III. 研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編者名	書籍名	出版社名	出版地	出版年	ページ
切替照雄	病院感染対策関連法規		エビデンスに基づいたICTのための感染対策トレーニングブック	メディカ出版	大阪	2005	244-251
倉辻忠俊 吉倉廣 宮崎久義 切替照雄 大西文子		倉辻忠俊 吉倉廣 宮崎久義 切替照雄 大西文子	改定・院内感染防止手順ーすぐ実践できる	メヂカルフレンド社	東京	2006	1-220

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Itoyama S, Keicho N, Hijikata M, Quy T, Phi NC, Long HT, HaLeD, Ban VV, Matsushita I, Yanai H, Kirikae F, Kirikae T, Kuratsuji T Sasazuki, T.	Identification of an alternative 5'-untranslated exon and new polymorphism of angiotensin-converting enzyne 2 gene: lack of association with SARS in the Vietnamese population.	Am J Med Genet A,	136	52-57	2005
Hamano E, HijikataM, Itoyama S, Quy T, Phi NC, Long TH, HaLeD, Ban VV, Matsushita I, Yanai H, Kirikae F, Kirikae T, Kuratsuji T, Sasazuki T, Keicho N.	Polymorphisms of interferon-inducible genes OAS-1 and MxA associated with SARS in the Vietnamese population.	Biochem. Biophysic. Res. Commun.	329	1234-39	2005

Xiao W, Hsu YP, Ishizaka A, Kirikae T, Moss RB.	Sputum cathelicidin, urokinase plasminogen activation system components and cytokines discriminate cystic fibrosis, COPD and asthma inflammation.	Chest	128	2316-2326	2005
Sekiguchi J, Asagi T, Miyoshi-Akiyama T, Fujino T, Kobayashi I, Morita K, Kikuchi Y, Kuratsuji T, Kirikae T.	Multidrug- resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6')-Iae* gene cassette encoding a novel amino-glycoside acetyltransferase.	Antimicrob. Agents Chemoth.,	49	3734-3742	2005
Sekiguchi J, Tharavichitkul P, Miyoshi-Akiyama T, Chupia V, Fujino T, Araake M, Irie A, Morita K, Kuratsuji T, Kirikae T.	Cloning and characterization of a novel trimethoprim-resistant dihydrofolate reductase from a nosocomial isolate of *Staphylococcus aureus* CM.S2 (IMCJ1454).	Antimicrob. Agents Chemoth	49	3948-3951	2005
Otsuka Y, Fujino T, Mori N, SekiguchiJ, Toyota E, Saruta K, Kikuchi Y, SasakiY, AjisawaA,OtsukaY, N agai H, Takahara M, Saka H, Shirasaka T, Yamashita Y, KiyosukeM, KogaH, Oka S, Kimura S, Mori T, Kuratsuji T, Kirikae T.	Survey of human immunodeficiency virus (HIV)-seropositive patients with mycobacterial infection in Japan.	J. Infect.	51	364-374	2005
Nishimura H, Kuratsuji T, Quy T, Phi NC, Ban VV, Ha	Rapid awareness and transmission of severe acute respiratory syndrome in Hanoi	Am. J. Trp. Med. Hyg.	73	17-25	2005

LeD, Long HT, Yanai H, Keicho N, Kirikae T, Sasazuki T, Anderson RM.	French Hospital, Vietnam., 2005				
Kawana A, Teruya K, Hama T, Kuroda E, Sekiguchi J, Kirikae T, Naka G, Kimura S, Kuratsuji T, Ohara H, Kudo K	Trial surveillance of cases with acute respiratory symptoms at IMCJ hospital.	Jpn J Infect Dis.	58	241-243	2005
Sekiguchi J, Fujino T, Araake M, Toyota E, Kudo K, Saruta K, Yoshikura H, Kuratsuji T, Kirikae T.	Emergence of rifampicin resistance in methicillin-resistant *Staphylococcus aureus* in tuberculosis wards.	J Infect Chemother.	12	47-50	2006
Obata S, Zwolska Z, Toyota E, Kudo K, Nakamura A, Sawai T, Kuratsuji T, Kirikae T.	Association of *rpoB* mutations with rifampicin resistance in *Mycobacterium avium*.	Int J Antimicrob Agents	27	32-39	2006
倉辻忠俊	ICT：組織としての対応を理解する	臨床医	31	1409-1410	2005

