

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

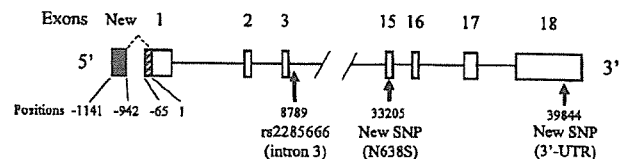


Fig. 1. A schematic diagram of the *ACE2* gene structure and the positions of SNPs. The known exons are depicted as open boxes. A solid box and a striped box indicate the new exon and the new extended region of the exon 1, respectively. The arrows represent locations of the SNPs analyzed in a case-control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

^aData 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
<i>Intron 3</i>	<i>8789</i>	<i>rs2285666</i>	<i>A/G</i>	—	<i>15</i>	<i>32^c</i>
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
<i>Exon 18 (3'-UTR)</i>	<i>39844</i>	<i>NEW</i>	<i>G/A</i>	—	<i>3</i>	<i>4^c</i>
					No. of samples tested = 20	No. of samples tested = 57

^aPosition numbers indicate distance from 5' end of the original exon 1.

^bNewly identified SNPs are shown as NEW.

^cMinor 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			Non-contacts
				SARS cases	Antibody (+)	Antibody (-)	
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)
		Allele no. (frequency)	Total no.	31	9	48	33
			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)
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)
		Allele no. (frequency)	Total no.	31	9	48	33
			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)
	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)
		Allele no. (frequency)	Total no.	31	9	48	33
			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)

^aGenotype 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.

ACKNOWLEDGMENTS

The authors thank Dr. Nguyen Le Hang, Pham Thi Phuong Thuy, and Nguyen Thi Thu Ha for their help in the management and coordination of this study in Vietnam and Dr. Shuzo

Kanagawa and Dr. Yukihiko Ishizaka in Japan. The authors also thank Kazuko Tanabe D.V.M. for her critical reading of this manuscript and Mei Murakami for her excellent technical support. This work was supported by grant for International Health Cooperation Research (14C-9) a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare in 2004.

REFERENCES

- Breathnach R, Chambon P. 1981. Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem* 50:349–383.
- Chiu RW, Tang NL, Hui DS, Chung GT, Chim SS, Chan KC, Sung YM, Chan LY, Tong YK, Lee WS, Chan PK, Lo YM. 2004. ACE2 gene polymorphisms do not affect outcome of severe acute respiratory syndrome. *Clin Chem* 50:1683–1686.
- Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, Goedert JJ, Buchbinder SP, Vittinghoff E, Gomperts E, Donfield S, Vlahov D, Kaslow R, Saah A, Rinaldo C, Detels R, O'Brien SJ. 1996. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* 273:1856–1862.
- Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. 2000. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. *Circ Res* 87:E1–E9.
- Douglas GC, O'Bryan MK, Hedger MP, Lee DK, Yarski MA, Smith AI, Lew RA. 2004. The novel angiotensin-converting enzyme (ACE) homolog, ACE2, is selectively expressed by adult Leydig cells of the testis. *Endocrinology* 145:4703–4711.
- Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW. 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 348:1967–1976.
- Guan M, Chen HY, Foo SY, Tan YJ, Goh PY, Wee SH. 2004. Recombinant protein-based enzyme-linked immunosorbent assay and immunochromatographic tests for detection of immunoglobulin G antibodies to severe acute respiratory syndrome (SARS) coronavirus in SARS patients. *Clin Diagn Lab Immunol* 11:287–291.
- Haagmans BL, Kuiken T, Martina BE, Fouchier RA, Rimmelzwaan GF, van Amerongen G, van Riel D, de Jong T, Itamura S, Chan KH, Tashiro M, Osterhaus AD. 2004. Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques. *Nat Med* 10:290–293.
- Hamming I, Timens W, Bulthuis ML, Lely AT, Navis GJ, van Goor H. 2004. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol* 203:631–637.
- Harmer D, Gilbert M, Borman R, Clark KL. 2002. Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. *FEBS Lett* 532:107–110.
- Ip M, Chan PK, Lee N, Wu A, Ng TK, Chan L, Ng A, Kwan HM, Tsang L, Chu I, Cheung JL, Sung JJ, Tam JS. 2004. Seroprevalence of antibody to severe acute respiratory syndrome (SARS)-associated coronavirus among health care workers in SARS and non-SARS medical wards. *Clin Infect Dis* 38:e116–118.
- Jeffers SA, Tusell SM, Gillim-Ross L, Hemmila EM, Achenbach JE, Babcock GJ, Thomas WD Jr, Thackray LB, Young MD, Mason RJ, Ambrosino DM, Wentworth DE, Demartini JC, Holmes KV. 2004. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci USA* 101:15748–15753.
- Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ, SARS Working Group. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 348:1953–1966.
- Kuiken T, Fouchier RA, Schutten M, Rimmelzwaan GF, van Amerongen G, van Riel D, Laman JD, de Jong T, van Doornum G, Lim W, Ling AE, Chan PK, Tam JS, Zambon MC, Gopal R, Drosten C, van der Werf S, Escriou N, Manuguerra JC, Stohr K, Peiris JS, Osterhaus AD. 2003. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* 362:263–270.
- Lechner JF, LaVeck MA. 1985. A serum-free method for culturing normal human bronchial epithelial cells at clonal density. *J Tissue Cult Methods* 9:43–48.
- Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426:450–454.
- Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, MacDonald ME, Stuhlmann H, Kouy RA, Landau NR. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 86:367–377.
- Martinson JJ, Chapman NH, Rees DC, Liu YT, Clegg JB. 1997. Global distribution of the CCR5 gene 32-basepair deletion. *Nat Genet* 16:100–103.
- Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, Nicholls J, Yee WK, Yan WW, Cheung MT, Cheng VC, Chan KH, Tsang DN, Yung RW, Ng TK, Yuen KY, SARS study group. 2003a. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 361:1319–1325.
- Peiris JS, Yuen KY, Osterhaus AD, Stohr K. 2003b. The severe acute respiratory syndrome. *N Engl J Med* 349:2431–2441.
- Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 382:722–725.
- Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. 2000. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* 275:33238–33243.
- Wang L, Hirayasu K, Ishizawa M, Kobayashi Y. 1994. Purification of genomic DNA from human whole blood by isopropanol-fractionation with concentrated NaI and SDS. *Nucleic Acids Res* 22:1774–1775.
- WHO. 2003. Global surveillance for severe acute respiratory syndrome (SARS). *Wkly Epidemiol Rec* 78:100–119.
- Woo PC, Lau SK, Tsui HW, Chan KH, Wong BH, Che XY, Tam VK, Tam SC, Cheng VC, Hung IF, Wong SS, Zheng BJ, Guan Y, Yuen KY. 2004. Relative rates of non-pneumonic SARS coronavirus infection and SARS coronavirus pneumonia. *Lancet* 363:841–845.



