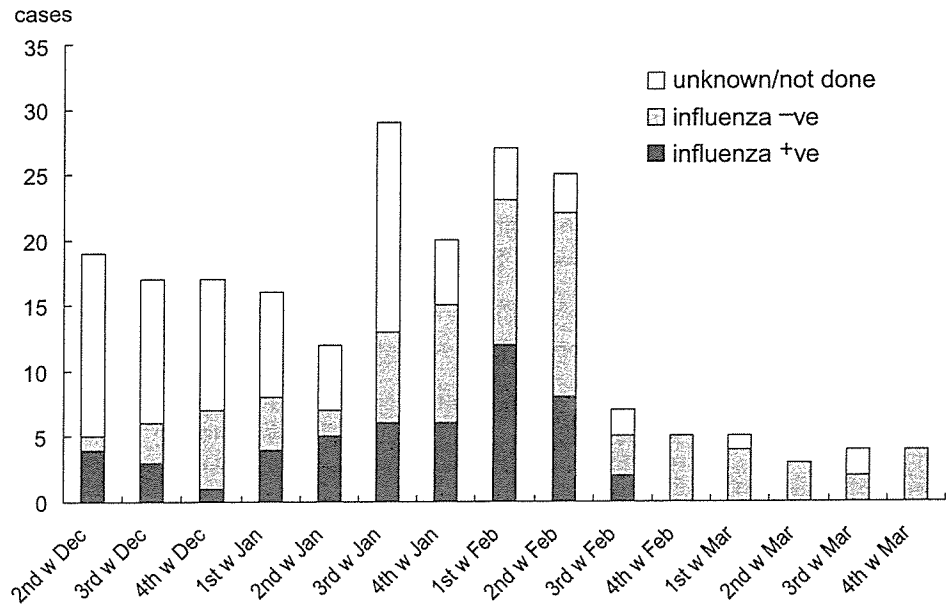

Fig. 1. Trend graph of reported case with acute respiratory symptoms between the 2nd week of December 2003 and the 4th week of March 2004.

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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Identification of an Alternative 5′-Untranslated Exon and New Polymorphisms of Angiotensin-Converting Enzyme 2 Gene: Lack of Association With SARS in the Vietnamese Population

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We analyzed genetic variations of angiotensin-converting enzyme 2 (ACE2), considering that it might influence patients' susceptibility to severe acute respiratory syndrome-associated coronavirus (SARS-CoV) or development of SARS as a functional receptor. By cloning of the full-length cDNA of the *ACE2* gene in the lung, where replication occurs on SARS-CoV, it was shown that there are different splicing sites. All exons including the new alternative exon, exon-intron boundaries, and the corresponding 5′-flanking region of the gene were investigated and 19 single nucleotide polymorphisms (SNPs) were found. Out of these, 13 SNPs including one non-synonymous substitution and three 3′-UTR polymorphisms were newly identified. A case control study involving 44 SARS cases, 16 anti-SARS-CoV antibody-positive contacts, 87 antibody-negative contacts, and 50 non-contacts in Vietnam, failed to obtain any evidence that the *ACE2* gene polymorphisms are involved in the disease process in the population. Nevertheless, identification of new 5′-untranslated exon and new SNPs is considered helpful in investigating regulation of ACE2 gene expression in the future. © 2005 Wiley-Liss, Inc.

KEY WORDS: **angiotensin-converting enzyme 2 (ACE2); severe acute respiratory syndrome (SARS); SARS associated coronavirus (SARS Co-V); virus receptor; polymorphism; association study**

INTRODUCTION

Severe acute respiratory syndrome (SARS) is an emerging infectious disease characterized by systemic inflammation followed by atypical pneumonia [Peiris et al., 2003b]. Shortly after the initial worldwide outbreak in 2003, SARS-associated coronavirus (SARS-CoV) was discovered as an etiological agent of SARS [Drosten et al., 2003; Ksiazek et al., 2003; Kuiken et al., 2003; Peiris et al., 2003a], and then angiotensin-converting enzyme 2 (ACE2) was identified as a functional receptor of this newly arrived virus [Li et al., 2003]. More recently, CD209L was reported as being another alternative receptor for the virus, but it appears to be a less efficient entry site than ACE2 [Jeffers et al., 2004].