IV.　研究成果の刊行物・別冊

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

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Cloning of ACE2 cDNA From the Lung

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

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

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

Genomic DNA Samples for the Association Study

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

Testing for Antibody Response to the SARS-CoV

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

Identification of Polymorphisms Within *ACE2* Gene

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

Genotyping of Identified Polymorphisms

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

Statistical Analysis

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

RESULTS

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

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

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

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

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

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

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

Identification of Polymorphisms Within *ACE2* Gene

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

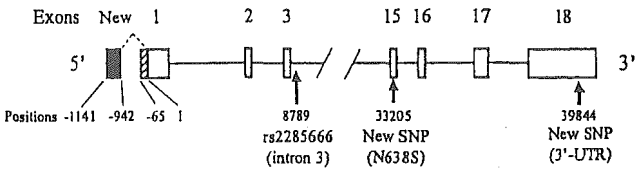

Fig. 1. A schematic diagram of the *ACE2* gene structure and the positions of SNPs. The known exons are depicted as open boxes. A solid box and a striped box indicate the new exon and the new extended region of the exon 1, respectively. The arrows represent locations of the SNPs analyzed in a case-control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

TABLE II. Demographic Findings of Subjects and Subgroups

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

[a]Data not available.

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

Genotype and Allele Frequency of Three SNPs

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

DISCUSSION

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

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

TABLE III. SNPs Within the *ACE2* Gene

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

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

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

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

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

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

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

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

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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, Koup 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, Tsoi 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.

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

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Abstract

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

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

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

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

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

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

Materials and methods

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

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

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

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

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

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

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

Results

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

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

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

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

The SNPs in *MxA* gene and *PKR* gene were not associated with the development of SARS (Table 2). However, on comparison of the *MxA* genotype between hypoxemic SARS patients requiring oxygen therapy and non-hypoxemic SARS patients who did not, GG genotype was found more frequently in patients of the former category (odds ratio 3.75; 95% CI 1.08–10.7; $p = 0.0346$). It was also shown that the G-allele was more frequent in the former group ($p = 0.0195$) (Table 4). The other SNPs did not show any significant p values between these two groups (data not shown).

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

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

Discussion

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

Table 1
Characteristics of SARS cases and healthy contacts

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

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

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

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

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

In the presence of double-stranded RNA (dsRNA), OAS-1 catalyzes the 2′,5′-oligomers of adenosine in order to permit the binding and activation of a latent ribonuclease, RNase L, which cleaves cellular and viral RNAs [11,25]. *OAS-1* gene has two major transcripts that are generated by alternative splicing at the last two exons [23]. E16 (NM_002534) is a short transcript with 5 exons and is translated to p40 isoform. E18 (NM_016816) is a long transcript with 6 exons and is translated to p46 isoform. Another transcript 9-2 is generated using a different splice acceptor site that comes from E18 at exon 6 and is translated to 9-2 protein [26]. The 9-2 protein has a unique property due to the Bcl-2 homology domain 3 present in its unique carboxyl-terminal region. This is also distinctive in causing cellular apoptosis by binding to the anti-apoptotic proteins of the Bcl-2 family [26]. Therefore, OAS-1 has dual functions representing the synthesis of 2′,5′-oligomers of adenosine and the promotion of cellular apoptosis.

Knapp et al. [22] described how the GG genotype in exon 6 of *OAS-1* gene was more frequent in persistent HCV infection than in self-limiting infection. In our study, the G-allele was more frequently observed in SARS-CoV infected individuals than in the uninfected group. In both these studies, the G-allele was susceptible to virus infection. The A/G polymorphism in exon 6 is located downstream of the stop codon for E18 transcript meaning therefore that it is included in the 3′-untranslated region. However, it is located upstream of the stop codon for 9-2, and the A/G SNP results in amino acid substitution Arg397Gly of 9-2 protein, which is located near the Bcl-2 homology domain (amino acid positions 372–393). It will be an interesting aspect if this phenomenon occurs with any functional importance. We also analyzed the A/G polymorphism in exon 3 of *OAS-1* gene and found that there was strong linkage disequilibrium between the two SNPs. The A/G polymorphism in exon 3 causes amino acid substitution Ser162Gly in three isoforms, which is located near the dsRNA binding domain (amino acid positions 104–158) of OAS-1 [27]. We are unable at this point to determine which SNP is directly related to susceptibility to SARS or SARS-CoV infection. One can also consider that the other unidentified polymorphism of strong linkage disequilibrium with these SNPs may serve as the basis for any functional difference. Judging from the results obtained in this study, polymorphisms in *OAS-1* gene are likely to be involved in SARS-CoV infection or the development of SARS, at least in part, bearing in mind the fact that OAS-1 might have antiviral potential against SARS-CoV.