Polymorphisms of interferon-inducible genes OAS-1 and MxA associated with SARS in the Vietnamese population

Emi Hamano ^a, Minako Hijikata ^a, Satoru Itoyama ^a, Tran Quy ^b, Nguyen Chi Phi ^b,
Hoang Thuy Long ^c, Le Dang Ha ^d, Vo Van Ban ^e, Ikumi Matsushita ^a,
Hideki Yanai ^f, Fumiko Kirikae ^g, Teruo Kirikae ^g, Tadatoshi Kuratsuji ^h,
Takehiko Sasazuki ⁱ, Naoto Keicho ^{a,*}

^a Department of Respiratory Diseases, Research Institute, International Medical Center of Japan, Japan

^b Bach Mai Hospital, Viet Nam

^c National Institute of Hygiene and Epidemiology, Viet Nam

^d Institute for Clinical Research in Tropical Medicine, Viet Nam

^e Hanoi-French Hospital, Viet Nam

^f The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Japan

^g Department of Infectious Diseases, Research Institute, International Medical Center of Japan, Japan

^h Research Institute, International Medical Center of Japan, Japan

ⁱ International Medical Center of Japan, Japan

Received 13 February 2005

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.

© 2005 Elsevier Inc. All rights reserved.

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.

* Corresponding author. Fax: +81 3 3207 1038.

E-mail address: nkeicho-1ky@umin.ac.jp (N. Keicho).

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

Table 2

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

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

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

Discussion

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

Table 1
Characteristics of SARS cases and healthy contacts

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

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

<i>OAS-1</i>	SARS infected (<i>n</i> = 60)	Uninfected (<i>n</i> = 87)	<i>p</i> value	Controls without contact (<i>n</i> = 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 (<i>n</i> = 44)		<i>p</i> value
	Non-hypoxemic group (<i>n</i> = 22)	Hypoxemic group (<i>n</i> = 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.

Acknowledgments

The authors thank Dr. Nguyen Le Hang, Ms. Pham Thi Phuong Thuy, and Ms. Nguyen Thi Thu Ha for their help in the management and coordination of this study in Vietnam. The authors also thank Kazuko Tanabe D.V.M. and Mr. John Crosskey for their critical reading of the manuscript and Dr. Goh Tanaka for his help in statistical analysis. This work was supported by a grant for International Health Cooperation Research (14C-9) and for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare in 2004.

References

- [1] World Health Organization (2003), Consensus document on the epidemiology of severe acute respiratory syndrome (SARS), WHO/CDS/CSR/GAR/2003.11, Geneva.
- [2] C.M. Booth, L.M. Matukas, G.A. Tomlinson, A.R. Rachlis, D.B. Rose, H.A. Dwosh, S.L. Walmsley, T. Mazzulli, M. Avendano, P. Derkach, I.E. Ephtimos, I. Kitai, B.D. Mederski, S.B. Shadowitz, W.L. Gold, L.A. Hawryluck, E. Rea, J.S. Chenkin, D.W. Cescon, S.M. Poutanen, A.S. Detsky, Clinical features and short-term outcomes of 144 patients with SARS in the greater Toronto area, *Jama* 289 (2003) 2801–2809.
- [3] J.W. Chan, C.K. Ng, Y.H. Chan, T.Y. Mok, S. Lee, S.Y. Chu, W.L. Law, M.P. Lee, P.C. Li, Short term outcome and risk factors for adverse clinical outcomes in adults with severe acute respiratory syndrome (SARS), *Thorax* 58 (2003) 686–689.
- [4] M. Lin, H.K. Tseng, J.A. Trejaut, H.L. Lee, J.H. Loo, C.C. Chu, P.J. Chen, Y.W. Su, K.H. Lim, Z.U. Tsai, R.Y. Lin, R.S. Lin, C.H. Huang, Association of HLA class I with severe acute respiratory syndrome coronavirus infection, *BMC Med. Genet.* 4 (2003) 9.
- [5] M.H. Ng, K.M. Lau, L. Li, S.H. Cheng, W.Y. Chan, P.K. Hui, B. Zee, C.B. Leung, J.J. Sung, Association of human-leukocyte-antigen class I (B*0703) and class II (DRB1*0301) genotypes with susceptibility and resistance to the development of severe acute respiratory syndrome, *J. Infect. Dis.* 190 (2004) 515–518.
- [6] S. Itoyama, N. Keicho, T. Quy, N.C. Phi, H.T. Long, D. Ha le, V.V. Ban, J. Ohashi, M. Hijikata, I. Matsushita, A. Kawana, H. Yanai, T. Kirikae, T. Kuratsuji, T. Sasazuki, ACE1 polymorphism and progression of SARS, *Biochem. Biophys. Res. Commun.* 323 (2004) 1124–1129.
- [7] J.S. Peiris, S.T. Lai, L.L. Poon, Y. Guan, L.Y. Yam, W. Lim, J. Nicholls, W.K. Yee, W.W. Yan, M.T. Cheung, V.C. Cheng, K.H. Chan, D.N. Tsang, R.W. Yung, T.K. Ng, K.Y. Yuen, Coronavirus as a possible cause of severe acute respiratory syndrome, *Lancet* 361 (2003) 1319–1325.
- [8] T.G. Ksiazek, D. Erdman, C.S. Goldsmith, S.R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J.A. Comer, W. Lim, P.E. Rollin, S.F. Dowell, A.E. Ling, C.D. Humphrey, W.J. Shieh, J. Guarner, C.D. Paddock, P. Rota, B. Fields, J. DeRisi, J.Y. Yang, N. Cox, J.M. Hughes, J.W. LeDuc, W.J. Bellini, L.J. Anderson, A novel coronavirus associated with severe acute respiratory syndrome, *N. Engl. J. Med.* 348 (2003) 1953–1966.
- [9] C. Drosten, S. Gunther, W. Preiser, S. van der Werf, H.R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R.A. Fouchier, A. Berger, A.M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J.C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H.D. Klenk, A.D. Osterhaus, H. Schmitz, H.W. Doerr, Identification of a novel coronavirus in patients with severe acute respiratory syndrome, *N. Engl. J. Med.* 348 (2003) 1967–1976.
- [10] T. Kuiken, R.A. Fouchier, M. Schutten, G.F. Rimmelzwaan, G. van Amerongen, D. van Riel, J.D. Laman, T. de Jong, G. van Doornum, W. Lim, A.E. Ling, P.K. Chan, J.S. Tam, M.C. Zambon, R. Gopal, C. Drosten, S. van der Werf, N. Escriou, J.C. Manuguerra, K. Stohr, J.S. Peiris, A.D. Osterhaus, Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome, *Lancet* 362 (2003) 263–270.
- [11] C.E. Samuel, Antiviral actions of interferons, *Clin. Microbiol. Rev.* 14 (2001) 778–809.
- [12] B.L. Haagmans, T. Kuiken, B.E. Martina, R.A. Fouchier, G.F. Rimmelzwaan, G. van Amerongen, D. van Riel, T. de Jong, S. Itamura, K.H. Chan, M. Tashiro, A.D. Osterhaus, Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques, *Nat. Med.* 10 (2004) 290–293.
- [13] J. Cinatl, B. Morgenstern, G. Bauer, P. Chandra, H. Rabenau, H.W. Doerr, Treatment of SARS with human interferons, *Lancet* 362 (2003) 293–294.
- [14] L.E. Hensley, L.E. Fritz, P.B. Jahrling, C.L. Karp, J.W. Huggins, T.W. Geisbert, Interferon-beta 1a and SARS coronavirus replication, *Emerg. Infect. Dis.* 10 (2004) 317–319.
- [15] M. Spiegel, A. Pichlmair, E. Muhlberger, O. Haller, F. Weber, The antiviral effect of interferon-beta against SARS-coronavirus is not mediated by MxA protein, *J. Clin. Virol.* 30 (2004) 211–213.
- [16] U. Stroher, A. DiCaro, Y. Li, J.E. Strong, F. Aoki, F. Plummer, S.M. Jones, H. Feldmann, Severe acute respiratory syndrome-