Virus receptors generally play a key role in the entry of the pathogen into the host cells and may influence development or progression of viral diseases. For example, it is well known that genetic polymorphism of chemokine receptor 5 (CCR5), a co-receptor for human immunodeficiency virus-1 (HIV-1), influences the natural history of HIV-1 infection. The mutant allele CCR5-Δ32 does not produce a functional protein and has been shown to protect host cells against HIV-1 infection, and progression into acquired immunodeficiency syndrome is delayed after seroconversion takes place [Dean et al., 1996; Liu et al., 1996; Samson et al., 1996]. By analogy with the above, we considered that genetic polymorphisms of ACE2 could influence SARS-CoV infection or clinical manifestations of SARS.

ACE2 is a homologue of ACE1 and exhibits 40% identity of amino acid sequence to its N- and C-terminal domains [Tipnis et al., 2000]. Similar to ACE1, ACE2 is a metalloprotease that constitutes a renin-angiotensin system. Human full-length ACE2 cDNAs have been cloned already from lymphoma (GenBank accession No. AF241254) [Tipnis et al., 2000], cardiac left ventricle (AF291820) [Donoghue et al., 2000] and testis (AY623811) [Douglas et al., 2004]. Based on published data, it has been said that the ACE2 gene (*ACE2*) contains 18 exons, and spans approximately 40 kb of genomic DNA on the human X-chromosome. Although *ACE2* mRNA expressions were demonstrated in the lung by the method of quantitative reverse transcription-PCR (RT/PCR) [Harmer et al., 2002] and its protein expression was obviously shown by immunohistochemistry [Hamming et al., 2004], full-length *ACE2* cDNA has not been cloned from the lung so far. This is considered to be

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very likely as being an important replication site of SARS-CoV [Haagmans et al., 2004].

In the present study, we attempted a full-length cloning of *ACE2* cDNA from the human lung and found a new alternative, the 5′-untranslated exon. During this process, an extended region of the original exon 1 was identified in the testis' RNAs. Then, we explored genetic polymorphisms within 19 exons including new regions and the 5′-flanking region of *ACE2* and tried to determine whether the polymorphisms of *ACE2* are associated with SARS in Vietnamese.

MATERIALS AND METHODS

Cloning of ACE2 cDNA From the Lung

Cloning was performed by combination of RT/PCR and 5′- and 3′- rapid amplification of cDNA ends (RACE) procedures, using human lung total RNA (Stratagene, La Jolla, CA) and human testis total RNA (Stratagene) as a control. The total RNAs were reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT)$_{12-18}$, and then cDNA was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) with primers ACE2-exon 1s (5′-CAA AGG CTG ATA AGA GAG AA-3′) and ACE2-exon 18 as (5′-GAA CAG AAG TCA AAT CCA GA-3′) to amplify the transcript of 2721 bp encompassing the original 18 exons of *ACE2* gene on database.

The First Choice RLM-RACE Kit (Ambion, Austin, TX) was used for 5′- and 3′-RACE procedures following the manufacturer's recommendation. Gene-specific primer sets for 5′-RACE were ACE2-5′Outer1 and ACE2-5′Inner1 (5′-GTG GAT ACA TTT GGG CAA GT-3′ and 5′-CCT AGA CTA AAA CCT CCT CA-3′), and ACE2-5′Outer2 and ACE2-5′Inner2 (5′-GAA GTA AGA AAG CCT CCA CA-3′ and 5′-CTC CTG ATC CTC TGT AGC CA-3′). Gene specific primer set for 3′-RACE was ACE2-3′Outer and ACE2-3′Inner (5′-CAA TGA TGC TTT CCG TCT GA-3′ and 5′-ACA CTT GGA CCT CCT AAC CA-3′). Nucleotide sequences of PCR products were directly determined by the automated DNA sequencer (PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA).

To investigate expression of the exons on the 5′ side, RT/PCR procedures were performed on the total RNAs of human lung, testis, trachea (Stratagene), primary-cultured bronchial epithelial cells [Lechner and LaVeck, 1985], small intestine (Ambion), and on the human major organ cDNAs (Bio Chain Institute) with the sense primer New-exon (5′-TTC TTA CTT CCA CGT GAC CT-3′) or Extended-exon 1 (5′-GCT CAG CAG ATT GTT TAC TG-3′) and the antisense primer ACE2-5′Outer1.

