表 1. HLA-A、B、Cw のタイプ別のアリル数と頻度

	SARS 2N = 88	接触非発症 2N = 200	非接触コントロール 2N = 100	p value
A*01	3 (3.4%)	4 (2.0%)	3 (3.0%)	
A*02	19 (21.6%)	45 (22.5%)	25 (25.0%)	
A*03	0 (0.0%)	2 (1.0%)	1 (1.0%)	
A*11	31 (35.2%)	57 (28.5%)	33 (33.0%)	
A*24	14 (15.9%)	33 (16.5%)	13 (13.0%)	
A*26	0 (0.0%)	4 (2.0%)	3 (3.0%)	
A*29	5 (5.7%)	18 (9.0%)	8 (8.0%)	
A*30	0 (0.0%)	1 (0.5%)	0 (0.0%)	
A*31	2 (2.3%)	1 (0.5%)	2 (2.0%)	
A*32	0 (0.0%)	1 (0.5%)	0 (0.0%)	
A*33	12 (13.6%)	32 (16.0%)	10 (10.0%)	
A*34	1 (1.1%)	1 (0.5%)	1 (1.0%)	
A*68	1 (1.1%)	0 (0.0%)	0 (0.0%)	
A*74	0 (0.0%)	1 (0.5%)	1 (1.0%)	
B*07	7 (8.0%)	20 (10.0%)	9 (9.0%)	
B*13	3 (3.4%)	6 (3.0%)	9 (9.0%)	
B*15	27 (30.7%)	54 (27.0%)	25 (25.0%)	
B*18	2 (2.3%)	6 (3.0%)	1 (1.0%)	
B*27	0 (0.0%)	5 (2.5%)	2 (2.0%)	
B*35	5 (5.7%)	10 (5.0%)	2 (2.0%)	
B*37	0 (0.0%)	2 (1.0%)	1 (1.0%)	
B*38	6 (6.8%)	15 (7.5%)	7 (7.0%)	
B*39	2 (2.3%)	4 (2.0%)	1 (1.0%)	
B*40	3 (3.4%)	11 (5.5%)	6 (6.0%)	
B*41	1 (1.1%)	0 (0.0%)	0 (0.0%)	
B*44	4 (4.5%)	9 (4.5%)	3 (3.0%)	
B*46	7 (8.0%)	19 (9.5%)	11 (11.0%)	
B*48	**5 (5.7%)**	3 (1.5%)	**0 (0.0%)**	**0.017**
B*49	0 (0.0%)	1 (0.5%)	0 (0.0%)	
B*51	**1 (1.1%)**	4 (2.0%)	**8 (8.0%)**	**0.026**
B*52	0 (0.0%)	0 (0.0%)	3 (3.0%)	
B*54	4 (4.5%)	5 (2.5%)	3 (3.0%)	
B*55	0 (0.0%)	2 (1.0%)	1 (1.0%)	
B*56	2 (2.3%)	3 (1.5%)	0 (0.0%)	
B*57	3 (3.4%)	5 (2.5%)	2 (2.0%)	
B*58	6 (6.8%)	16 (8.0%)	6 (6.0%)	
Cw*01	12 (13.6%)	27 (13.5%)	13 (13.0%)	
Cw*03	13 (14.8%)	36 (18.0%)	21 (21.0%)	
Cw*04	10 (11.4%)	18 (9.0%)	7 (7.0%)	
Cw*05	0 (0.0%)	0 (0.0%)	1 (1.0%)	
Cw*06	3 (3.4%)	8 (4.0%)	3 (3.0%)	
Cw*07	19 (21.6%)	45 (22.5%)	17 (17.0%)	
Cw*08	22 (25.0%)	38 (19.0%)	18 (18.0%)	
Cw*12	1 (1.1%)	4 (2.0%)	3 (3.0%)	
Cw*14	0 (0.0%)	2 (1.0%)	4 (4.0%)	
Cw*15	7 (8.0%)	21 (10.5%)	13 (13.0%)	
Cw*16	0 (0.0%)	1 (0.5%)	0 (0.0%)	
Cw*17	1 (1.1%)	0 (0.0%)	0 (0.0%)	