- related coronavirus is inhibited by interferon-alpha, *J. Infect. Dis.* 189 (2004) 1164–1167.
- [17] E.L. Tan, E.E. Ooi, C.Y. Lin, H.C. Tan, A.E. Ling, B. Lim, L.W. Stanton, Inhibition of SARS coronavirus infection in vitro with clinically approved antiviral drugs, *Emerg. Infect. Dis.* 10 (2004) 581–586.
- [18] B. Zheng, M.L. He, K.L. Wong, C.T. Lum, L.L. Poon, Y. Peng, Y. Guan, M.C. Lin, H.F. Kung, Potent inhibition of SARS-associated coronavirus (SCOV) infection and replication by type I interferons (IFN-alpha/beta) but not by type II interferon (IFN-gamma), *J. Interferon Cytokine Res.* 24 (2004) 388–390.
- [19] B. Sainz Jr., E.C. Mossel, C.J. Peters, R.F. Garry, Interferon-beta and interferon-gamma synergistically inhibit the replication of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), *Virology* 329 (2004) 11–17.
- [20] M. Hijikata, Y. Ohta, S. Mishiro, Identification of a single nucleotide polymorphism in the MxA gene promoter (G/T at nt -88) correlated with the response of hepatitis C patients to interferon, *Intervirology* 43 (2000) 124–127.
- [21] F. Suzuki, Y. Arase, Y. Suzuki, A. Tsubota, N. Akuta, T. Hosaka, T. Someya, M. Kobayashi, S. Saitoh, K. Ikeda, M. Matsuda, K. Takagi, J. Satoh, H. Kumada, Single nucleotide polymorphism of the MxA gene promoter influences the response to interferon monotherapy in patients with hepatitis C viral infection, *J. Viral Hepat.* 11 (2004) 271–276.
- [22] S. Knapp, L.J. Yee, A.J. Frodsham, B.J. Hennig, S. Hellier, L. Zhang, M. Wright, M. Chiaramonte, M. Graves, H.C. Thomas, A.V. Hill, M.R. Thursz, Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR, *Genes Immun.* 4 (2003) 411–419.
- [23] P. Benech, Y. Mory, M. Revel, J. Chebath, Structure of two forms of the interferon-induced (2'-5') oligo A synthetase of human cells based on cDNAs and gene sequences, *Embo J.* 4 (1985) 2249–2256.
- [24] R.C. Lewontin, On measures of gametic disequilibrium, *Genetics* 120 (1988) 849–852.
- [25] D. Rebouillat, A.G. Hovanessian, The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties, *J. Interferon Cytokine Res.* 19 (1999) 295–308.
- [26] A. Ghosh, S.N. Sarkar, T.M. Rowe, G.C. Sen, A specific isozyme of 2'-5' oligoadenylate synthetase is a dual function proapoptotic protein of the Bcl-2 family, *J. Biol. Chem.* 276 (2001) 25447–25455.
- [27] S.K. Ghosh, J. Kusari, S.K. Bandyopadhyay, H. Samanta, R. Kumar, G.C. Sen, Cloning, sequencing, and expression of two murine 2'-5'-oligoadenylate synthetases. Structure-function relationships, *J. Biol. Chem.* 266 (1991) 15293–15299.
- [28] J.D. Mosca, P.M. Pitha, Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis, *Mol. Cell. Biol.* 6 (1986) 2279–2283.
- [29] M.O. Diaz, S. Ziemn, M.M. Le Beau, P. Pitha, S.D. Smith, R.R. Chilcote, J.D. Rowley, Homozygous deletion of the alpha and beta 1-interferon genes in human leukemia and derived cell lines, *Proc. Natl. Acad. Sci. USA* 85 (1988) 5259–5263.
- [30] J. Cinatl Jr., G. Hoever, B. Morgenstern, W. Preiser, J.U. Vogel, W.K. Hofmann, G. Bauer, M. Michaelis, H.F. Rabenau, H.W. Doerr, Infection of cultured intestinal epithelial cells with severe acute respiratory syndrome coronavirus, *Cell. Mol. Life Sci.* 61 (2004) 2100–2112.
- [31] M. Hijikata, S. Mishiro, C. Miyamoto, Y. Furuichi, M. Hashimoto, Y. Ohta, Genetic polymorphism of the MxA gene promoter and interferon responsiveness of hepatitis C patients: revisited by analyzing two SNP sites (-123 and -88) in vivo and in vitro, *Intervirology* 44 (2001) 379–382.
- [32] N. Fernandez-Arcas, A. Blanco, M.J. Gaitan, M. Nyqvist, A. Alonso, A. Reyes-Engel, Differential transcriptional expression of the polymorphic myxovirus resistance protein A in response to interferon-alpha treatment, *Pharmacogenetics* 14 (2004) 189–193.

SARS コロナウイルス

SARS-Corona virus

倉辻忠俊 切替照雄

Key words: SARS, 呼吸器感染症, 血清抗体, 診断, バイオセーフティー

1. 概 説

重症急性呼吸器症候群(severe acute respiratory syndrome: SARS)は, 2003年2月末から(発端は2002年11月)東アジアを中心に, 短期間に全世界へと流行拡散した, SARS コロナウイルスによる急性呼吸器感染症である。感染者の20%前後が発症後数日以内に乾性咳嗽, 呼吸困難, 低酸素血症へと急速に重症化する進行性肺炎(呼吸窮迫症候群)で, 死亡率は10%前後である。飛沫感染, 接触感染によりヒト-ヒト感染が主であるが, まれに糞口感染, 空気感染の可能性を示唆する例もある。20-50%が病院感染で, 早期診断, 院内感染対策が重要である。

