structure; positive ions, pH, and its own concentration affect the structure and change its antimicrobial activity.[54] It is unknown how changes in pH, ion concentration, sputum viscosity, and other factors in patients with chronic airways disease may affect the bioactivity of CAP18. We found that increases in CAP18 correlated with IL-10 and IFN-γ rather than IL-8 or TNF-α levels in CF, suggesting a possibly compensatory antiinflammatory rather than proinflammatory role. However, high concentrations of CAP18 may be cytotoxic to eukaryotic cells, and a deleterious effect certainly cannot be excluded.[54]

Sputum levels of PAI-1 also correlated negatively with pulmonary function. This suggests a relationship between PAI-1 and the degree of inflammation and tissue remodeling. PAI-1 binds uPA/uPAR, forming uPA/uPAR/PAI-1 complexes that are internalized across the cell membrane together with low-density lipoprotein receptor-related protein and degraded in the lysosome. uPAR is recycled to the cell membrane. PAI-1 thus not only controls the proteolytic activity of uPA but also modulates the number of uPAR on the cell surface.[15,55] The parallel increase of PAI-1 with uPAR suggests a finely tuned proteolytic balance is critical in the airway plasminogen activator system.[56] The dissolution and remodeling of extracellular matrix depends on a tightly controlled dynamic that maintains a proper balance between uPA/uPAR and PAI-1.[57] The increase in the PAI-1 may be homeostatic for the proproteolytic activity of increased uPAR.

In conclusion, comparison of CF to COPD and asthma as well as normal control subjects revealed interesting differences in innate immune factor levels. CAP18 is elevated in mild CF as well as COPD and inversely related to lung function, but correlation to IL-10 and IFN-γ suggests it may homeostatic rather than proinflammatory. The low levels of CAP18 in asthma are unexpected, perhaps reflecting the eosinophilic character of asthmatic inflammation or metabolic differences. Elevations of IL-8 in CF and COPD confirm prior studies, while levels of IL-10 have been variously reported to be low, normal, or elevated. TNF-α is only modestly increased in CF sputum, and IFN-γ levels are normal. CF and COPD, as noted above, share a common elevation of uPAR that is greater than that seen in asthma, while all three diseases show comparable increases in PAI-1. Neutrophils seem to be a prominent source of CAP18, IL-8, and PAI-1 in these diseases, These differing patterns allow may allow disease differentiation from a pathobiologic perspective and offer avenues for further research on pathogenetic pathways. For example, the striking differences observed in sputum CAP18 (elevated in CF and COPD, depressed in asthma) suggest further studies on the role of cathelicidins in specific forms of airway inflammation. The practical implications of such studies will likely emerge only after considerably more investigation.

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Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation

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RAPID AWARENESS AND TRANSMISSION OF SEVERE ACUTE RESPIRATORY SYNDROME IN HANOI FRENCH HOSPITAL, VIETNAM

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Abstract. A case-control study was conducted to examine the relationship between severe acute respiratory syndrome (SARS) and the time-dependent precautionary behaviors taken during an outbreak of SARS in Hanoi French Hospital (HFH), Vietnam. Masks (odds ratio [OR] = 0.3; 95% confidence interval [CI]: 0.1, 0.7) and gowns (OR = 0.2; 95% CI: 0.0, 0.8) appeared to prevent SARS transmission. The proportion of doctors and nurses who undertook each measure significantly improved (χ^2 = 9.8551, P = 0.043) after the onset of secondary cases. The impact of individual behaviors on an outbreak was investigated through mathematical approaches. The reproduction number decreased from 4.1 to 0.7 after notification. The basic reproduction number was estimated, and the use of masks alone was shown to be insufficient in containing an epidemic. Intuitive results obtained by means of stochastic individual-based simulations showed that rapid improvements in behavior and isolation would increase the probability of extinction.

INTRODUCTION

Notwithstanding the announcement of containment by the World Health Organization (WHO) in 2003,[1] severe acute respiratory syndrome (SARS) has remained a matter of concern worldwide, and it is not surprising that several cases of SARS have reemerged, for example, in China in April 2004.[2] Although the mode of transmission remains partially unclear, especially with regard to airborne transmission[3] and superspreading events,[4,5] it appears to occur predominantly by large droplets, direct contact with infectious material, or contact with fomites contaminated with infectious material.[6,7] The most effective containment measures identified to date include the tracing of contacts,[8] quarantine,[9] triage and early case detection,[10,11] and isolation.[12] Further, because the close contact required for transmission easily occurs in hospital settings,[13–15] nosocomial spread was determined as one of the major epidemiologic features of SARS.[7,16,17] The elimination of hospital transmission through enhanced infection control practices is therefore a crucial control measure.