表 2. HLA-DRB1、DQB1 の型別のアリル数と頻度

	SARS 2N = 88	接触非発症 2N = 200	p value	非接触コントロール 2N = 100	p value	ベトナム一般集団[4]
DRB1*01	0 (0.0%)	1 (0.5%)		0 (0.0%)		
DRB1*03	6 (6.8%)	13 (6.5%)		2 (2.0%)		4.1%
DRB1*04	**2 (2.3%)**	18 (9.0%)		**12 (12.0%)**	**?**	8.8%
DRB1*07	5 (5.7%)	14 (7.0%)		5 (5.0%)		4.6%
DRB1*08	**1 (1.1%)**	6 (3.0%)		**9 (9.0%)**	**0.015**	5.3%
DRB1*09	6 (6.8%)	26 (13.0%)		10 (10.0%)		13.6%
DRB1*10	5 (5.7%)	15 (7.5%)		8 (8.0%)		4.4%
DRB1*11	2 (2.3%)	5 (2.5%)		1 (1.0%)		0.5%
DRB1*12	**41 (46.6%)**	**58 (29.0%)**	**?**	**27 (27.0%)**	**0.008**	31.8%
DRB1*13	**0 (0.0%)**	**14 (7.0%)**	**?**	**6 (6.0%)**	**0.018**	5.8%
DRB1*14	**9 (10.2%)**	**9 (4.5%)**	**?**	7 (7.0%)		4.4%
DRB1*15	11 (12.5%)	20 (10.0%)		9 (9.0%)		12.2%
DRB1*16	0 (0.0%)	1 (0.5%)		4 (4.0%)		2.5%
DQB1*02	**11 (12.5%)**	21 (10.5%)		**5 (5.0%)**	**?**	5.8%
DQB1*03	49 (55.7%)	109 (54.5%)		51 (51.0%)		50.5%
DQB1*04	1 (1.1%)	7 (3.5%)		4 (4.0%)		4.6%
DQB1*05	23 (26.1%)	41 (20.5%)		26 (26.0%)		19.6%
DQB1*06	**4 (4.5%)**	22 (11.0%)		**14 (14.0%)**	**0.025**	9.0%

D. 考察

ベトナム一般集団の HLA のアリル頻度の情報は限られているが、HLA-DRB1 と HLA-DQB1 については、過去に報告 4) された頻度分布と本研究の非接触コントロール群の結果は近似しており、本研究のコントロール集団と型判定は妥当であると推測される。

過去の SARS と HLA の関連解析の報告によると、Lin らは台湾において HLA-B*4601 と B*5401 が SARS 患者群でハイリスク医療従事者群より有意に多く、特に重症患者で B*4601 が有意に多いと報告しており 1)、Ng らは香港において、B*0703 と DRB1*0301 が SARS 発症に強く関連していることを報告 2)、さらに Chen らは台湾で Cw*0801 が SARS-CoV 感染と関連があると報告しており 3)、報告により SARS に関連するとされる HLA の型は異なっている。我々がベトナム人集団から得た結果は、過去の報告の結果とはまた異なっており、SARS 患者群と接触非発症者群との間と、SARS 患者群と非接触コントロール群との間のいずれの比較においても、DRB1*12 のアリル頻度が SARS 感受群で有意に高く、DRB1*13 の頻度が SARS 患者群で有意に低かった。すなわち、DRB1*12 は SARS に対して感受性であり、DRB1*13 は抵抗性である可能性が示された。ベトナム一般集団での DRB1*12 と DRB1*13 のアリル頻度は、台湾や香港からの報告と大きく異なっておらず、今回得られた結果はベトナム人集団の HLA アリル頻度がアジア人集団の中で特殊であるためとは思われない。一方、DRB1*13 アリルの感染症に対する抵抗性は他の疾患でも示されており、慢性 B 型肝炎について HBV 感染に対して抵抗性に働く可能性や 5)、慢性 B 型肝炎における HBV の人体からの排除に貢献する可能性が報告されている 6, 7)。また DRB1*13 を含むハプロタイプは、重症のマラリア感染に対して抵抗性であることも報告されている 8)。DRB1*13 アリルが感染症に対して抵抗性に働く機序は不明であり、DRB1*13 自体に抵抗性の機能があるのか、あるいは DRB1*13 と連鎖不平衡状態にある別の抵抗性の因子が近傍にあるのかはわからない。しかし、これらの報告と、我々が SARS で得た結果は、いくつかの感染症に共通する宿主側の遺伝的抵抗性因子の存在を示唆している。