診断はウイルス分離, RT-PCR, LAMP法などによる遺伝子診断, 血清抗体検査などによる。

ウイルスのレザポアはハクピシンなど野生の動物が考えられているが, まだ確定していない。

2. 検査の目的

ウイルス分離および遺伝子診断は治療および疫学的対策, 鑑別診断のために行う。ベア血清の抗体検査は確認のために行う。

3. 試料の採取方法

飛沫感染するので, 試料採取の際はN95マスク, ゴーグル, 手袋などの个人防护具を必ず着用する。

ウイルス分離, 遺伝子診断のための試料は, 鼻咽頭拭い液, 喀痰などの気道分泌液, 便, 尿などが用いられる。ウイルス分泌は発症後10

日頃が最大となるので, 発症初期の診断が陰性でも, 10日余り後にもう一度採取することが望ましい。

血清抗体価検査は急性期(10病日以内)および20病日以降のベアとして採取する。20病日前後の血清が陰性の場合, 30病日以降の血清を採取して確認することが望ましい。

4. 測定法

日本におけるSARS コロナウイルスの標準的確定診断は確立されていない。また, 施設内感染が多いため, 患者(疑いを含む)の診療, 試料の取り扱いの際は原則としてN95マスク, ゴーグル, 手袋など个人防护具の着用ばかりでなく, バイオセーフティレベル2以上の部屋で, 安全キャビネット内で, 十分な準備と細心の注意をもって実施する。

a. ウイルス分離

BSL3対応の施設が必要で, 通常の病院検査室では行ってはならない。したがって, 試料は保健所に連絡のうえ, 指定の地方衛生研究所あるいは国立感染症研究所へ, 万国郵便条約に則って送付する。すなわち, 試料の入ったスクリュエーキャップで密閉できる第1次容器を吸収性の良い布あるいは紙タオルで包み, WHOの基準を満たす耐圧の第2次容器に入れる。第3次容器に試料の内容, 患者情報, 依頼元を記した封筒, 第2次容器, 保冷剤を入れて封をし, 外側に宛先, 依頼元を記し, 更にバイオハザードのシールを貼る。発送の前に受け入れ先(地方衛生研究所あるいは国立感染症研究所)に連絡をしておく。

Tadatashi Kuratsuji, Teruo Kirikae: Research Institute, International Medical Center of Japan 国立国際医療センター研究所

0047-1852/05/¥40/頁/JCLS

b. ウイルスの遺伝子検出(LAMP法)^{1,2)}

現在のところ、日本で臨床診断薬として承認されているものは、栄研化学のLoopamp SARS コロナウイルス検出試薬キットのみである。SARS コロナウイルスの replicase 領域内に設定した6種類のプライマーを使用しているため、他のヒトコロナウイルスなどと交差反応を起こさず、特異性が高い。

汚染を防ぐために安全キャビネット内で操作、またマイクロ遠心機もバイオシールドのローターを使用する(バイオセーフティレベルBSL2)。また、他試料とのコンタミネーションを防ぐための注意は、他の遺伝子検査と同様である。

直前にバッファーをエタノールなどで調整しておく。

1.5ml チューブに buffer AVL/carrier RNA 540 μ l, 試料 140 μ l を入れ、キャップを閉めボルテックス攪拌混和、10分間室温静置後、遠心、エタノール、バッファーなど添加、遠心など繰り返し、RNAを抽出する。

以下の操作は循環型クリーンベンチを使用、手袋を着用する。

試料、陽性、陰性コントロール分のマスターミックスを調整し、使用まで氷上保存する。試料、マスターミックスを混合し、遠心する。チューブを 62-63°C, 45分間反応させ、次に 80°C, 5分間加温して反応を停止させる。

LA-320C 制御ソフトを起動し、測定条件を設定し、濁度を測定する。簡易にチューブ底面から紫外線(254-366nm)を照射し、目視してもよい。

c. 血清 IgG 抗体価(ELISA法)³⁾

血清を熱非動化した後はBSL2で施行してよい。血清を段階希釈してリコンビナントのSARS-CoV ウイルス N 蛋白と U274 でコートしたマイクロプレートに蒔いて 37°C に静置する。抗ヒト IgG および基質(3,3',5,5'-tetramethylbenzidine)を加えて反応させた後、450nm, 620nm の吸光度を測定する。我が国では臨床診断用には販売されていないが、Generalabs Diagnostics Pte. Ltd. (Singapore) 社製の診断キットは、抗原としてリコンビナント SARS-CoV 蛋白 N,

U274 を用いている。本キットは、同患者の急性期ウイルス分離、RT-PCR による遺伝子診断の結果および著者らの6カ月後の血清抗体価の結果と一致し、特異性、感度とも十分に診断に堪える。

5. 基準値

正常：ウイルス分離陰性、RT-PCR によるウイルス検索陰性、血清抗体価陰性。

動物のコロナウイルスには交差反応はないとされている。

6. 生理的変動(測定に影響を及ぼす因子)

試料の採取時期がキーポイントである。潜伏期(無症状期)にはウイルスは分泌されない。発熱、筋肉痛、全身倦怠などの前駆期もウイルス分泌は微量で、これらの時期のウイルス分離や RT-PCR が陰性でも、SARS は否定できない。

7. 臨床的意義(異常値を示す疾患)

当初は通常 SARS の保因者はないと考えられていた。したがって陽性の場合には SARS の確定診断となる。

LAMP 法の場合、上述のように他のヒトコロナウイルス、腸コロナウイルス、ネコ、イヌ、ウシ、ウサギ、ラットなど他種のコロナウイルス、マウス肝炎ウイルス、ブタ伝染性胃腸炎ウイルスなどとも交差反応は起こさず、また陽性反応的中率(positive predictive value)も高いため、陽性と判定された場合は確定診断である。しかし、negative predictive value は低いため、陰性であっても SARS は否定できない。したがって、Loopamp SARS コロナウイルス検出試薬キットは SARS を否定するための鑑別診断に用いてはならない。WHO の定義による SARS 患者の糞便では 81.0%、鼻咽頭拭い液で 56.3% の陽性率であった。

ELISA による抗体検査の場合は、特異性、感度の点から、ベア血清の場合、否定診断にも用いられる。抗体は長期にわたる持続が確認されているので、回復期以降の血清であれば、1ポイントでも診断し得る。また1シーズン前の呼

吸器疾患の鑑別にも用いることができる。著者らの調査では、濃厚接触で感染予防策を講じた場合でも6.3%程度の不顕性感染を認めた³⁾(表1)。また不顕性感染は濃厚接触しなくてもあり得るが、その場合の血清抗体価は低い。

8. 関連検査項目

10日以内の流行地の渡航歴、検査室・研究室勤務歴、発熱、乾性咳嗽、呼吸困難など主要症状、胸部X線写真などが診断の基本である。

急性呼吸器症状を呈するマイコプラズマ、インフルエンザ、RSウイルスなど他の病原微生物の抗原あるいは抗体検査を行う。

表1 ELISAによる血清抗SARS-IgG抗体³⁾

施設(名)	検査人数	陽性人数	陽性率
A病院	148	60	40.5%
(発症者)	44	44	100%
B病院	127	8	6.3%
C病院	50	1	2.0%
合計	325	69	21.2%

注：A病院は輸入SARS患者1人に端を発し、職員は濃厚接触あり、病院感染が多発した。B病院はA病院から転送されたSARS患者を除く。職員は濃厚接触あるも、感染予防策を講じた。C病院はB病院と同じ敷地内であるが、SARS患者の入院はない。

文献

- 1) Loopamp SARS コロナウイルス検出試薬キット説明書, 栄研化学, 2003.
- 2) 田代真人: 迅速診断法の開発, 科学技術振興調整費「SARSの診断及び検査手法等に関する緊急調査研究」成果報告書, 2004.3.
- 3) Kirikae T, et al: Detection of antibodies against SARS-associated coronavirus in patients and healthcare workers at three hospitals following the 2003 Hanoi outbreak. FEMS Microbiology letters, 2004.