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

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

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

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

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

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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. Ephtimios, 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. Ziemin, 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.

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

Wei Xiao, Yao-Pi Hsu, Akitoshi Ishizaka, Teruo Kirikae and Richard B. Moss
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Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation*

Wei Xiao, MD; Yao-Pi Hsu, MS; Akitoshi Ishizaka, MD; Teruo Kirikae, MD, PhD; and Richard B. Moss, MD

Background: **Interest in airways inflammatory disease has increasingly focused on innate immunity. We investigated several components of innate immunity in induced sputum of patients with cystic fibrosis (CF), COPD, and asthma, and healthy control subjects.**
Methods: **Twenty eight patients with mild CF lung disease (age ≥ 12 years; FEV_1, 74 ± 3% predicted [mean ± SE]), 74 adults with COPD (FEV_1, 55 ± 2% of predicted), 34 adults with persistent asthma (FEV_1, 66 ± 2% of predicted), and 44 adult control subjects (FEV_1, 85 ± 1% of predicted) were studied while in stable clinical condition. Levels of sputum interleukin (IL)-8, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, human cationic antimicrobial protein 18 (CAP18), urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), and plasminogen activator inhibitor (PAI)-1 were determined. Cell sources were investigated by flow cytometry and immunohistochemistry. Spirometry was performed prior to sputum induction.**
Results: **CF patient sputum showed greatest increase in IL-8 compared to that of patients with COPD and asthma (which were also greater than control subjects), and elevated levels of TNF-α and IL-10 compared to other groups. There were no differences in IFN-γ. CAP18 levels were elevated in CF and COPD patients compared to control subjects, while asthma patients had reduced CAP18 levels. uPA levels were similar but uPAR was elevated in CF and COPD patients more so than in asthma patients, while PAI-1 levels were elevated in all three disease groups. CAP18 localized to neutrophil secondary granules; neutrophils were also sources of IL-8 and PAI-1. CAP18 and PAI-1 negatively correlated with pulmonary function.**
Conclusion: **Induced-sputum innate immune factor levels discriminate inflammatory changes in CF, COPD, and asthma, suggesting potential roles in pathophysiology and as well as providing disease-specific biomarker patterns.** ***(CHEST 2005; 128:2316–2326)***

Key words: cathelicidin; cystic fibrosis; cytokine; innate immunity; urokinase plasminogen activator system

Abbreviations: BSA = bovine serum antigen; CAP18 = human cationic antimicrobial protein 18; CF = cystic fibrosis; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate; IFN = interferon; IL = interleukin; PAI = plasminogen activator inhibitor; PBS = phosphate-buffered saline solution; PE = phycoerythrin; TNF = tumor necrosis factor; uPA = urokinase-type plasminogen activator; uPAR = uPA receptor

The pathogenesis of cystic fibrosis (CF) lung disease is characterized by compromised local innate immunity, which permits microbial colonization and chronic infection.[1] Current thinking emphasizes the primary role of volume depletion of airway surface liquid and resulting compromise of mucociliary clearance.[2] Additional innate defense mechanisms may also be involved, as a primary proinflam-