B*48、B*51、DRB1*04、DRB1*08、DRB1*14、DQB1*06 のアリル頻度は、二通りの群間の比較のうちで一方しか有意差を示さなかった。これはサンプル数が限られているためかもしれず、さらなる検討が必要と考えられる。しかし、これらの中で、DQB1*06 は DRB1*13 と連鎖不平衡状態にあることがアジア人集団で知られており、DQB1*06 のアリル頻度が SARS 患者群で低いことは、DRB1*13 で得られた結果と一致する傾向であり、今後更にハプロタイプ解析を行うなど検討をすすめたい。

結果を示していないが、SARS 患者群を重症度により中～重症患者群と軽症患者群に分類して、HLA の各アリルと SARS 重症化との関連を検討したが、群間

の有意差は観察されなかった。

今後、SARS に関連する宿主側因子としての HLA-DRB1*12 と DRB1*13 の意義を、さらに検討していきたい。

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分担研究報告書

研究課題：SARS の感染、発症、重症化の分子機構

活性化マクロファージにおける SARS RNA による炎症性サイトカインの発現誘導
SARS RNA induces inflammatory cytokines in activated macrophage

小笠原康悦　国立国際医療センター研究所、難治性疾患研究部、臨床免疫研究室

研究要旨
身体全体に存在しているマクロファージは、生体防御機構において、自然免疫系に重要な役割を担っており、細菌感染、ウイルス感染、真菌感染などにおいて、Toll-like receptor (TLR) family を用いて、それら病原体を認識していることが知られている。SARS ウイルスは RNA ウイルスであり、細胞内で増殖することが知られている。我々は、SARS 病原体モデルの刺激によって、活性化マクロファージが多量の炎症性サイトカイン TNF-alpha を発現誘導することを明らかにした。このことは、SARS ウイルス感染時において、マクロファージが産生する多量の TNF-alpha が患者の炎症を増悪させている可能性がある。

Macrophages are distributed throughout the body where they are poised to alert the innate immune systems to infection. Macrophages recognize the presence of infection using the Toll-like receptor (TLR) family of proteins that detect a variety of ligands on bacterial, viral and fungal pathogens. SARS virus is a RNA virus that proliferate in infected cells. Macrophages activated with pathogen products known to signal through TLRs express ligands. We show that SARS RNA stimulation induces a large amount of TNF-alpha in macrophages. These results suggest that TNF-alpha which is produced by SARS infected macrophages induce severe inflammation in patients.

A．研究目的

SARS の感染、発症の阻止には免疫系の果たす役割が大きいと考えられる。一般的にウイルス感染の防御には、初期感染時、自然免疫系が効率よく働くことが必須である。自然免疫系で重要な役割を果たすマクロファージは、ウイルス感染細胞の除去、獲得免疫系の活性化に重要であることが知られているが、その分子機構はよくわかっていない。我々は、SARS 感染の重症化機構を解明する目的で、SARS 感染において、マクロファージの果たす役割を解明するため研究を行ってきた。マクロファージは、細菌感染、ウイルス感染、真菌感染などにおいて、Toll-like receptor (TLR) family を用いて、それら病原体を認識していることが知られている(1)。現時点では、SARS 感染のマウスモデルが確立されていないため、直接的に SARS ウイルス感染時における解析は困難である。本年度は、ウイルス感染類似のモデルとして SARS RNA を用いて実験系を構築し、マクロファージにおける炎症性サイトカインの発現を検討した。