SARS のウイルス学

切 替 照 雄

要旨 SARSの原因は、新型のSARSコロナウイルス(SARS-CoV)であることが明らかにされた。SARS-CoVはこれまで知られているコロナウイルスと同様に特徴的なスパイクを持ち、エンベロープを有するRNAウイルスである。ゲノム構造解析から、これまで知られているコロナウイルスとは異なるグループのウイルスであることがわかった。SARS-CoVの受容体がアンギオテンシン変換酵素2(ACE2: Angiotensin-converting enzyme 2)であることが明らかとなってきたが、いまだウイルスの起源やワクチン開発など大きな課題が残されている。

(キーワード: SARS-CoV, コロナウイルス, ゲノム, 自然宿主, ウイルス受容体, アンギオテンシン変換酵素2(ACE2: Angiotensin-converting enzyme 2))

VIROLOGY OF SARS CORONAVIRUS

Teruo KIRIKAE

Abstract A novel corona virus, SARS-corona virus was discovered in association with cases of severe acute respiratory syndrome (SARS). SARS-CoV is an enveloped RNA virus with characteristic spikes like other corona viruses. Genome sequence analysis revealed that SARS-CoV belongs to a group different from previously known corona viruses. Angiotensin-converting enzyme 2 (ACE2) were found to be a functional receptor for SARS-CoV. The origin and natural reservoir of SARS-CoV should be identified and the development of vaccine and anti viral compounds against the virus will be required for successful control of the global SARS epidemic.

(Key Words: SARS-CoV, corona virus, genome, natural reservoir, a receptor for SARS-CoV, Angiotensin-converting enzyme 2 (ACE2))

2002年11月中国広東省広州市の隣の仏山(ふざん)市在住の男性が広州医学院第一附属医院に異型肺炎で入院した。この患者がSARSの第1症例とされている。以来、この新興感染症は院内感染を中心に広州市で拡大するとともに、香港、ベトナム、シンガポール、カナダ、台湾、北京などを中心に世界30ヵ国以上に伝播し、患者数8,000人と死亡約800人にのぼる大流行となった。WHOの多施設共同研究ネットワークの参加研究施設を中心とした世界規模の研究協力体制によって、SARSの原因ウイルスがこれまでに報告のない新しいタイプのコロナウイルス、すなわちSARSコロナウイルス(SARS-CoV)であることが明らかにされた。また、この発見後数週間

でSARSコロナウイルスの全ゲノムが解読されている。本稿では、ウイルス学的な側面から、SARS-CoVを概説する。

分 類

コロナウイルスは動物細胞を宿主としてその細胞質内で増殖するエンベロープウイルスに属する¹⁾。このウイルスを電子顕微鏡で観察すると、特徴的な突起(スパイク)が観察できる。このスパイクが太陽のコロナに似ていることから、コロナウイルスと命名された。コロナウイルス科(*Coronaviridae*)のウイルスは、コロナウイルス属(*Coronavirus*)とトロウイルス属(*Torovirus*)に

国立国際医療センター研究所 Research Institute, International Medical Center of Japan 感染・熱帯病研究部

Address for reprints: Teruo Kirikae, Department of Infectious Diseases and Tropical Medicine, Research Institute, International Medical Center of Japan, 1-21-1, Shinjuku, Tokyo 162-8655 JAPAN
e-mail: tkirikae@ri.imcj.go.jp

Received December 18, 2003

Accepted December 19, 2003

表 1 コロナウイルス、自然宿主と疾患

分類	ウイルス 略式名称	ウイルス名	宿主	疾患のタイプ	
I	TGEV	伝染性胃腸炎ウイルス	ブタ	呼吸器感染, 胃腸炎, 全身性感染	
	FECoV	ネコ消化器ウイルス	ネコ	軽い腸炎	
	FIPV	ネコ伝染性腹膜炎ウイルス	ネコ	腹膜炎, 呼吸器感染, 脳炎, 全身性感染	
	CCoV	イヌコロナウイルス	イヌ	下痢症	
	HCoV-229E	ヒトコロナウイルス229E	ヒト	鼻風邪	
II	PECV	ブタ伝染性下痢症ウイルス	ブタ	下痢症	
	MHV	マウス肝炎ウイルス	マウス	呼吸器感染, 脳炎, 胃腸炎, 肝炎	
	SDAV	ラットコロナウイルス	ラット	唾液腺, 涙腺, 扁桃腺炎	
	BCoV	ウシコロナウイルス	ウシ	下痢	
	HCoV-OC43	ヒトコロナウイルスOC43	ヒト	鼻風邪	
III	HEV	血球凝集性脳炎ウイルス	ブタ	脳炎, 腸炎, 呼吸器感染	
	IBV	伝染性気管支炎ウイルス	ニワトリ	気管支炎, 肝炎, 全身性感染	
	TCov	七面鳥コロナウイルス	七面鳥	下痢症	
	IV(?)	HCoV-SARS	重症急性呼吸器症候群ウイルス	ヒト	重症肺炎

分類されて、SARS-CoV はコロナウイルス属に分類される。ゲノムは1本鎖(ss) RNA, レトロウイルスのような逆転写酵素 (RT) を用いる複製過程がなく、ゲノムセンスがプラス (+) 鎖 (mRNA と同様の塩基配列) をもつ、すなわちss (+) RNA ウイルスである。コロナウイルス属のウイルスは、抗原性、アミノ酸配列や塩基配列の相同性から、3つのグループに分けられている (表1)。SARS-CoV のポリメラーゼなどの遺伝子の保存領域の配列を比較すると、SARS-CoV はこれら3つのグループのウイルスとも相同性が低く、第4番目のグループに属すると考えられる。しかし、スパイク, Nsp1, マトリックスやカプシドといった比較的多様性のある領域のアミノ酸配列を比較すると、SARS-CoV はグループ2に相対的に近縁であることが示唆されている。グループ2に属するウイルスはトリ由来であり、グループ3は中国で食用として使用される動物で見出される。このことは、SARS-CoV の起源がこれらの動物であるという可能性を遺伝学的にも支持しているかもしれない。