An early study in Hong Kong showed that the practice of droplet and contact precautions was adequate in most clinical settings in significantly reducing the risk of infection after exposure to patients with SARS,[18] and if practiced by a high proportion of susceptible individuals, precautionary measures are expected to significantly reduce transmission.[19] The adoption of routine preventive behaviors based on appropriate training and control among health care workers (HCWs), undertaken prior to the isolation of SARS patients, was shown to be one of the most crucial control measures.[20–22]

In this context, Vietnam is considered to have achieved the first highly successful containment of SARS during the early phase of the outbreak.[23] One reason for this rapid containment is thought to be the prevention of infection leakage from hospitals back into the general community.[24] A second is the successful discontinuation of the chain of nosocomial transmission several days after onset based on the radical control measures of the Ministry of Health, Vietnam.[25] Although several nosocomial transmissions were observed in Hanoi French Hospital (HFH) in the early days of the outbreak,[26,27] none were identified in HFH or another local hospital in the latter phase.[28] In both hospitals, staff instituted stringent precautions, strict isolations, and quarantines under the encouragement of Dr. Carlo Urbani (Dr. Urbani died of SARS before seeing the success of the containment).[29] We therefore consider that a comprehensive understanding of the successful containment measures adopted by HFH and their theoretical underpinnings are crucial to the success of control strategies for any future recurrence. Here, we use a case-control study design to time-dependently examine the relationship between SARS and the precautionary behaviors undertaken by those exposed in HFH. We then use mathematical approaches to develop intuitive analyses of the impact of individual behaviors on the control of a SARS epidemic.

MATERIALS AND METHODS

Case-control study. HFH is a 56-bed secondary care hospital. After the admission of an index case on February 26, 2003, 38 cases in total were confirmed to have symptomatic SARS infection. The occurrence of newly diagnosed SARS cases due to local transmission continued until April 7, 2003, 3 weeks before the date when the Vietnamese government and WHO declared the outbreak successfully contained (April 28, 2003) (Table 1). The duration of the HFH outbreak was analyzed by separating it into three phases: Stage 1, February 26–March 4, from admission of the index case to the onset of secondary cases; Stage 2, March 5–March 10, from the suspicion of nosocomial spread to closure of the hospital; and Stage 3, from March 11 on, from strict isolation to local eradication.

A case-control study of 29 of the 38 laboratory-confirmed SARS cases and 98 controls was performed in HFH. The case group included 22 of 28 (78.6%) individuals admitted and retained in HFH and 7 of 10 (70.0%) individuals transferred to another hospital after first being admitted to HFH (total N = 29). The reasons for nonparticipation were death due to

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TABLE 1
Chronology of the outbreak of SARS in Hanoi French Hospital (HFH), Vietnam

Stage 1		
26-Feb-03	Day 0*	An index case complaining of fever, dry cough, and headaches was admitted to HFH.
2-Mar-03	Day 3	After intubation, the index case was isolated in ICU the following day.
4-Mar-03	Day 6	Nine secondary cases were suspected.
Stage 2		
5-Mar-03	Day 7	Seven additional cases were suspected. HFH informed the Ministry of Health, Vietnam, of the strange influenza. The health minister and experts from the World Health Organization (WHO) held a meeting. Dr. Carlo Urbani informed all staff to perform stringent precautions.
8-Mar-03	Day 10	HFH decided to close all outpatient/inpatient services. Visitors were not allowed to enter HFH. The hospital board of directors held an emergency meeting. Dr. Carlo Urbani explained the necessity of precautions and possibility of contamination as a mode of transmission. Health care workers were advised not to return home.
Stage 3		
11-Mar-03	Day 13	All inpatients were transferred to other hospitals. The 2nd floor of HFH was allocated to SARS patients only and strict isolation was enforced. ● Three zones were allocated according to symptoms. ● Nonmission individuals including health care workers were not allowed to enter.
13-Mar-03	Day 15	A special committee for SARS control and prevention was established. WHO issued a "global alert" to worldwide health authorities.
28-Apr-03	Day 60	The Vietnamese government and WHO declared successful containment of SARS in Vietnam.

* Day, days after onset of the outbreak. SARS, severe acute respiratory syndrome; ICU, intensive care unit.

SARS and/or respiratory failure ($N = 5$, 13.2%), refusal to take part ($N = 1$, 2.6%), or relocation ($N = 3$, 7.9%). The case group included 28 HFH employees (3 doctors, 13 nurses and nursing assistants, 10 radiologists and other co-medical workers, and 2 receptionist and administrative staff) and 1 relative of a patient. A further 23 Vietnamese patients who were directly admitted to another hospital were excluded because the detailed source of infection was unknown, although several cases were thought to have been infected in HFH. Detailed descriptions of the laboratory diagnoses were given previously.[28] They were confirmed through serological studies using an indirect enzyme-linked immunosorbent assay (ELISA) (Kirikae T, et al., unpublished data).

Controls were nominated based on employment in HFH and exposure among patients' relatives through HFH. The selection criteria included i) Vietnamese individuals more than 20 years old, ii) those who provided written informed consent based on explanation of our methods and purposes, and iii) those thought to have had contact with confirmed cases inside the hospital based on contact investigations. In total, 98 individuals were included as controls; most were HFH employees (13 doctors, 20 nurses and nursing assistants, 13 radiologists and other co-medical workers, and 11 receptionists and administrative staffs) or relatives of patients ($N = 41$). Although we investigated certain known contacts for inclusion as controls, namely individuals who took care of cases or entered cases' room, those who might have had trivial contact, such as possible exposure outside the hospital during, for example, transportation of SARS cases or in the casualty reception room, were not followed and included. The number of hospital employees investigated represented approximately 55.9% of the total employees used during the outbreak.