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Drs. Moss, Ishizaka, and Kirikae have assigned entire right, title, and interest in the CAP18 immunoassay described here to Seikagaku Corporation, Tokyo, Japan.
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matory bias of the CF epithelia has been posited,[3-4] and activity of endogenous antimicrobial peptides produced by airway epithelia and glands may be altered in CF.[5-6] While some epithelial antimicrobials have received much attention in CF, in particular the β-defensins, others have not.[7-8] In particular, the role of the only human cathelicidin, the 18-kd, 140 amino acid cationic antimicrobial protein (human cationic antimicrobial protein 18 [CAP18]), has not been investigated in sputum from patients with CF. It is produced by respiratory epithelia as well as stored in secondary (specific) granules of polymorphonuclear leukocytes, and in a model system restores deficient antimicrobial activity in the CF airway milieu.[9-10] Proteolytic cleavage of CAP18 by proteinase 3 yields a potent antimicrobial peptide (carboxy terminal 37 amino acid fragment of CAP18).[11] Via its action on the formyl peptide receptor-like 1 expressed on several cell types, carboxy terminal 37 amino acid fragment of CAP18 is also an important regulator of macrophage function; has potent chemotactic activity for neutrophils, monocytes, and T cells; and possesses angiogenesis activity.[12-14] Abnormalities in CAP18 could therefore profoundly affect the pathophysiology of CF by its ability to link innate to adaptive immunity and its neovascularizing effect.

Another innate defense pathway recently found active in the airway is the plasminogen activator system constituted by its local serine protease activator urokinase-type plasminogen activator (uPA), the uPA-specific cell surface receptor (uPA receptor [uPAR]) [CD87], and an arginine-specific serine protease inhibitor (serpin), plasminogen activator inhibitor (PAI)-1.[15-16] uPA binding to uPAR results in enhanced activation of cell-bound plasminogen with subsequent effects on cell adhesion, chemotactic migration, and tissue remodeling.[16-20] The uPA system is involved in a number of pathologic states, including inflammation after tissue injury as key participants in the enzymatic modification of the extracellular matrix, resulting in cell recruitment, migration, adhesion, and mitogenesis.[15,19] In addition to proteolytic activity, plasmin activates metalloproteinases that degrade extracellular matrix.[20] To maintain normal lung function and integrity in the host response, tight control of the proteolytic enzymes and their inhibitors is needed to maintain proper function of neutrophils, macrophages, and mesenchymal cells. The uPA system is one of the components that act on neutrophils and macrophages to facilitate the interaction between cells and matrix.[21-22] A critical innate defense role for the uPA/uPAR/PAI-1 system has recently been described in murine models of acute pulmonary infection as well as in the pathogenesis of interstitial lung diseases.[23-26] The role of this system in human chronic airway inflammatory lung diseases is just emerging.[26-27]

CF, COPD, and asthma are chronic airway diseases with different causes that share some features in pathologic changes and clinical syndrome. In these diseases, there is chronic mucosal and airway inflammation with distinct pathophysiologic features in each but a common increase in the infiltration of neutrophils and a variety of inflammatory mediators including interleukin (IL)-8. The pathologic processes in these diseases all seem to involve progressive inflammatory responses with elements of tissue remodeling, airway obstruction, and reduction in expiratory flow rates. In the present study, we determined and compared the levels and sources of uPA/uPAR/PAI-1, CAP18, and several cytokines (IL-8, tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and IL-10) in induced sputum of CF, COPD, and asthma patients, and healthy control subjects to see if differing local inflammatory patterns can be discerned noninvasively and related to airflow obstruction as measured by expiratory flow rates.

Materials and Methods

Subjects

We recruited 28 patients with CF (age, 12 to 50 years) followed up at the CF Center clinic at Stanford University Medical Center. CF diagnoses in all patients were made by positive (> 60 mEq/L) pilocarpine iontophoresis sweat test results, with homozygous or compound heterozygous for ΔF508 CF transmembrane conductance regulator mutations. All patients had chronic infection with *Pseudomonas aeruginosa* by serial sputum culture and were in stable clinical condition (no pulmonary exacerbation within previous month). CF patients were also excluded for FEV_1 values < 40% predicted, oxyhemoglobin saturation < 92% on room air, pneumothorax, hemoptysis, or history of *Burkholderia cepacia* in sputum. None were receiving regular inhaled or systemic corticosteroids. Seventy-four patients (age 38 to 79 years) with previously diagnosed COPD were recruited from the Respiratory Clinic of the Hospital of Shandong Medical University, Peoples Republic of China. All were in stable condition and not receiving antibiotics for at least 2 weeks prior to testing. Thirty-four patients with asthma (age, 14 to 75 years) followed up in Shandong were also studied. Finally 44 healthy nonsmoking subjects without reported respiratory symptoms (age, 20 to 60 years; 20 patients at Stanford and 24 patients at Shandong) were also studied. All participants gave written informed consent with protocols approved by the Institutional Review Boards at Stanford and Shandong.