ウイルス粒子の構造

一般的にウイルス粒子は、遺伝情報を担うウイルス核酸とそれを包んで保護するタンパク質の殻から成り立っている。このタンパク質の殻のことをカプシド、またゲノムとともにヌクレオカプシドとよぶ。ウイルスによっては、ヌクレオカプシドの外側にエンベロープとよばれる糖タンパクと脂質の膜が覆っている。コロナウイルス粒子の直径は120-160 nm である。ゲノムの大きさは約30,000塩基 (30 kb) である。コロナウイルスのカプシ

ドは管状でらせん対称な構造である (図1)。その周りにエンベロープを持つが、コロナウイルス粒子は特徴的なスパイクを有する。SARS-CoV も同様なスパイクを持つエンベロープウイルスであることが電子顕微鏡で観察されている。ウイルス表面のスパイクを形成するのはSタンパク質である。この他、エンベロープには、膜 (M) タンパク質、エンベロープ (E) タンパク質が存在する。エンベロープの内側にゲノムRNAがあり、それに核 (N) タンパク質が結合し、らせん状のヌクレオカプシドを形成している。

ゲノム構造

SARS-CoV を含むコロナウイルスのゲノムは、約30 kb の (+) 鎖 RNA からなる。これは、RNA としてはもっとも大きく、レトロウイルスのその約3-4倍である。ゲノムの5'末端には cap 構造が、3'末端には poly (A) が存在する。5'末端にはコロナウイルス特異的な配列 (5'-CUAAAC-3') のコアをもつ leader sequence があり、その下流にRNAPolymerase (replicase 1a, replicase 1b) 遺伝子がある。非構造タンパク質遺伝子はゲノム全体の約70%を占めている。ウイルス構造タンパク質は、これらの下流で、S, E, M,

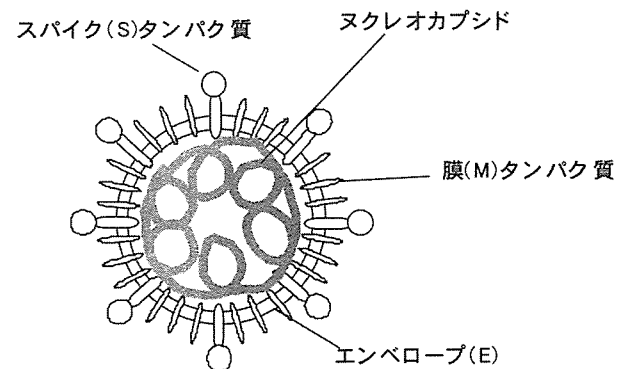


図 1 SARS-CoV 粒子の模式図
エンベロープには、ウイルス表面のスパイクを形成するSタンパク質、膜 (M) タンパク質、エンベロープ (E) タンパク質が存在する。エンベロープの内側にゲノム RNA があり、それに核 (N) タンパク質が結合し、らせん状のヌクレオカプシドを形成している。

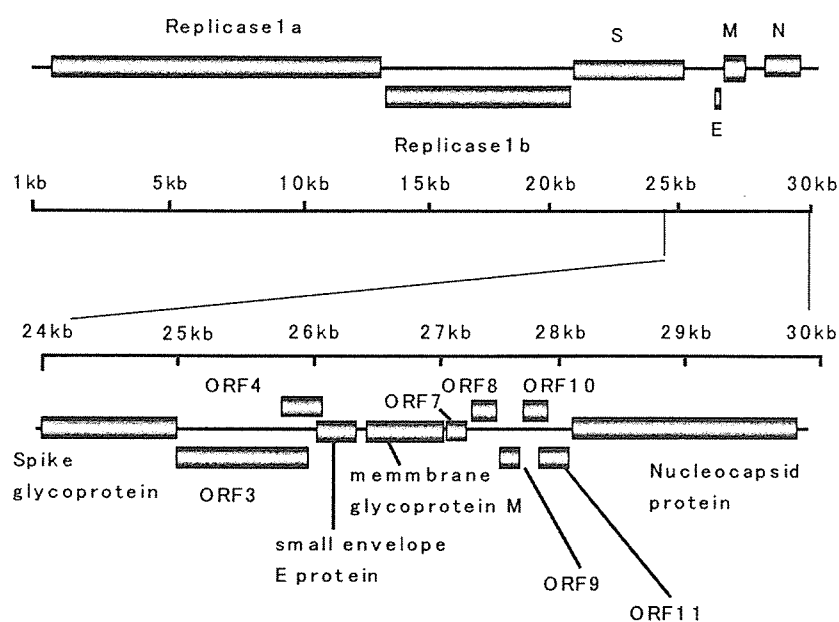


図 2 SARS-CoV のゲノム

SARS-CoV のゲノムは、約30kb の (+) 鎖 RNA からなる。ゲノムの 5' 末端は cap 構造を、3' 末端には poly (A) が存在する。5' 末端にはコロナウイルス特異的な配列 (5'-CUAAC-3') のコアをもつ leader sequence があり、その下流に RNA polymerase (replicase 1a, replicase 1b) 遺伝子がある。ウイルス構造タンパク質は、これらの下流で、S, E, M, N 遺伝子の順で存在する

N 遺伝子の順で存在する (図 2)。グループ 2 に属するウイルスには、RNA polymerase 遺伝子のすぐ下流に Hemagglutinin-esterase タンパク質をコードする HE 遺伝子が存在するが、他のグループと SARS-CoV には HE 遺伝子はない。

SARS-CoV の ORFs (open reading frame) は、既知のコロナウイルスタンパク質の相同性から決定している。その結果、主要なタンパク質、replicase 1a と 1b, S, E, M, N タンパク質が同定された。そのほかに 9 個の ORFs が推定されているが、生物学的関連性は不明である。

ウイルスタンパク質の構造と機能

replicase 1a と 1b は巨大タンパク質として翻訳され、内在するタンパク分解酵素によって分解され、RNA polymerase や helicase などの機能タンパク質が作られる。これらのタンパク質はウイルスの複製に関与する。

S タンパク質は、ウイルス粒子表面のスパイクを構成する分子量180-200 kDa、1,255アミノ酸残基のタイプ I の糖タンパク質で、3量体で1つのスパイクを形成する。S タンパク質は、外側、N 末端側の頭部に当たる S1 領域とその内側で C 末端で膜に貫通する S2 領域からな

る。S タンパク質は、コロナウイルスのもつ多くの生物活性に関与している。すなわち、受容体への結合活性、カプシドを宿主細胞内へ侵入させる働き、細胞融合活性などである。また、ウイルス抗原の主要エпитープとなり、宿主による中和抗体産生を引き起こす。

E タンパク質は、分子量約 8 kDa で、エンベロープ内に埋もれている。M タンパク質は、25 kDa の 3 回膜貫通型タンパク質で、細胞外に短い N 末端領域、細胞内に比較的長い末端 C 領域を持つ。この C 末端領域にウイルスのヌクレオカプシドが結合する。ウイルスタンパク質としては最も多く、ウイルス粒子の形態形成に関与し、粒子が組み立てられる過程で S タンパク質とゲノムを取り込む (図 3)。