B．方法

1）oligo SARS RNA の合成

Database より SARS 全塩基配列から、GU rich の領域を選び出し、２０塩基を単位として oligo SARS RNA を合成した。また、positive control として、HIV ウイルス RNA 配列（RNA40）：5'-GCCCGUCUGUUGUGUGACUC-3' (2)および、R837: Imiquimod (invivogen)を、negatuve control として HIV ウイルス RNA 配列（RNA41）：5'-GCCCGACAGAAGAGAGACAC-3' (2)を用いた。

2）oligo SARS RNA を用いた SARS 感染モデル

マウスマクロファージ cell line である RAW 細胞を用い、SARS 感染モデルを構築するため、oligo SARS RNA (7.5 ug/ml) を遺伝子導入試薬 DOTAP (45 ug/ml) (Roche)を用いて細胞内へ導入した。oligo SARS RNA を導入して１６時間後に培養上清を回収し、TNF-alpha の産生を ELISA (Pharmingen) により検出した。

C．結果

1）SARS virus における GU rich 配列の検索

マクロファージは、ウイルス等の病原体を Toll like receptor を介して認識することが知られている。TLR の一つ TLR-7 は、RNA ウイルスの GU rich 配列を認識することが報告された(2, 3)。我々は、TLR-7 に着目し、SARS ウイルス中の GU rich 配列を検索した。SARS ウイルス全配列の中で、任意の２０塩基中の G または U の配列の割合を調べた(図１)。

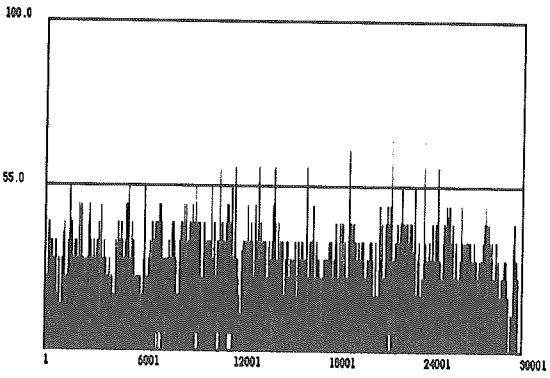

図１　SARS virus における GU rich 配列の検索

sequence position	region	GU%
1474~1493	replicase 1AB	90%
11451~11476	replicase 1AB	90%
14363~14382	replicase 1AB	90%
19088~19107	replicase 1AB	95%
19091~19110	replicase 1AB	95%
21772~21791	Spike glycoprotein	95%
23810~23829	Spike glycoprotein	90%
24694~24718	Spike glycoprotein	90%

表１　SARS ウイルス中の GU rich 配列領域

表１で示すように、９０%以上 GU を含む GU rich の配列領域は８カ所あり、それに対応する oligo RNA を合成した（表２）。

Sample number	sequence position	region	GU%
1	1474~1493	replicase 1AB	90%
2	11451~11470	replicase 1AB	90%
3	11453~11472	replicase 1AB	90%
4	11454~11473	replicase 1AB	90%
5	11455~11474	replicase 1AB	90%
6	11456~11475	replicase 1AB	90%
7	11457~11476	replicase 1AB	90%
8	14363~14382	replicase 1AB	90%
9	19088~19107	replicase 1AB	95%
10	19091~19110	replicase 1AB	95%
11	21772~21791	spike glycoprotein	95%
12	23810~23829	spike glycoprotein	90%
13	24694~24713	spike glycoprotein	90%
14	24699~24718	spike glycoprotein	90%