Pulmonary Function

Pulmonary function tests were performed according to American Thoracic Society guidelines for performance and acceptance prior to sputum induction.

Sputum Induction and Processing

Sputum was collected from each patient as previously described using 3% hypertonic saline solution (at Stanford) or 3.5% hypertonic saline solution (at Shandong) via an ultrasonic nebulizer with 2-min collections of sputum, which were pooled for analysis.[28–29] All subjects underwent sputum induction regardless of history of ability to expectorate. Subjects were encouraged to cough, and sputum was collected into polypropylene cups. The induced-sputum samples were weighed, and an equal volume of Sputolysin (Calbiochem-Novabiochem; San Diego CA) diluted 10% in normal saline solution was added. Samples were vortexed 3 seconds and incubated for 5 min at 37°C in a water bath with vigorous shaking (160 rotations per minute). Samples were further mixed by aspirating up and down 20 times in a transfer pipette. Five-minute incubations were then repeated two more times. Finally, the samples were centrifuged at 2,000 revolutions per minute (800g) for 5 min at 4°C, and the sol phase was used for analysis.

Soluble Mediators of Innate Immunity

Human IL-8, IL-10, IFN-γ, TNF-α, CAP18, uPA, uPAR, and PAI-1 levels in the supernatant sol phase of sputum were determined by enzyme-linked immunosorbent assay (ELISA) using a standardized format. Wells of microtiter plates (polypropylene 96-well culture clusters, Catalog No. 3598; Costar; Pleasanton CA) were coated with 50 μL per well capture antibody (see below for specific reagents) diluted in phosphate-buffered saline solution (PBS) [P-4417; Sigma Chemical; St. Louis, MO], incubated overnight at 4°C, and washed three times with wash buffer (PBS 0.01%, Thimersol; Sigma Chemicals; 0.05% Tween 20 [polyoxyethylene sorbitan mono-oleate]; Sigma Chemical). Blocking solution (1% bovine serum albumin [BSA], A-2153; Sigma Chemical; 5% sucrose PBS) 200 μL per well was added, incubated at room temperature for 1 h, and the wells were washed three times with washing buffer. Samples and standards diluted in diluting solution (0.1% BSA-0.05% Tween 20-Tris-buffered saline solution) were then added (50 μL per well), incubated overnight at 4°C, and washed three times with washing buffer. Biotinylated detection antibody (see below for specific reagents) in dilution buffer was then added (50 μL per well) and incubated 2 h at room temperate with gentle mixing. Plates were then washed four times with washing buffer. Avidin-peroxidase-conjugated secondary antibody (see below for specific reagent) diluted in dilution buffer was then added (50 μL per well), and incubated 1 h at room temperature with gentle mixing. Plates were then washed four times with washing buffer. Developing solution (75 μL per well *o*-phenylenediamine [P-6912; Sigma Chemical] in citrate-phosphate buffer pH 6.0 with 4 μL 30% H_2O_2 [H-1009; Sigma Chemical]) per 10-mL buffer was added, the reaction stopped with 25 μL per well 2 N H_2SO_4, and the well color was read at an optical density of 492 with an automated microplate reader (Molecular Devices; Mountain View, CA).

Specific Reagents for the ELISA

IL-10: For IL-10, the capture antibody was from Pharmingen (Catalog No. 18551D; BD Pharmingen; San Diego, CA), the primary antibody was purified rat anti-human IL-10 diluted to 4 μg/mL, the detection antibody was biotinylated rat anti-human IL-10 (Catalog No. 18562D; BD Pharmingen) diluted to 4 μg/mL, the detector was horseradish peroxidase-streptavidin (Catalog No. 43–4323; Zymed Laboratories; San Francisco, CA) diluted 1:1000, and the standard was recombinant human IL-10 (Catalog No. 19701N; BD Pharmingen) diluted to 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156, 78, and 39 pg/mL.