ヌクレオカプシドタンパク質である N タンパク質は、約 50 kDa の塩基性タンパク質で、一部 C 末端が酸性である。中心部の領域に RNA 結合部位を、C 末端領域に M タンパク結合領域を持ち、機能として mRNA の翻訳に関与しているようである。

ウイルス受容体

ごく最近、SARS-CoV の受容体がアンギオテンシン変換酵素 2 (ACE2: Angiotensin-converting enzyme 2) であることが明らかとなってきた²⁾。SARS-CoV は、アフリカミドリザルの腎臓細胞株である VeroE6 細胞に感染することができるが、ヒトの腎臓細胞株 293T 細胞には感染できない。そこで SARS-CoV の S タンパク質の S1 タンパク質にヒト免疫グロブリンの Fc 部分を融合したタンパク質を作成した。フローサイトメトリーで解析すると、この融合タンパク質は、VeroE6 細胞には結合するが、293T 細胞には結合しない。免疫沈降で解析すると、このタンパク質と結合するタンパク質が同定でき、アミノ酸配列の検討から、ACE2 が受容体候補タンパク質として同定されてきた。可溶性の ACE2 は SARS-CoV が VeroE6 細胞に結合するのを抑制するが、ACE1 は抑制しない。ACE2 を発現していない 293T 細胞に ACE2 遺伝子を導入して ACE2 を発現させると、

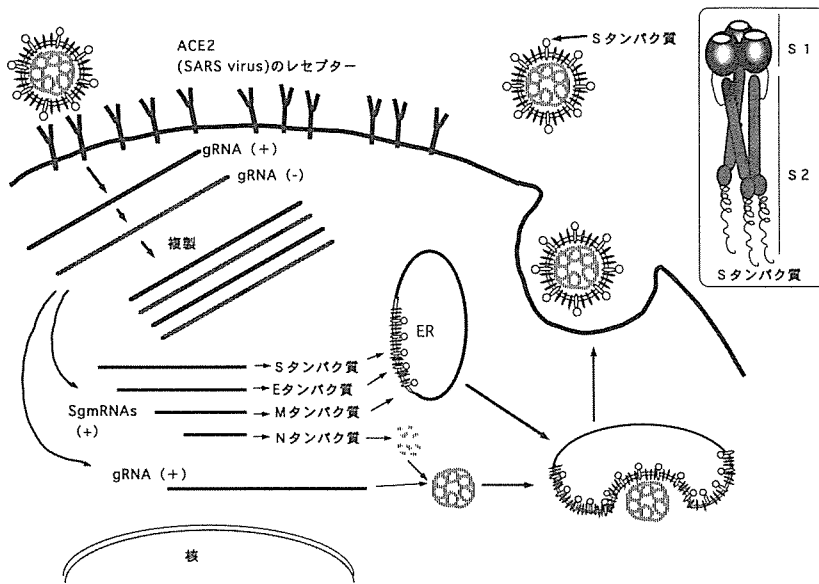


図3 SARS-CoVの増殖環(模式図)とSARS-CoV Sタンパク質の構造
受容体に結合後、Sタンパク質の細胞融合活性を介して、細胞内に侵入したコロナウイルス(+)鎖ゲノムの5'末端にコードされているRNA polymeraseが翻訳され、この酵素を利用して、ゲノムと相補的な(-)鎖RNAが合成される。さらに、この(-)鎖RNAから幾種類かの subgenomic (sg) mRNAが合成される。数種類の sg mRNAから、タンパク質が合成される。合成されたNタンパク質はゲノムRNAと結合しヌクレオカプシドを形成する。これがMタンパク質と internal compartments で会合して出芽し、その後 exocytosis によって細胞外に放出される。

SARS-CoVが感染して、ウイルスが増殖するようになる。ACE2に対する抗体は、SARS-CoVのVeroE6細胞への感染を抑制するが、ACE1に対する抗体は阻止しない。以上の実験から、ACE2がSARS-CoVの機能的な受容体である可能性が強く示唆されている。

他のコロナウイルスに関する2つの型の受容体が同定されている。ひとつは、グループIIに属するMHVでCEACAM1 (carcinoembryonic antigen cell adhesion) と呼ばれる細胞接着分子である。これは標的細胞にも発現しているが、非標的細胞にも発現しており、機能的な受容体かどうか疑問が残っている。もうひとつは、グループIコロナウイルスで見られる受容体として亜鉛メタロペプチダーゼN (APN, CD13)が同定されている。これはウイルスが標的細胞に侵入するのに必須のタンパク質である。ACE2がSARS-CoVの機能的な受容体である可能性が強いが、ACE2もAPN (CD13)と同様にメタロペプチダーゼ活性があり、この酵素活性がウイルスの細胞侵入に関与していることが考えられる。ACE2の活性部位の2つのヒスチジンをアスパラギンに置換しても、ウイルス受容体活性が変化しない。同様の実験結果

がCD13でもでている。酵素活性と、ウイルス感染は直接は関連しないかもしれない。

ウイルスの宿主細胞内増殖

コロナウイルスは、受容体に結合後、Sタンパク質の細胞融合活性を介して、直接細胞膜から侵入すると考えられている。細胞内に侵入したコロナウイルス(+)鎖ゲノムの5'末端にコードされているRNA polymeraseが翻訳され、この酵素を利用して、ゲノムと相補的な(-)鎖RNAが合成される。さらに、この(-)鎖RNAから幾種類かの subgenomic (sg) mRNAが合成される。sg mRNAは、ゲノムの3'末端から異なる長さで5'方向に延びている。いずれの長さの sg mRNAもゲノムに存在する leader sequenceがある。これらの mRNA セットは nested set とよばれ、これがコロナウイルスを含むニドウイルス目の特徴となっ

ている¹⁾。

数種類の sg mRNA から、原則として5'末端のORFが翻訳されタンパク質が合成される。コロナウイルスのタンパク質の集合や出芽は、細胞膜から直接細胞外へ放出されるのではなく、ERからゴルジ装置を介して、internal compartments 内腔に出芽される。合成されたNタンパク質はゲノムRNAと結合しヌクレオカプシドを形成する。これが親和性の高いMタンパク質と internal compartments で会合して出芽し、その後 exocytosis によって細胞外に放出される。

病原性

ある病原体が疾患の原因であることを科学的に実証するための要件として、有名な「コッホの4原則」が知られている。この原則は、ウイルスが病原体の場合改変されて、6つの要件を満たすものとされている。SARS-CoVの場合、この6原則を満たす³⁾。すなわち、1)患者からウイルスが分離される、2)宿主細胞で培養できる、3)ろ過可能である、4)宿主もしくはそれに近縁の動物で同様な疾患を引き起こすことができる、5)再び同じウ