All participants were surveyed with regards to their use of personal protective equipment (PPE) and hygiene habits when in contact with patients with SARS; that is, the use of masks, gloves, and gowns, and the practice of hand washing, which were specifically recommended as droplet and contact precautions. In this paper, masks denote surgical masks; N95 masks were not available in the early stage of the outbreak in Vietnam. Individual behaviors were investigated mainly in two separate phases according to time-dependency (in Stage 1 and after entering Stage 2; i.e., Stages 2 and 3) (Table 1) to clarify any behavioral changes that occurred. Standardized questionnaires requiring one of two possible answers for each precaution ("performed" or "not performed") were given to each subject, and all responses were collected. Answers of "sometimes" or "seldom" were defined as "not performed" due to imperfect efficacy. In addition, the frequency of contact with infected individuals was investigated to represent the number of exposures per day. An exposure result of "many times" was recorded for those who had close contact with SARS patients, that is, those who cared for or lived with SARS patients, and those likely to have come into direct contact with the respiratory secretions or body fluids of SARS patients, for example, during close conversation (within 3 feet).[30] After completing the initial primary survey, an identical confirmation survey was performed to confirm the validity of the answers. These surveys were conducted along with other epidemiologic studies (Nishiyama A, et al., unpublished data) until mid-March 2004, almost 1 year after onset of the epidemic. No blood test results showing possible asymptomatic infections were available during the survey period. The participants were informed of how the information would be used and assured of the confidentiality of their responses. The purpose of the study was explained in Vietnamese, and written informed consent was obtained.

Statistical analyses were performed as follows. First, univariate associations between precautionary behaviors and infection were investigated in two separate stages (Stage 1 or Stages 2 and 3). Comparisons between groups were made using the χ^2 or Fisher's exact test for univariate analysis. Multivariate logistic regression was done in Stage 1 using forward stepwise selection (Waldesian) to determine the most significant variable associated with protection among those studied. Significant steps were taken to minimize recall bias with Stages 2 and 3 data. Analysis was restricted to those who had probable contact in these stages. It was further restricted to those cases developing symptoms whose incubation period

was within the greater than 95% confidence interval (95% CI) of having occurred after the beginning of Stage 2; and finally to medical doctors and nurses only, for both cases and controls. Second, univariate associations between sociodemographic variables (sex, age, and occupation) and SARS were investigated, with age and occupation categorized into four different groups each. Third, interactions between the identified most significant protective behavior and other variables significantly associated in univariate analysis were investigated through the use of crosstabs statistics, in which the odds of being infected were stratified according to a comparison of variables, and interactions were sought through the different odds ratio in each strata. Finally, multiple logistic regression analysis was used to determine the protective effect and eliminate confounding variables. As described in the next section, all variables significantly associated in univariate analyses, as well as sociodemographic variables, were selected and entered together in the final model. All data were entered into Microsoft Excel 2000 (Microsoft Co., Redmond, WA), and the statistical data were analyzed using the statistical software "R" (R Development Core Team, Vienna).[31]

Mathematical methods. The predictive effects of the behavioral changes were simulated using an individual-based stochastic model. For ease of understanding, a compartmental model, a type of SEIR (susceptible [S], exposed [E], infected [I], and recovered/removed [R]) model, which considered the process of transmission according to the protective behaviors taken against infectious contact among susceptible individuals, was applied. Instead of assuming "exposed (latent)" and "infectious" periods, E and I were defined as "incubation" and "symptomatic" periods, respectively, as the infectious period of SARS has not been fully clarified. Although SEIR models are usually deterministic and use mean estimations as model parameters, even with regard to SARS,[12,32] stochastic simulations were performed in this study because of the need to consider the stochasticity of each protective behavior, and also because of the small sample population size. The infectious lifetime of each individual was presented as an absorbing Markov chain. The simulations start with an individual index case (Day 0) in a population of 300 in which all individuals are susceptible.

Of the total 127 subjects studied (29 cases and 98 controls), 62.2% ($N = 79$) were considered to have had casual contact and 37.8% ($N = 48$) to have had close contact with SARS patients. The number of casual contacts (κ_1) was directly obtained ($= 0.7 \pm 0.2$ [day^{-1}]), while the mean of close contacts ($\kappa_2 = 0.4$ [day^{-1}]) was determined with the following equation:

$$\kappa_2 = \kappa_1 \ln(\mathrm{OR}_{\mathrm{closed}}) \quad (1)$$

where $\mathrm{OR}_{\mathrm{closed}}$ (= 2.5; 95% CI: 1.1–5.9) denotes the odds ratio (OR) of getting infected as a result of close contact. In other words, to quantify close contact, we assumed that the frequency of infection is mainly determined by the frequency of contact, so that the ratio of the frequency of close to casual contact becomes proportional to the logarithm of the OR of transmission. The protective effect of precautionary behavior was approximated by:

$$\beta = 1 - \mathrm{RR} = 1 - \frac{a(c+d)}{c(a+b)} \approx 1 - \frac{ad}{bc} \approx 1 - \mathrm{OR} \quad (2)$$

where RR and OR denote the relative risk and odds ratio, respectively, of becoming infected while performing a protective behavior (with precaution = with exposure). Here, a is the number of exposed ill people; b, the number of exposed healthy people; c, the number of unexposed ill people; and d, the number of unexposed healthy people. If the outcome (i.e., disease investigated) is a rare event, that is, if a and c are very small compared with b and d, respectively, $(a + b)$ and $(c + d)$, respectively, would be closely similar to b and d alone. In this case, OR would approximate RR.