表２　合成 oligo RNA の SARS ウイルス sequence position

2）origo SARS RNA を用いた SARS 感染モデルにおけるサイトカイン産生

SARS 感染モデルを in vitro で確立するため、表２で示すように oligo RNA を合成し、マクロファージ cell line である RAW 細胞に oligo RNA を細胞内へ導入してウイルスが細胞内に侵入している状況を作り出した。oligo RNA を細胞内へ導入して、１６時間後に培養上清を回収し、ELISA 法により TNF-alpha の産生を検出した。oligo RNA を加えないものでは（None）、TNF-alpha の産生はほとんど認められず、positive control（p. c)あるいは、R837 では TNF-alpha が多量に産生されることが観察され、実験系が確立していることが確認された（図２）。また、SARS oligo RNA は、全て TNF-alpha の産生を誘導し、産生量も R837 や positive control の oligo RNA をしのぐほど多量であった。

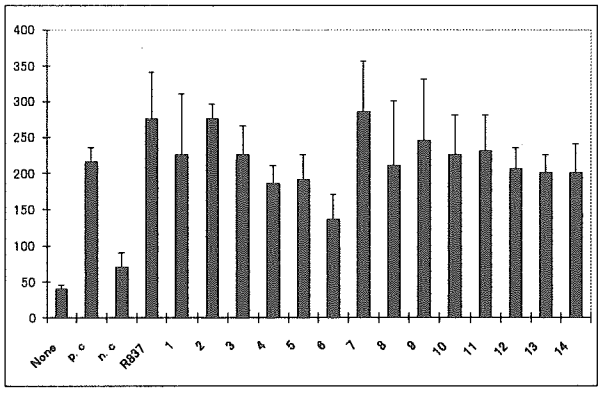

図２　マクロファージにおける TNF-alpha の産生（単位 pg/ml）

D. 考察

本研究において、in vitro で RNA ウイルス感染モデルが確立された。本実験モデルにおいて SARS ウイルス RNA は、マクロファージを活性化して、炎症性サイトカインである TNF-alpha を多量に産生させることが明らかとなった。また、SARS ウイルス RNA は、Toll like receptor を関してシグナルを伝え、TNF-alpha の産生を誘導している可能性が示唆された。

以上の結果は、SARS ウイルス感染時、生体防御機構が働いてマクロファージ等の自然免疫担当細胞が SARS ウイルスを取り込んだ際に多量の炎症性サイトカインを放出することが、過剰な免疫反応、炎症を誘導して、病態を悪化させている可能性が考えられた。今後、Toll like receptor と SARS ウイルス RNA との関係を詳細に解析し、その分子機構について追究することで、SARS 感染の重症化機構に迫りたいと考えている。

E.結論

本研究において、SARS 等の RNA ウイルス感染モデルを細胞培養系で確立した。本実験モデルにおいて SARS ウイルス RNA は、マクロファージを活性化して、炎症性サイトカインである TNF-alpha を多量に産生させることが明らかとなった。本研究から、SARS 感染時に炎症性サイトカインが多量に産生されることで、過剰な炎症、免疫反応が引き起こされ、病態が悪化している可能性が示唆された。

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F.研究発表

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分担研究報告書

重症急性呼吸器症候群（SARS）とSP－B遺伝子多型の関連性について

	大河内雅子	国立国際医療センター研究所呼吸器疾患研究部
分担研究者	慶長直人	国立国際医療センター研究所呼吸器疾患研究部
	土方美奈子	国立国際医療センター研究所呼吸器疾患研究部
	櫻田紳策	国立国際医療センター研究所呼吸器疾患研究部
	松下育美	国立国際医療センター研究所呼吸器疾患研究部
主任研究者	笹月健彦	国立国際医療センター