ウイルスが再分離できる、6) ウイルスに対する特異的な免疫反応が惹起できる。後半の3つは、サルを用いた感染実験で証明されている。サルの感染では、感染後3日で嗜眠傾向と一過性の発疹を示し、感染後4日で呼吸困難を示すようになる。感染後2-6日で鼻腔や咽頭拭い液中のPCRが陽性となり、ウイルスが分離できるようになる。また、ウイルスは糞便中にも排出される。病理解剖では、重症の多巣性の肺炎が見られ、同時にウイルスが病巣からも分離できる。組織像では、肺胞広範な障害、しばしば多核細胞をともなった肺胞と気管支上皮の壊死など、ヒトSARSと同様な所見が見られる。

コロナウイルスはヒトや家畜などの動物に感染し、SARS以外にもさまざまな疾患を引き起こす(表1)。ヒトでは、2種類のコロナウイルス(229E株とOC43株)が知られていた。いずれも軽症のかぜを引き起こし、かぜの20-30%の原因と考えられていた。これらはしかしSARSのような重症の呼吸器疾患を引き起こすことはない。SARS-CoVを含めコロナウイルスの主要な標的組織は、消化器と呼吸器である。多くの場合、年齢の若い動物が重篤な症状を示す。また、一般的にコロナウイルスは、種特異性が高く、固有宿主を超えた動物に感染することは少ないと考えられてきた。SARS-CoVはヒト以外にもサル、ネコ、イタチ、マウスやラットなどにも感染する。SARS-CoVが有する高い病原性には、宿主の免疫の過剰反応が関与しているであろうことは推定できる。しかし、SARS重症化の分子機作は解明されていない。

起 源

SARS-CoVの起源や自然宿主を知ることは、この疾患の根本的な予防・撲滅につながる非常に重要な研究課題である。しかし、SARS-CoVの起源はまだ明らかになっていない。SARS-CoVの起源は、中国広東省を中心とした南部中国であろう。この地域では、多くの種類の野生動物が食用として市場にでまわり、ヒトと野生動物や野生動物間での接触がきわめて濃厚である。初期のSARS患者に野生動物を解体する料理人が含まれており、このような生活環境からSARS-CoVがヒトに伝播した可能性が高い。新型インフルエンザの出現も、中国南部地帯であり同様な背景の関与が考えられる⁴⁾。広東省ではハクビシンやタヌキなどの野生動物から、SARS-

CoVゲノムや抗体が検出される。これらの動物から分離されたSARS-CoV様ウイルスは、SARS-CoVと99%以上の相同性をもち、SARS-CoVであると考えられる。ハクビシンから分離されたSARS-CoVは、ヒト由来株と比べるとN遺伝子上流に29塩基が余分に存在する。動物由来のSARS-CoVは、ヒトには感染しないが、変異や組換えにより、ヒトに感染するようになった可能性が考えられる。しかし、広東省で初期の患者から分離されたウイルスは、ハクビシンのそれと同じ29塩基を持っている。また、ヒトと野生動物由来SARS-CoV間の違いは、S遺伝子領域でも存在することから、S遺伝子が宿主を決定する因子となっていることも考えられる。

お わ り に

SARS-CoVの研究が始まって、まだ1年もたっていない。その間にこれまで考えられなかったほどの速さでSARS-CoVのウイルス学が明らかにされてきた。これを可能にした要因のひとつとして、これまでのコロナウイルスでの地味な研究が基盤となっていることを強調したい。ウイルスの起源、ワクチン開発、重症化の機序の解明など解明すべき問題はまだまだ多い。

文 献

- 1) Lai MMC, Cavanagh D: The molecular biology of coronaviruses. *Adv Virus Res* 48: 1-100, 1997
- 2) Li W, Moore J, Vasilieva N et al: Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 426: 450-454, 2003
- 3) Fouchier RAM, Kuiken T, Schutten M et al: Koch's postulates fulfilled for SARS virus. *Nature* 423: 240, 2003
- 4) Guan y, Zheng BJ, He YQ et al: Isolation and characterization of viruses related the SARS coronavirus from animals in southern china. *Science* 302: 276-278, 2003

(平成15年12月18日受付)

(平成15年12月19日受理)

検査法の現状と今後の展望

切替 照雄*

SARS コロナウィルスの検査法として、ウィルス分離法、PCR (polymerase chain reaction)法による遺伝子診断及び抗体検査がある。ウィルス分離法は感受性細胞株など標準プロトコールが既に公表されており、国立感染症研究所や全国の地方衛生研究所でウィルス分離が可能となっている。PCR 法による遺伝子診断法は、WHO が標準的なプライマー配列を公表している。これらのプライマーは、特異性の高いものであるが、感度を高める必要がある。ほかに LAMP 法と呼ばれる簡便な遺伝子増幅法、定量化が可能なりアルタイム PCR 法も含め感度の高い検査キットが開発されている。ビーズアレーを用いた PCR 法や SARS 全ゲノムの DNA マイクロアレーなども開発されている。免疫学的検査法は、IFA (免疫蛍光抗体法) と ELISA (固相酵素結合免疫測定法) が開発され、流行地では SARS に対する抗体検出法として使用された。しかし、一般検査キットとしての開発は遅れていたが、ようやく ELISA とイムノクロマトを用いた抗 SARS 抗体 (IgG) を検出するキットが入手可能になってきた。

Key Words : SARS 検査法 / ウィルス分離 / PCR 法 / 免疫検査法

I はじめに

SARS 診断のための検査法は基本的には他のウィルス性疾患のそれと変わらない。ウィルスの検査法としては、ウィルス分離法、PCR 法による遺伝子診断、抗体検出及び抗原検出が一般的である。このうち SARS ウィルスの検査法としては、現在抗原検出以外の方法が実際に実施されている¹⁾。ウィルス分離法は感受性細胞株など標準プロトコールが既に公表されており、国立感染症研究所 (感染研) や全国の地方衛生研究所 (地衛研) でウィルス分離が可能となっている。PCR 法による遺伝子診断法は、WHO が標準的なプライマー配列を公表している²⁾。これらのプライマーは、WHO 共同研究ネットワークに属する研究施設で開発、評価され特異性の高いものであるが、感度

を高める必要がある。このほかに LAMP 法と呼ばれる簡便な PCR 法、定量化が可能なりアルタイム PCR 法も含め感度の高い検査キットが使用可能になってきた。また、in situ ハイブリダイゼーション法を用いて、組織中のウィルス遺伝子の同定も可能になってきた。ビーズアレーを用いた PCR 法や SARS 全ゲノムの DNA マイクロアレーなども開発されている。免疫学的検査法は、IFA (免疫蛍光抗体法) と ELISA (固相酵素結合免疫測定法) が開発され、流行地では SARS に対する抗体検出法として使われた。しかし、感染初期には検出できないことなど早期診断には問題があった。さらにまた陽性コントロールとなる抗血清やウィルス粒子 (不活化ウィルスも含め) が簡単には入手できないなどの理由で、一般検査キットとしての開発は遅れていたが、ようやく ELISA とイ

Laboratory testing for SARS

* Teruo Kirikae 国立国際医療センター研究所感染・熱帯病研究部長

(63) 63