The lengths of the incubation and symptomatic periods were both assumed to be independently and identically distributed random variables with a probability density function of γ distribution, the mean and variance of which were defined as 3.8 [days] and 8.3 [days^2], and 16.2 [days] and 7.9 [days^2], respectively.[24,33] These distributions were applied to difference equations (as a discrete time model) by discretizing the probability density functions by day (for a detailed description of the simulation algorithm, see the Appendix).

The first simulation scenario hypothetically investigated the unchanged coverage and mean protective effects of a behavioral measure throughout the epidemic. Primary information on protective behaviors was obtained from our Stage 1 survey. Estimates for the extent of a protective effect, the associated causative behavior of which was found in forward stepwise logistic regression to be the most significantly associated with protection (as described above), were obtained through the use of further multivariate logistic regression analysis. This analysis incorporated all variables significantly associated with SARS on univariate analysis (i.e., other precautionary behavior, gender, age and occupation). To investigate the impact of the coverage of a protective measure on the trajectory of an outbreak, sensitivity of the cumulative number of SARS cases at Day 30 to the coverage of masks was investigated in the mean field equation. In the second scenario, it was assumed that coverage improved dramatically after entering Stage 2 (Day 7) due to an awareness of transmission. Further, in Stage 3 (Day 13), the hospital implemented not only stringent precautions but also strict isolations. To understand the trajectory of transmission in detail, the number of incubating as well as symptomatic individuals was investigated. As was in fact seen during Stage 3 of the outbreak, it was also assumed that all cases who became symptomatic were immediately isolated and that nobody except a limited number of healthcare workers were permitted to have contact with them. Because the greatest uncertainty applies to the time taken to increase coverage of a protective measure and to implement strict isolations, sensitivity analyses comparing the cumulative number of SARS cases up to Day 30 were performed with the time to change both protective measures set simultaneously on the same day. Finally, the basic reproduction number was estimated using the (effective) reproduction number obtained in Stage 1 (see Appendix).

RESULTS

Table 2 shows the univariate association between the precautionary behaviors taken (SARS and non-SARS [control] cases) in Stage 1 and SARS. The use of masks ($P = 0.011$) and gowns ($P = 0.012$) appeared to prevent infection, whereas handwashing and the use of gloves were less likely to provide protection. Only two subjects who performed all pro-

TABLE 2
Precautionary measures taken by all participants in Stage 1

	SARS cases (N = 25)	Non-SARS (N = 90)	P value*	Odds ratio† (95% CI)‡
All measures	2	44	0.059	0.2 (0.0–1.0)
Handwashing before§	12	51	0.937	1.0 (0.4–2.3)
Handwashing after¶	15	56	0.766	1.1 (0.5–2.8)
Masks‖	8	35	0.011	0.3 (0.1–0.7)
Gloves	8	30	0.643	0.7 (0.3–1.9)
Gowns	2	25	0.012	0.2 (0.0–0.8)

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.
‡ 95% CI: 95% confidence interval.
§ Hands washed before having contact with a patient.
¶ Hands washed after having contact with a patient.
‖ Only those who always used a mask.

tective measures developed symptomatic infections (P = 0.059). Forward stepwise logistic regression of the five protective measures (0.05 for entry and 0.10 for removal probability) showed that only the use of masks was significant in the final model (OR, 0.29, 95% CI; 0.11-0.73, P = 0.009). In Stages 2 and 3, the use of masks (P = 0.001) and gowns (P = 0.010) was significantly associated with non-infection among doctors and nurses still not infected after Stage 1 (Table 3). Most performed all the personal protective measures recommended, and only one individual who wore masks was infected. The comparative results of the behaviors of all participants at Stage 1 and after entering Stage 2 are shown in Figure 1a. The proportions of individuals who performed the investigated protective behaviors increased after entering Stage 2. However, these behavioral changes were not significantly different between the two phases (P = 0.960). The behaviors performed by the doctors and nurses (N = 48; Figure 1b) who had the closest contact with the SARS patients drastically and significantly improved after entering Stage 2 (χ^2 = 9.855, P = 0.043).

The univariate associations between socio-demographic variables and SARS throughout the epidemic are shown in Table 4. Females were more likely to become infected than males (P = 0.011), and a significant association of SARS with nurses (P = 0.008) was observed. In HFH, infection was frequent in the 40–49 age strata (P = 0.015). Among all study subject, relatives of patients ($P < 0.001$) appeared to be the least frequently infected. Table 5 shows the interaction between the use of masks and other significantly associated variables in univariate analyses. Even though we saw no significant difference in the OR of using masks versus the use of gowns, females (OR = 0.2) and nurses (OR = 0.1) were more effectively protected by the use of masks than others in Stage 1. In Stages 2 and 3, the use of gowns showed overall reasonable OR (= 0.2), whereas most other interactions could not be calculated due to the scarcity of cases.

TABLE 3
Precautionary measures taken by health care workers in Stages 2 and 3

	SARS cases (N = 4)	Non-SARS (N = 26)	P value*	Odds ratio† (95% CI)‡
All measures	1	25	0.001	< 0.1 (0.0–0.3)
Handwashing before§	4	25	1.000	NC
Handwashing after¶	4	25	1.000	NC
Masks‖	1	25	0.001	< 0.1 (0.0–0.3)
Gloves	4	25	1.000	NC
Gowns	3	26	0.010	NC

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.
‡ 95% CI: 95% confidence interval.
§ Hands washed before having contact with a patient.
¶ Hands washed after having contact with a patient.
‖ Only those who always used a mask.