Genomic DNA Samples for the Association Study

An association study between SARS patients and controls was reviewed and approved by local ethics committees. Of 62 cases fulfilling the World Health Organization case definition of probable SARS in Vietnam [WHO, 2003], 5 fatal cases and 3 non-Vietnamese cases were excluded from this study. In the remaining 54 cases, 44 individuals agreed to participate in this study as cases. One hundred and three Vietnamese staff members, who did not develop SARS but may have come in contact with SARS patients in the hospital where nosocomial infection of SARS had arisen, were enrolled as contacts. Furthermore, 50 medical staff members who had been working in a separate building and those considered having no history of contact with SARS patients joined in this study as non-contacts, according to information obtained by questionnaire. Peripheral blood samples of all the subjects were collected and genomic DNA was extracted from the blood cells by a method described elsewhere [Wang et al., 1994].

Testing for Antibody Response to the SARS-CoV

To detect the antibody to the SARS-CoV in serum, all the blood samples were tested with SARS ELISA (Genelabs Diagnostics Pte. Ltd., Singapore Science Park, Singapore) in accordance with the manufacturer's recommendation [Guan et al., 2004].

Identification of Polymorphisms Within *ACE2* Gene

Of the 44 SARS cases and 103 contacts recruited, a half of the samples were randomly selected for searching polymorphisms within the *ACE2* gene. PCR primers were designed to amplify 19 exons including the new alternative exon, exon-intron boundaries and approximately 1,000 bp of the 5′-flanking region of the new exon, reaching 2,000 bp upstream of the 5′-end of the original exon 1 (Table I). Genomic DNA of each sample was subjected to PCR amplification followed by direct sequencing.

Genotyping of Identified Polymorphisms

Non-synonymous nucleotide substitutions and other variations with a minor allele frequency higher than 0.05 were subjected to genotyping in all SARS cases, contacts and non-contacts. Consequently, one novel non-synonymous substitution, two possible non-synonymous polymorphisms in the database (dbSNP identification nos. rs4646116 and rs11798104), and variations of 3′-UTR in exon 18 (position 39844) and of intron 3 (rs2285666, position 8789) were genotyped by the combination of direct sequencing method and single-strand conformation polymorphism (SSCP) analysis or PCR-based restriction fragment length polymorphism (RFLP) analysis.

Statistical Analysis

Disease associations were assessed by the chi-square test. The P values less than 0.05 were considered significant in all the tests and data analysis was carried out using JMP version 5 (SAS Institute, Inc., Cary, NC).

RESULTS

Full-Length ACE2 cDNAs From the Lung and Expression of the Transcripts

By the use of the RT/PCR encompassing all known exons of *ACE2* and 3′-RACE method, we could amplify *ACE2* cDNA as PCR fragments completely corresponding to the published sequence of *ACE2* cDNA (AF241254). The 5′-RACE procedure on the total RNA of the lung demonstrated the presence of a new alternative exon (registered as AB193259), which consisted of a segment between position −1141 and −942 and was connected to the 5′-end of the original exon 1. The 5′-end of transcripts was extended to position −1141 repeatedly by both sets of gene-specific primers. In addition, novel 65 nucleotides on the 5′-side (registered as AB193260), extending the 5′-end of the original exon 1 upstream, were amplified from the total RNA of testis. A schematic diagram of the exon-intron structure is shown in Figure 1.

RT-PCR revealed that the expression of the new alternative exon could be seen not only in the lung but also in the testis, trachea, bronchial epithelial cells, small intestine, and various major organs (data not shown). The new extended region was expressed not only in the testis but also in other organs including bronchial epithelial cells and the small intestine (data not shown).

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

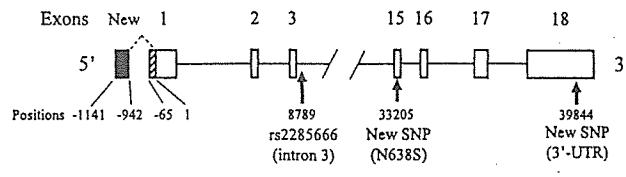

Fig. 1. A schematic diagram of the *ACE2* gene structure and the positions of SNPs. The known exons are depicted as open boxes. A solid box and a striped box indicate the new exon and the new extended region of the exon 1, respectively. The arrows represent locations of the SNPs analyzed in a case-control 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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Polymorphisms of interferon-inducible genes OAS-1 and MxA associated with SARS in the Vietnamese population

