(corresponding to 2 pg of reverse-transcribed RNA) was used to quantitatively target the housekeeping gene *B2M*. The three assays were highly linear over the examined range (see the online Data Supplement). Likewise, in a 10-fold dilution series of a *FLJ10350* cDNA clone ranging from 10^9 to 10 copies per reaction, assayed by all three assays, real-time quantification was linear, although it showed more deviation among the triplicates performed on low amounts of template (see the online Data Supplement).

We analyzed expression of three genes that had been found, by SYBR Green I analyses, to be differentially expressed in patients with congenital heart defects *(17)*. The results confirmed the previously published data, with *FLJ10350* and *TNNI1* being significantly up-regulated and *PIPPIN* being significantly down-regulated (see the online Data Supplement). Throughout our assays, we saw no amplification of the no-template controls (see the online Data Supplement).

For normalization of the target genes analyzed in the course of this study, the housekeeping gene *B2M* was simultaneously assayed with the genes of interest. The obvious sample-to-sample variations (see the online Data Supplement) stress the importance of effective systems for normalization, as achieved with the multiplexed cr-real-time PCR assay.

In summary, we have described a single-step method for real-time PCR that is sensitive, robust, and requires minimal optimization effort. Because the system uses nonmodified, tailed amplification primers and universal reporting reagents, it is characterized by a flexible and low-cost format. The use of differentially labeled reporting reagents enables multiplexing approaches for monitoring of more than one target per well, e.g., both a candidate and housekeeping gene. Therefore, the cr-real-time PCR assay appears suitable for the broad spectrum of real-time PCR applications.

This work was funded by EU FP6 Grant 503155 (Moltools). We are grateful to Martin Lange for providing the *FLJ10350* cDNA clone and Bogac Kaynak for providing the cDNA samples. We thank Maike Tribbels and Christina Grimm for critical reading of the manuscript.

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DOI: 10.1373/clinchem.2004.034512

ACE2 Gene Polymorphisms Do Not Affect Outcome of Severe Acute Respiratory Syndrome, *Rossa W.K. Chiu,[1,2] Nelson L.S. Tang,[1,2] David S.C. Hui,[1,3] Grace T.Y. Chung,[1,2] Stephen S.C. Chim,[1,2] K.C. Allen Chan,[1,2] Ying-man Sung,[2] Louis Y.S. Chan,[4] Yu-kwan Tong,[1,2] Wing-shan Lee,[1,2] Paul K.S. Chan,[1,5] and Y.M. Dennis Lo[1,2*]* ([1] The Centre for Emerging Infectious Diseases, and Departments of [2] Chemical Pathology, [3] Medicine and Therapeutics, [4] Obstetrics and Gynaecology, and [5] Microbiology, The Chinese University of Hong Kong, Shatin, Hong Kong; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Room 38023, 1/F Clinical Sciences Bldg., Prince of Wales Hospital, 30-32 Ngan Shing St., Shatin, New Territories, Hong Kong Special Administrative Region, China; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

Severe acute respiratory syndrome (SARS) is the first pandemic of the 21st century *(1)*. Since its recognition, 8437 individuals have been affected and 813 have died *(2)*. Approximately 20–30% of patients required intensive care admission *(1)*. Although there was a slight predom-

Table 1. Allele and genotype frequencies for the studied groups.