研究要旨

重症急性呼吸器症候群（SARS）患者の重症例において、ほぼ全例が急性呼吸促迫症候群（ARDS）に発展し、10%以上の死亡率を示すという報告がある。ARDSは通常の肺機能に不可欠な肺サーファクタント（surfactant protein）と関連している。肺サーファクタントにはAからDの４種類があり、そのうち肺サーファクタントB（SP-B）遺伝子多型とARDSにおける高い関連性が知られている。そこで本研究ではSP-B遺伝子とSARSの関連性をベトナム人SARS患者44人、非接触対照者50人、接触対照者として患者収容病院職員の非発症者103人、計197人を対象とし、ダイレクトシークエンス法により検討した。得られたデータを χ 二乗検定に用いたところ、解析を行った８SNPs全てにおいて有意差は認められなかった。供試サンプル数が少ないこともその原因として挙げられるが、SP-BがSARSへ及ぼす影響は、あるとしても弱いものではないかと考えられる。

英文抄録

Advanced cases of severe acute respiratory syndrome (SARS) were presented as acute respiratory distress syndrome (ARDS). ARDS is influenced by the activity of surfactant proteins , essential factors for normal lung function. We hypothesized that surfactant protein B (SP-B) might affect SARS. We analyzed single nucleotide polymorphisms (SNPs) of the SP-B gene ([A/C(-18)](5' flanking), [A/C 1013] (intron2), [C/T 1580] (exon4), [A/G 9306] (3' UTR)). 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. As the results of χ^2 test, we failed to obtain any significant differences in allele frequency. Possible effects of SP-B polymorphism on SARS might be weak, if any, though the number of samples is small.

A. 研究目的

重症急性呼吸器症候群(SARS)は2002年から2003年にかけて世界的に流行した、SARSコロナウィルス（SARS-CoV）の感染により発症する新興感染症である。重症例では、ほぼ全例が急性呼吸促迫症候群（ARDS）の症例定義を満たし、10%以上の死亡率を示す[1]。

ARDSは通常の肺機能に不可欠な肺サーファクタント（surfactant protein）と関連しており、肺組織に不可逆的変化を生じる高致死率を示す。肺サーファクタントはAからDの4種類あり、そのうち肺サーファクタントB（SP-B)遺伝子では、A/C(-18) SNP (5' flanking)、 A/C 1013 SNP(intron2)、C/T 1580 SNP（exon4)、 A/G 9306 SNP(3' UTR)の遺伝子型においてARDSとの関連性が報告されている[2, 3]。そこで本研究ではSP-B遺伝的多型とARDSの関連性に着目し、ベトナム人SARS患者を対象としてSP-B遺伝子多型とSARSの関連性をダイレクトシークエンス法により検討した。

対象

ベトナム人SARS患者44例、非接触対照者として50例、接触対照者として患者収容病院職員の非発症者103例、計197例を本研究の対象とした。

本研究は、ベトナム保健省および当センターの倫理委員会の承認を受けて行われた。また、全対象者からの書面による同意が取得されている。

B. 方法

最初にPCR法によりSP-Bの全11エクソンを含めた領域の増幅を行った。次にその増幅産物を鋳型とし、dbSNPで既に報告されている非同義SNP 3部位（C/T(1580).rs1130866, C/T(2536).rs3024801, A/G(4593).rs3024809）を含めた6SNPをターゲットとして再度増幅を行い、ダイレクトシークエンス法を用いてスクリーニングを行った。SP-B遺伝子とSNP位置及び増幅部位の略図を図1に、本研究に用いたプライマー一覧を表1にそれぞれ示す。

各genotype頻度及びallele頻度とSARSの関連性については、χ二乗検定を用いて解析し、0.05以下のp値を有意とした。このとき、SARS患者44例をSARS群、接触対照者103例を接触対象群、非接触対照者50例を非接触対象群とした。