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Abstract

We hypothesized that host antiviral genes induced by type I interferons might affect the natural course of severe acute respiratory syndrome (SARS). We analyzed single nucleotide polymorphisms (SNPs) of 2′,5′-oligoadenylate synthetase 1 (OAS-1), myxovirus resistance-A (MxA), and double-stranded RNA-dependent protein kinase in 44 Vietnamese SARS patients with 103 controls. The G-allele of non-synonymous A/G SNP in exon 3 of OAS-1 gene showed association with SARS ($p = 0.0090$). The G-allele in exon 3 of OAS-1 and the one in exon 6 were in strong linkage disequilibrium and both of them were associated with SARS infection. The GG genotype and G-allele of G/T SNP at position −88 in the MxA gene promoter were found more frequently in hypoxemic group than in non-hypoxemic group of SARS ($p = 0.0195$). Our findings suggest that polymorphisms of two IFN-inducible genes OAS-1 and MxA might affect susceptibility to the disease and progression of SARS at each level.

Keywords: Severe acute respiratory syndrome; SARS associated coronavirus; Association study; Polymorphism; Oligoadenylate synthetase 1; Myxovirus resistance-A; Interferon; Vietnam

Severe acute respiratory syndrome (SARS) is a new infectious disease that emerged towards the end of 2002, spreading from China to countries in Asia, Europe, and North America. During the outbreak, a total of 8098 cases of SARS were diagnosed and the mortality rate was 9.6% [1]. Risk factors for exacerbation of the clinical progress in SARS have been reported as being patients in excess of 60 years of age, or having diabetes mellitus or other comorbid medical conditions [2,3]. However, little is known about host genetic factors associated with the development or progression of SARS, excepting human leukocyte antigens [4,5] and insertion/deletion polymorphism in the angiotensin converting enzyme 1 gene whose association with the disease [6] our research group had identified.

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It has been shown that SARS is caused by a newly identified SARS coronavirus (SARS-CoV) [7–10]. Among innate immunity against viral infection, type I interferons (IFN-α/β) induced by virus infection generally play an important role in the first line of defense, inducing intracellular antiviral proteins, such as 2′,5′-oligoadenylate synthetase 1 (OAS-1), myxovirus resistance-A (MxA), and double-stranded RNA-dependent protein kinase (PKR) [11]. Although the induction of endogenous type I IFNs in the SARS-CoV infection in vivo has not yet been clarified, recent studies have shown that administration of exogenous type I IFNs could inhibit SARS-CoV replication both in vivo [12] and in vitro [13–19]. Investigations into the role of the IFN system against SARS-CoV infection are important, not only to understand the mechanisms of viral pathogenesis but also to adopt effective therapeutic strategies against SARS.

Host genetic factors that influence antiviral effects of IFNs have been well studied in the field of viral hepatitis. Type I IFNs have been widely used as antiviral agents, mainly to treat hepatitis C virus (HCV) infection. Host genetic factors that affect the outcome of IFN treatment in chronic hepatitis C have been investigated, and a single nucleotide polymorphism (SNP) in the promoter region of IFN-inducible *MxA* gene was associated with the response to IFN treatment in the Japanese [20,21] and Caucasian populations [22]. The SNP in *MxA* gene and SNPs in *OAS-1* gene and in *PKR* gene were also shown to be associated with self-limiting infection of HCV by Knapp et al. [22]. Their report indicated that the SNPs in IFN-inducible genes were not only associated with the result of IFN treatment but also with the natural course of HCV infection.

It has been highly suspected that host genetic factors affect the course of various viral infections, including cases of SARS-CoV infection. In the present study, we have tried to determine whether the polymorphisms in IFN-inducible genes are associated with SARS-CoV infection, development, and progression of SARS. This was carried out by investigating 44 Vietnamese SARS cases, with 103 controls of individuals with a history of contact with SARS patients and 50 controls of individuals with no such contact history.

Materials and methods

Subjects. This study was reviewed and approved by ethics committees in the Ministry of Health in Vietnam as well as the International Medical Center in Japan. Written informed consent had been obtained from all subjects and detailed characteristics of the subjects had been described beforehand [6]. In short, the study population comprised 44 SARS patients in Vietnam, 103 staff members of the same hospital as control subjects, who had come into contact with SARS patients but had not developed SARS, and 50 individuals reflecting the general Vietnamese population, having had no contact history with SARS patients. Out of 44 SARS patients, 22 required oxygen therapy because of hypoxemia, with the other 22 cases, not being hypoxemic, not receiving any such oxygen therapy. There was a significant correlation between the degree of lung involvement in chest radiographs and the requirement of supplementary oxygen. Because of this finding, the progression of SARS in the lung could be reasonably determined from the status of supplementary oxygen ascertained in our previous study [6]. Peripheral blood samples were obtained in all subjects and the genomic DNAs were subsequently extracted [6]. Anti-SARS-CoV antibodies in the blood samples were tested by SARS ELISA (Genelabs Diagnostics, Singapore).