Figure 2a shows the mean and corresponding 95% CI of the trajectory (shown as prevalence) of an epidemic from 250 simulation runs which hypothetically assumed unchanged coverage as well as the protective effects of the precautionary measures observed in Stage 1. The precautionary measure in this simulation was based on a multivariate logistic regression which included all variables showing significant associations in univariate analyses, and focused on the impact of the use of masks, given the identification of this behavior as the most important protective measure (β = 0.6 obtained from OR = 0.4, P = 0.020). The coverage of masks was obtained as 52.0% from Table 2. If an outbreak was simply allowed to continue growing under these conditions, the results showed that approximately 50 to 90 symptomatic cases would occur by Day 30. The reproduction number (R) was estimated as 4.1 (95% CI; 1.9–6.4), and from this estimate the basic reproduction number was estimated as 6.0. Sensitivity of the cumulative number of cases to the coverage of masks, in the mean field, is shown in Figure 2b. Certain reduction in the cumulative number of cases was observed with significant improvements in coverage.

Figures 2c and 2d shows the outbreak trajectory of 250 simulations assuming improved coverage (from 52.0 to 81.5%) among susceptible individuals on Day 8 and restriction of contact with symptomatic individuals to health care workers on Day 13. The protective effect obtained from multivariage regression was 0.9 (OR = 0.1, P = 0.955). The reproduction number in Stage 2 was estimated as 0.7 (95% CI; 0.0–2.3). The number of incubating individuals began to show a decreasing trend after these events (Figure 2c), followed by a declining trend in the number of symptomatic cases (Figure 2d). Most of the simulated outbreaks eventually declined to extinction before Day 120. The sensitivity of the final size of an epidemic, evaluated through observations of the cumulative numbers of cases, to the timing of drastic changes in protective behaviors accompanied by strict isolation is shown in Figure 2e. When the stochastic effects are taken into account together with the effects of single precautionary measures and isolation, the rapid implementation of combined measures reduces the number of transmissions and increases the probability of extinction.

DISCUSSION

The findings of this case-control study indicate that the use of masks was significantly associated with the prevention of SARS transmission and that precautions against droplet contamination and contact were adequate in preventing transmission; this implies mainly to in-hospitals. The results are roughly consistent with those of previous reports.[18,20,22] Although a number of exceptions were seen with regard to protective effects during patient intubation, during which transmission to staff occurred even when droplet and contact precautions were taken,[7,34] one of the most important lessons from the SARS outbreak is the need to enhance infection control programs in hospitals.[13,35] Even though the use of

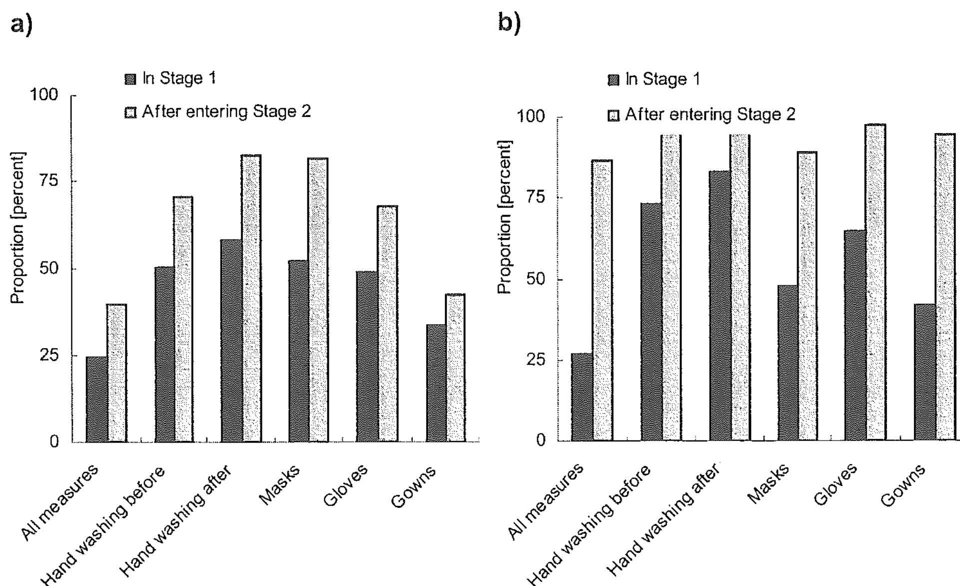

FIGURE 1. Protective behavioral changes defined by stage. **a**, Proportion of participants (SARS and non-SARS [control] cases) who performed each precautionary measure in Stage 1 ($N = 127$) and after entering Stage 2 ($N = 108$). Handwashing "before" and "after" denote before and after contact with a patient, respectively. **b**, Proportion of health care workers who performed each precautionary measure in Stage 1 ($N = 48$) and after entering Stage 2 ($N = 37$).

masks was the most effective precautionary measure, masks alone together with the observed coverage did not reduce the reproduction number below unity ($R_0 = 6.0$ and R with the protective effects of masks $= 4.1$). Put simply, the use of masks alone was shown to be insufficient to contain the epidemic. Further, it was shown that the coverage of precautionary behaviors among the study subjects increased with the progression of the outbreak, and this was especially obvious among doctors and nurses. In HFH, remarkable changes occurred in the very early phase of the outbreak before detailed information about SARS was available. According to the stochastic simulations, an increased probability of extinction would be observed if the combined measures of precaution and isolation were rapidly implemented.