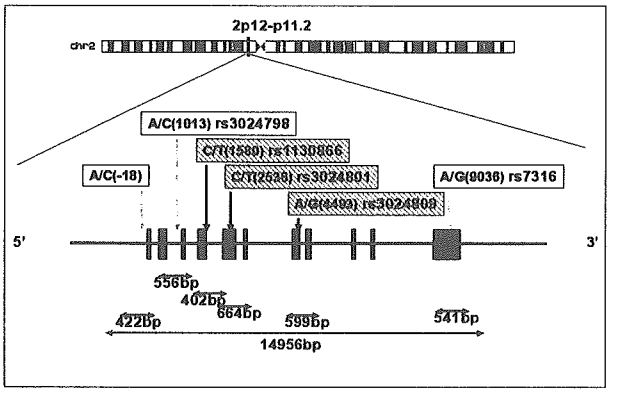

図1. SP-B遺伝子とSNP位置及び増幅部位　非同義SNPを斜線で示す。

表1. プライマー一覧

primer name	配列(5'――――――3')	増幅bp
SPB_LF2	GTA GGC CAG GAG AAG AAC CGT CAG TAG AGG	14956bp
SPB_LR4	GCT CTT CTG CAA CCA CAT CAC ACT GGG AAC	
SPB_(-18)F1	CTT GAG AGC CCC TGG TTG GA	422bp
SPB_(-18)R2	GAC AGA CTT GGG TTA ATG CC	
SPB_(+1013)F1	GTG CCT TGG AGT GCT CTG TT	556bp
SPB_(+1013)R1	CTT CCT CAT CGT GTC CTG GG	
SPB_(+1580)F1	GGT CAT GGC CCT GAG CTC AA	402bp
SPB_(+1580)R2	CCA TGG GTG GGC ACA GGG GC	
SPB_(+2536)F1	GCT TCC TCC CAT TCC AGT GG	664bp
SPB_(+2536)R1	CAG GGA GCT ACA GGT ATG CG	
SPB_(+4993)F1	AGT GAA GGT CCC ATG CTG GC	599bp
SPB_(+4993)R1	CAG CCT TCT GCC TTG AAC GG	
SPB_(+9036)F1	TGC TGT GAT TTA TCT GCT GA	541bp
SPB_(+9036)R1	CCA AGG GAG AGA GTG AGG TA	

C. 結果

今回対象とした全6SNP及び、それ以外に検出されたSNPで頻度の高かったもの(A/G4563.rs2304566, C/T4568.rs762548)のgenotype頻度と allele頻度を表2に示す。検討した全SNPにおいて、SARS患者群・接触対照群・非接触対照群における両頻度の傾向が類似していた。統計学的にもこれらの数値から有意差が認められるものはなかった。

表２．各 SNP における genotype 頻度及び allele 頻度

		SARS 群	接触対照群	非接触対照群
A/C(-18)	解析可能個体数	44	100	50
	A/A	9(20.5%)	23(23%)	14(28%)
	A/C	23(52.3%)	49(49%)	28(56%)
	C/C	12(27.2%)	28(28%)	8(16%)
	Aallele	0.466	0.475	0.56
	Callele	0.534	0.525	0.44
A/C1013	解析可能個体数	44	101	50
rs3024798	A/A	8(18.2%)	13(12.9%)	3(6%)
	A/C	21(47.7%)	41(40.6%)	24(48%)
	C/C	15(34.1%)	47(46.5%)	23(46%)
	Aallele	0.42	0.331	0.3
	Callele	0.58	0.669	0.7
C/T1580	解析可能個体数	43	101	50
rs1130866	C/C	23(53.5%)	66(65.3%)	34(68%)
	C/T	16(37.2%)	29(28.7%)	14(28%)
	T/T	4(9.3%)	6(6%)	2(4%)
	Callele	0.721	0.797	0.82
	Tallele	0.279	0.203	0.18
C/T2536	解析可能個体数	44	101	50
rs3024801	C/C	44(100%)	101(100%)	50(100%)
	C/T	0	0	0
	T/T	0	0	0
	Callele	1	1	1
	Tallele	0	0	0
A/G4493	解析可能固体数	44	101	50
rs3024809	A/A	0	0	0
	A/G	0	0	0
	G/G	44(100%)	101(100%)	50(100%)
	Aallele	0	0	0
	Gallele	1	1	1
A/G4563	解析可能固体数	44	101	50
rs2304566	A/A	32(72.7%)	75(74.3%)	32(64%)
	A/G	11(25%)	23(22.7%)	17(34%)
	G/G	1(2.3%)	3(3%)	1(2%)
	Aallele	0.852	0.856	0.81
	Gallele	0.148	0.144	0.19
C/T4568	解析可能固体数	44	101	50
rs762458	C/C	27(61.4%)	67(66.3%)	27(54%)
	C/T	16(36.4%)	33(32.7%)	20(40%)
	T/T	1(2.2%)	1(1%)	3(6%)
	Callele	0.795	0.827	0.74
	Tallele	0.205	0.173	0.26
A/G9306	解析可能個体数	44	99	46
rs7316	A/A	30(68.2%)	67(67.7%)	25(54.4%)
	A/G	13(29.5%)	31(31.3%)	18(39.1%)
	G/G	1(2.3%)	1(1%)	3(6.5%)
	Aallele	0.83	0.833	0.739
	Gallele	0.13	0.167	0.261