Genotyping of allelic variants of the OAS-1, MxA, and PKR genes. The SNPs analyzed in this study were all genotyped utilizing PCR and restriction fragment length polymorphism (RFLP) methods.

It was once held that *OAS-1* gene consisted of 8 exons [23]. However, according to the current database of RefSeq gene NM_016816, it comprises six exons. As a result, the A/G SNP (rs#2660) in exon 8 of *OAS-1* gene associated with outcome of HCV infection in the previous report by Knapp et al. [22] should have been located in exon 6, which falls on the 3′-untranslated region of long transcript E18 (NM_016816). To detect the SNP, genomic DNA was amplified by AmpliTaq Gold DNA polymerase (Applied Biosystems) with primers 25AS-e6F (5′-GAG GAC TGG ACC TGC ACC ATC CTC-3′) and 25AS-e6R (5′-AGA AAG TCA AGG CTG GAA TTT CAT-3′), and the PCR products of 309 bp were digested with *Mbo*II (New England Biolabs) at 37 °C for 1 h. The 309 bp product was not cut in the presence of G-allele, but was cut into fragments in the presence of A-allele. Subsequently, the fragment was separated into 188 and 121 bp units on 2% agarose gels with ethidium bromide.

We found a non-synonymous SNP in exon 3 of the *OAS-1* gene registered in the JSNP database (No. IMS-JST093062, i.e., rs#3741981). The A/G SNP in exon 3 was genotyped by PCR with primers 25AS-e3F (5′-ATC AGG AAT GGA CCT CAA GAC TTC-3′) and 25AS-e3R (5′-CGG ATG AGG CTC TTG AGC TTG GT-3′), and RFLP with *Aci*I (New England Biolabs). The PCR products of 306 bp were digested with *Aci*I and electrophoresed on 3% agarose gels to analyze undigested 306 bp band and digested parts of 159 and 147 bp bands.

The G/T SNP at position −88 in the promoter region of *MxA* gene was analyzed by PCR–RFLP methods as described previously [20]. The G/T SNP at position −88 was associated with the result of IFN treatment in chronic hepatitis C [20–22] and with the result of HCV infection [22].

The T/C SNP at position −168 in the promoter region of *PKR* gene, associated with result of HCV infection [22], was genotyped as follows. PCR was carried out with primers PKR-pF (5′-GTG GAA CCC TTG ATT CGA GAA CCT AGT-3′) and PKR-pR (5′-GCG GCT TCG GGA GAG CTG GTT CTC AGT-3′) using TaKaRa Ex *Taq* with GC buffer I (TaKaRa). The cycling condition is 45 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and digested with *Sgr*AI (New England Biolabs). Digested DNA was electrophoresed on a 5% agarose gel. The presence of T-allele was demonstrated by 169 and 155 bp fragments, and the presence of C-allele was indicated by 169, 136, and 19 bp fragments.

Statistical analysis. Possible differences deriving from the distribution of age and gender between two groups were evaluated with the unpaired t test and χ^2 test, respectively. Disease associations were assessed by the χ^2 test. p values less than 0.05 were considered significant in all the tests, and data analysis was carried out using JMP version 5 (SAS Institute). Genotype distribution of tested polymorphisms in the control population was in Hardy–Weinberg equilibrium. We calculated Lewontin's $|D'|$ and r^2 to assess the extent of pairwise linkage disequilibrium between polymorphisms [24]. These indices were calculated with the use of haplotype frequencies estimated by the PHASE algorithm (PHASE, version 2.1.1) based on Bayesian methods.

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 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).

Table 2
Genotype and allele frequencies in SARS cases and controls with contact history

	SARS cases ($n = 44$)	Controls ($n = 103$)	p value
OAS-1 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	
OAS-1 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	
MxA −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	
PKR −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	

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

OAS-1	SARS infected ($n = 60$)	Uninfected ($n = 87$)	p value	Controls without contact ($n = 50$)
Exon 6				
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
Exon 3				
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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