With regard to sociodemographic variables, females were more frequently infected than males. Given that transmission was most frequently observed among nurses, a plausible explanation for this finding would be occupational background. Although the 40–49 age group was frequently infected, we have no persuasive explanation for this apart from occupation: 61.9% of this stratum was medical doctors or nurses. Considering that nurses were more effectively protected from transmission by the use of masks, the control measures taken by them within HFH from early in the epidemic were admirable. The lowest frequency of infection was seen in relatives of patients, showing that our study included many relatives who remained uninfected but were nevertheless believed to have had contact. Because nonmatched case-control designs such as this are vulnerable to selection bias, we obtained estimates of the protective effect of masks by means of multivariate logistic regression analysis which entered all other variables significantly associated with infection in univariate analysis. After adjustment for internal confounding variables, the estimated reproduction number was given as 0.7 in Stages 2 and 3. Previous studies have shown that the (effective) reproduction number, defined as the average number of secondary cases generated by one index case in a susceptible population under certain restrictions and interventions, decreases with increasing awareness of the epidemic combined with several public health measures.[36,37] Using reasonable estimation procedures, another study showed that R significantly decreased after a global alert in most affected countries.[38] The current study showed that the estimated R decreased below unity after notification of a hospital outbreak, although the estimates were obtained using rough assumptions and the process of estimation was biased by various factors.

TABLE 4
Univariate associations between age-class/occupational categories and SARS

	Category	N	P value*	Odds ratio (95% CI)†
Sex	Male	47	0.011	0.3 (0.1–0.8)
	Female	70	0.011	3.3 (1.2–9.0)
Age class	29 y/o	29	1.000	0.9 (0.3–2.3)
	30–39 y/o	44	0.080	0.4 (0.2–1.1)
	40–49 y/o	42	0.015	2.8 (1.2–6.6)
	50 y/o	12	0.733	0.7 (0.1–3.2)
Occupation	Medical doctors	16	1.000	0.8 (0.2–2.9)
	Nurses	33	0.008	3.2 (1.3–7.7)
	Other co-medicals	36	0.076	2.2 (0.9–5.2)
	Relatives of patients	42	< 0.001	< 0.1 (0.0–0.4)

* Two-tailed.
† Odds ratio of being infected while taking specific precautions.

In HFH, the rapid increase in awareness, which led to not only strengthened precautionary measures and isolation but also quarantining of health care workers, seems to have been the greatest contributor to successful containment. One reason for this quick response could be attributed to the background of secondary cases that arose mainly from health care workers who had close contact with the index case. Almost all staff members working or on duty in the earliest days of the

TABLE 5
Interactions between wearing masks and other variables on the infection

	In stage 1			In stages 2 and 3		
	Odds for masks (+)	Odds for masks (−)	Odds ratio*	Odds for masks (+)	Odds for masks (−)	Odds ratio*
Gowns						
(+)	0.3	0.6	0.5	< 0.1	2.0	0.2
(−)	0.3	0.5	0.6	NC	NC	NC
Sex						
(male)	0.1	0.2	1.0	0.0	0.0	NC
(female)	0.2	0.8	0.2	0.1	NC	NC
Age class						
29 y/o	0.1	0.4	0.3	0.0	NC	NC
30–39 y/o	0.1	0.3	0.5	0.0	NC	NC
40–49 y/o	0.3	0.8	0.3	0.1	1.0	0.1
50 y/o	0.2	0.2	1.0	0.0	NC	NC
Occupation						
(Medical doctors)	NC	0.6	NC	0.0	0.0	NC
(Nurses)	0.2	1.6	0.1	0.1	0.0	NC
(Other co-medicals)	0.5	0.5	1.2			
(Relatives of patients)	NC	0.1	NC			

NC = not calculable.
* Odds ratio of being infected while taking specific precautions.

outbreak (in Stage 1) were severely infected.[39,40] Another reason might be due to the efforts led mainly by Dr. Carlo Urbani, who suggested quick improvements in the precautionary measures taken and isolation.[29] As a result, transmission leakage into the community was prevented, thus having a huge impact on the chains of transmission.[24] In HFH, those who were exposed implemented precautionary and other controlling measures quickly and efficiently, and the epidemic consequently declined to extinction.

In the interests of objective interpretation, the limitations of our study design must be addressed, as follows:

1) A study such as ours in which exposure has a strong intuitive causal link with outcome (i.e., mask usage) is vulnerable to recall bias. Even though we limited our subjects in Stages 2 and 3 to medical doctors and nurses, and cases were appropriately selected according to the probable date of infection and incubation period, our estimates are likely less accurate than would be obtained by blinded or matched case-control study. In addition to this directional bias, further bias may have been introduced by random misclassification, as our records were completed 1 year after the outbreak, and it is therefore possible that some of the precautions were uncertain exposures. The frequent use of masks among controls may have reduced the strength of the associations.
2) Model-generated results must be interpreted cautiously. Although the simulations shown here included only the effect of masks and were considered according to the results of multivariate logistic regression adjusted for internal factors, unknown external confounding factors likely exist. For example, in Stages 2 and 3, although multivariate logistic regression was performed with other variables, the P value obtained was 0.955, and overall the model was weak. Owing to the scarcity of case records, stratification in this stage failed to separate the effects of masks. Thus, the estimates of the protective effect of masks and reproduction number in this stage may include the effects of other concomitant changes, such as the reduced frequency of contacts and quarantine.
3) There are limitations concerning the simplicity of our model; for example, we neglected the possible differential susceptibility of humans to asymptomatic infections,[41,42] individual variance in severity and/or prognosis,[23,43,44] and the highly heterogenous transmission of SARS.[4,5,45] Theoretical exercises never replace reality.
4) Finally, because our model was based on a case-control study, the estimates of coverage were biased; principally, coverage in a case-control design is taken from a nonrepresentative sample. Although this study was conducted as a first attempt to incorporate the effect of behavioral factors, which change time-dependently, to model building strategies for the control of directly transmitted airborne diseases, further studies incorporating a number of methodological improvements are required.