IFN-γ: For IFN-γ, the capture antibody was purified mouse anti-human INF-γ (N1B4, Catalog No. 18891D; BD Pharmingen) diluted to 2 μg/mL, the detection antibody was biotinylated mouse anti-human IFN-γ (4SB3, Catalog No. 18902D; BD Pharmingen) diluted to 2 μg/mL, and the standard was recombinant human IFN-γ (Catalog No. 19751N; BD Pharmingen), diluted to 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156, 78, and 39 pg/mL.

IL-8: For IL-8, the capture antibody was purified monoclonal antibody to human IL-8 (Catalog No. MAB208; R&D Systems; Minneapolis, MN) diluted to 4 μg/mL, the detection antibody was biotinylated goat anti-human IL-8 (Catalog No. BAF208; R&D Systems) diluted to 200 ng/mL, and the standard was recombinant human IL-8 (Catalog No. 208-IL; R&D Systems) diluted to 5,000, 2,500, 1,250, 625, 312.5, 156, and 78 pg/mL.

TNF-α: For TNF-α, the capture antibody was mouse anti-human TNF-α (Part 840119; R&D Systems) diluted to 4 μg/mL, the detection antibody was biotinylated goat anti-human TNF-α (Part 840120; R&D Systems) diluted to 300 ng/mL, the detector was horseradish peroxidase-streptavidin (Part 89080; R&D Systems) diluted to 1:200, and the standard was recombinant human TNF-α (Part 840121; R&D Systems) diluted to 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 16 pg/mL.

uPA, PAI-1, and uPAR: For uPA, PAI-1, and uPAR, ELISA kits (Imubind; American Diagnostics; Greenwich, CT) were used according to the instructions of the manufacturer (Catalog Nos. 894, 821, and 893, respectively).

CAP18: For CAP18, the capture antibody was rabbit polyclonal antibody to human lipopolysaccharide-binding domain of CAP18 diluted to 1:200; the detection antibody was a mouse IgG1 monoclonal antibody to CAP18 diluted to 1:1000; the detector was horseradish peroxidase-conjugated goat anti-mouse IgG (Catalog No. 115-367-5296; Jackson Immuno Research Lab Inc.; West Grove, PA) diluted 1:2500; and the standard was a synthetic 27 amino acid peptide fragment of CAP18 (amino acids 109–135) diluted to 5,000, 2,500, 1,250, 625, 312.5, 156, 78, 39, and 20 ng/mL. The capture antibody, detection antibody and standard were obtained from Dr. Yoshikazu Naiki and Teruo Kirikae, Japan. In a pilot study, the same CAP18 ELISA was used to measure CAP18 levels in serum, BAL fluid, and expectorated sputum of patients with CF (see "Results").

Immunhistochemistry of Induced-Sputum Cells

Adherence of Sputum Cells to Glass Slides: After removal of the supernatant of centrifuged sputum samples, cell pellets were each suspended in 20 mL of PBS (P-4417; Sigma Chemical) and centrifuged at 1,200 revolutions per min for 10 min. After removal of the supernatant, the cell pellet was resuspended in 1% BSA (A-2153; Sigma Chemical)-PBS. Dead cells were excluded by Trypan Blue (T-9520; Sigma Chemical) cell count. Cells were diluted to approximately 2.5×10^5 in 1% BSA-PBS. Following cytocentrifuge (700 revolutions per minute for 5 min) 100 μL of cell suspension per slide were air dried and freezer stored at −20°C.

Staining: Frozen slides were thawed at room temperature. Cells were fixed by incubating slides in 4% formaldehyde (Catalog No. 16220; Electron Microscopy Sciences; Fort Washington PA)-PBS for 15 min at room temperature to fix cells. After three washes in PBS, slides were incubated in 1% H_2O_2 (H-1009; Sigma Chemical)-PBS 10 min at room temperature, washed three times in PBS, and stained. Slides were permeabilized by incubation with 0.5% saponin (S-2149; Sigma Chemical)-PBS for 10 min at room temperature. Slides were then washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal (T-5125; Sigma). Once the cells were permeabilized, the same buffer was used after this step. According to the protocol of the Vectastain Elite ABC kit