D.考察

今回の遺伝子型頻度とアリル頻度の結果からはSARSとSP-B遺伝子多型における関連性を見出すことは出来なかった。解析に用いたサンプル数が少なかったということもその理由となるかもしれないが、同サンプルを用いた報告でSARSとⅠ型インターフェロン（IFN）誘導性抗ウイルス蛋白遺伝子との関連性が示されている[４]。また、SARS患者群・接触対照群・非接触対照群におけるアリル頻度の傾向が類似していたことから、用いた集団に遺伝的な偏りはないといえる。これらのことから、SP-BがSARSに与える影響は、あるとしても弱いものではないかと考えられる。

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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

Satoru Itoyama,[1] Naoto Keicho,[1]* Minako Hijikata,[1] Tran Quy,[2] Nguyen Chi Phi,[2] Hoang Thuy Long,[3] Le Dang Ha,[4] Vo Van Ban,[5] Ikumi Matsushita,[1] Hideki Yanai,[6] Fumiko Kirikae,[7] Teruo Kirikae,[7] Tadatoshi Kuratsuji,[8] and Takehiko Sasazuki[9]

[1]*Department of Respiratory Diseases, Research Institute, International Medical Center of Japan, Tokyo, Japan*
[2]*Bach Mai Hospital, Hanoi, Vietnam*
[3]*National Institute of Hygiene and Epidemiology, Hanoi, Vietnam*
[4]*Institute for Clinical Research in Tropical Medicine, Vietnam*
[5]*Hanoi-French Hospital, Vietnam*
[6]*The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Japan*
[7]*Department of Infectious Diseases, Research Institute, International Medical Center of Japan, Japan*
[8]*Research Institute, International Medical Center of Japan, Japan*
[9]*International Medical Center of Japan, Japan*

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.

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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*Correspondence to: Dr. Naoto Keicho, Department of Respiratory Diseases, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. E-mail: nkeicho-tky@umin.ac.jp

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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

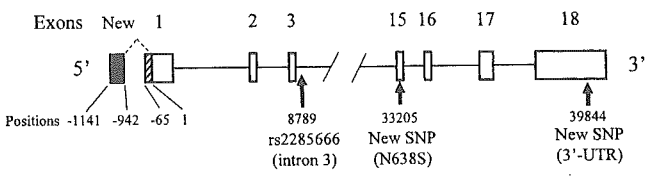

Fig. 1. A schematic diagram of the *ACE2* gene structure and the positions of SNPs. The known exons are depicted as open boxes. A solid box and a striped box indicate the new exon and the new extended region of the exon 1, respectively. The arrows represent locations of the SNPs analyzed in a case-control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