In conclusion, given that early recognition that leads to the implementation of protective behaviors and effective control strategies is crucial in hospitals,[46] we believe our model provides intuitive results that at least partly satisfy the need to evaluate outbreak trajectories based on individual behaviors.

APPENDIX

Each simulation starts with one index case and is based on a model constructed as follows:

i) The expected number of people who used protection on each subsequent day was determined by the number of susceptible individuals (S), number of contacts per day (κ), proportion of individuals who performed the protective behavior (p), and the protective effect of the precautionary measure (β), which were obtained based on our survey. The number of infectious contacts, denoted by the product of the number of susceptible individuals (S) and the mean number of contacts (κ), was divided into two subgroups: one that represents protection due to precautionary behaviors against infection with SARS-CoV (SARS-associated coronavirus) and another that does

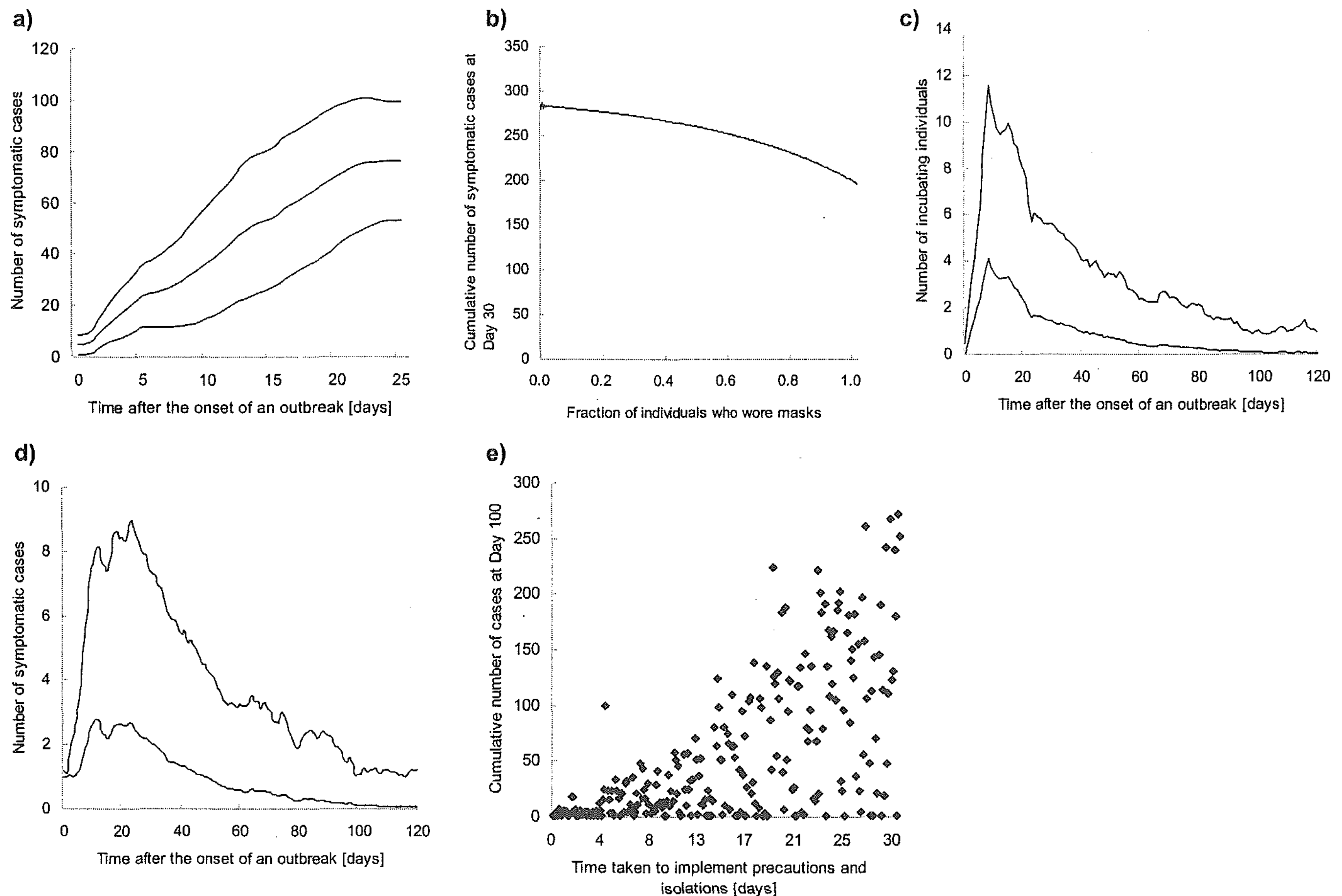

FIGURE 2. Stochastic simulations of a SARS outbreak with dependency on the coverage and protective effect of precautionary behaviors. **a**, Predicted number of symptomatic cases and corresponding 95% confidence interval (95% CI) given by 250 simulation runs assuming unchanged (stable) protective behaviors for the entire period. The reproduction number (R) was 4.1 ± 1.1. **b**, Sensitivity of the cumulative number of cases at Day 30 to the coverage of masks. The obtained line represents the simulation based on mean field (without assuming random function with binomial distribution in each transition probability). The protective effect of wearing a mask was fixed ($\beta = 0.6$). **c** and **d**, Stochastic simulations of a SARS outbreak with dependency on a combination of precautionary measures and strict isolation. **c**, The mean number of incubating individuals and corresponding 95% CI from 250 runs with changes in protective behaviors combined with strict isolation (lower 95% CI is x-axis). At Day 7, the effectiveness/coverage of precautionary measures used improved from 0.6/52.0 to 0.9/89.2, respectively. At Day 13, the number of susceptible individuals decreased from 300 to 20. The reproduction number decreased from 4.1–0.7 ± 1.1–0.8. **d**, The mean ± 95% CI of symptomatic cases given by 250 runs assuming changes in protective behaviors combined with strict isolation. The conditions were the same as those in **c**. **e**, Sensitivity of the size of an outbreak (represented by the cumulative number of cases) to the time taken to enhance precautionary measures and implement strict isolation; the combined measures are started at the same time and under the same conditions as in **c**.

not, according to $(1 - p\beta)$. However, these groups were not permanently fixed. The mean of the number of contacts based on our survey was approximated by:

$$\kappa = \kappa_1\pi_1 + \kappa_2\pi_2 = \kappa_1\pi_1 + \kappa_1 \ln(\mathrm{OR}_{\mathrm{close}})\pi_2 \quad \text{(A1)}$$

where κ_1, κ_2, π_1, and π_2 denote the number of casual and close contacts and the fraction of individuals who had casual and close contacts, respectively, while the odds ratio of getting infected with close contact is represented by $\mathrm{OR}_{\mathrm{close}}$ and N, respectively.

ii) Both the incubation (E) and symptomatic (I) periods were assumed to be independently and identically distributed following an approximated probability density function with gamma distributions[33] (denoted by γ_k and c_l for the discretized stages [days] k and l, respectively). We divided the probability density functions into k ($i = 14$) and l ($j = 12$) stages; the methodology of approximation by date was previously reported.[24] The relative measure of infectiousness for the incubation (E) period (q) was assumed to be 0.1.[12]

iii) Based on realistic settings in Vietnam, it was assumed that all individuals were isolated with the onset of early signs of clinical symptoms under the isolation measures; and for simplicity, the effect of quarantine was neglected. When considering strict isolation, the number of susceptible individuals having contact with SARS patients was limited to 20 (which is the approximate number of ward workers); the number of susceptible individuals was treated as being stable (always $S = 20$) so that S would not be exhausted thereafter; without isolation there were assumed to be 300 susceptible individuals (which is roughly the total number of people involved in possible contacts in HFH). $N = S + E + I + R$, and background mortality was neglected. The resulting simplest difference equations were formulated as follows:

$$S(t+1) = \exp\left[-\kappa(1-p\beta)\frac{I+qE}{N}\right]S(t)$$
$$E_1(t+1) = \left\{1-\exp\left[-\kappa(1-p\beta)\frac{I+qE}{N}\right]\right\}S(t)$$
$$E_k(t+1) = (1-\gamma_{k-1})E_{k-1}(t) \quad (A2)$$
$$I_1(t+1) = \sum_{k=1}^{i}\gamma_k E_k(t)$$
$$I_l(t+1) = (1-c_{l-1})I_{l-1}(t)$$
$$R(t+1) = R(t) + \sum_{l=1}^{j} c_l I_l(t)$$

Based on the forward stepwise logistic regression result in the case-control study, and to facilitate understanding, p and β were used only to represent the use of masks. However, the protective effect, β, was obtained from the result of further multiple logistic regression which entered all other significantly associated variables (in univariate analysis). All terms shown here as products of a probability and a state variable were generated in our simulations by using random variables with binomial distributions. Under these assumptions and using mean length of incubation and symptomatic periods, the reproduction number (R) is given by:

$$R = \kappa(1-p\beta)\left(\frac{q}{\gamma}+\frac{1}{c}\right) \quad (A3)$$

where γ^{-1} and c^{-1} are the means of the incubation and symptomatic periods in days, respectively. The basic reproduction number was estimated by

$$R_0 = \frac{R}{(1-p\beta)} \quad (A4)$$

For the purpose of mathematical convenience, although unrealistic, our model assumed homogenous mixing as well as all infectious individuals being equally infectious.

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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

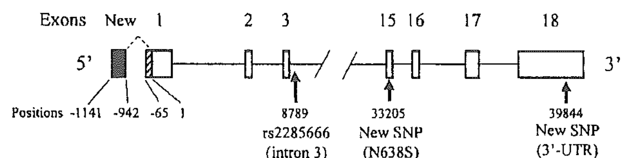

Fig. 1. A schematic diagram of the *ACE2* gene structure and the positions of SNPs. The known exons are depicted as open boxes. A solid box and a striped box indicate the new exon and the new extended region of the exon 1, respectively. The arrows represent locations of the SNPs analyzed in a case-control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

				SARS cases	Contacts: Antibody (+)	Contacts: 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.
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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.