A. Allele frequencies and χ^2 analyses (*df* = 1)

	SNP locus[a] (dbSNP ID)									
	1848 (rs2106809)		9570[a] (rs2285666)		16854 (rs4646142)		36024[b] (rs714205)		37138 (rs2074192)	
	Frequency, n (%)	χ^2	Frequency, n (%)	χ^2	Frequency, n (%)	χ^2	Frequency, n (%)	χ^2	Frequency, n (%)	χ^2
Minor allele	T		G		C		G		A	
Controls (174 M/154 F)	228 (47)		224 (46)		223 (46)		215 (45)		206 (43)	
SARS patients[c] (81 M/87 F)	114 (45)	$\chi^2 = 0.354$; $P = 0.552$	111 (44)	$\chi^2 = 0.470$; $P = 0.493$	110 (43)	$\chi^2 = 0.539$; $P = 0.463$	111 (44)	$\chi^2 = 0.047$; $P = 0.840$	105 (41)	$\chi^2 = 0.109$; $P = 0.741$
SARS patients with poor outcome[c] (30 M/16 F)	30 (48)	$\chi^2 = 0.000$; $P = 0.979$	27 (44)	$\chi^2 = 0.090$; $P = 0.765$	27 (44)	$\chi^2 = 0.072$; $P = 0.788$	26 (42)	$\chi^2 = 0.069$; $P = 0.793$	25 (40)	$\chi^2 = 0.051$; $P = 0.821$
Male SARS patients with poor outcome[d] (n = 30)	13 (43)	$\chi^2 = 0.590$; $P = 0.443$	12 (40)	$\chi^2 = 0.488$; $P = 0.485$	12 (40)	$\chi^2 = 0.410$; $P = 0.522$	11 (37)	$\chi^2 = 0.746$; $P = 0.388$	11 (37)	$\chi^2 = 0.401$; $P = 0.527$
Male controls (n = 174)	92 (53)		85 (49)		84 (48)		82 (47)		78 (45)	
Female SARS patients[d] (n = 87)	78 (45)	$\chi^2 = 0.002$; $P = 0.962$	77 (44)	$\chi^2 = 0.008$; $P = 0.928$	77 (44)	$\chi^2 = 0.008$; $P = 0.928$	78 (45)	$\chi^2 = 0.064$; $P = 0.799$	73 (42)	$\chi^2 = 0.000$; $P = 0.991$
Female controls (n = 154)	136 (44)		139 (45)		139 (45)		133 (43)		128 (42)	

B. Genotype frequencies and χ^2 analyses (*df* = 2)

Genotype	CC/CT/TT		AA/AG/GG		GG/GC/CC		CC/GC/GG		GG/AG/AA	
Female controls (n = 154)	48/76/30	$\chi^2 = 0.280$; $P = 0.878$	44/81/29	$\chi^2 = 0.099$; $P = 0.952$	44/81/29	$\chi^2 = 0.099$; $P = 0.952$	44/87/23	$\chi^2 = 1.102$; $P = 0.576$	48/84/22	$\chi^2 = 2.315$; $P = 0.314$
Female SARS patients[d] (n = 87)	25/46/16		25/47/15		25/47/15		26/44/17		1/39/17	

[a] The SNP loci are identified according to the University of Washington *ACE2* sequence coordinates *(14)*, and the relevant identification numbers at the SNP database *(15)* are in parentheses.

[b] Genotyping performed with Applied Biosystems Assays-on-Demand™, C2551616_1 and C2551626_1.

[c] The combined male (M) and female (F) control group is used as the comparison group.

[d] The control group of the same gender is used as the comparison group. Because males are hemizygous for *ACE2*, the number of alleles is equivalent to the number of males. Because females have two X chromosomes, the number of alleles is twice the number of females. At each SNP locus, only the minor allele frequency is listed.

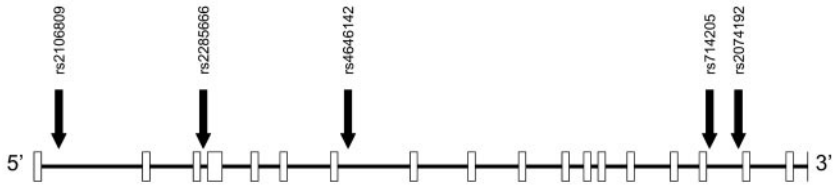

Fig. 1. Schematic illustration of the genomic organization of *ACE2* and positions of the studied SNP loci.

ACE2 contains 18 exons. Each exon is represented by an *open box*. The *arrows* mark the positions of the five verified SNPs. All five SNPs are located within noncoding regions of *ACE2*. The individual SNPs are named according to their identification numbers registered at the SNP database (dbSNP) *(15)*.

inance of female SARS patients, possibly because of the overrepresentation of female healthcare workers *(1)*, male SARS patients were more likely to suffer poor outcomes *(3)*. In a major hospital outbreak in Hong Kong *(4)*, 32% of male and 15% of female SARS patients required intensive care or died. Remarkably, similar demographic data were seen among SARS patients in the greater Toronto area, Canada, where 32% of males and 14% of females with SARS required intensive care or died *(5)*. Karlberg et al. *(3)* studied the case fatality rates among all confirmed SARS patients documented in the Hong Kong SARS epidemic in 2003. The authors concluded that the mortality rates differed significantly between males and females, being 21.9% and 13.2%, respectively. The relative risk for death in males was 1.62 after adjustment for age. It is thus an intriguing coincidence that *ACE2*, the gene for the newly identified functional receptor for the SARS coronavirus, angiotensin-converting enzyme 2, maps to the X-chromosome (Xp22) *(6)*.

ACE2 was first identified as a homolog of angiotensin-converting enzyme with zinc metalloproteinase activity *(7)*. Many of its activities differ from those of angiotensin-converting enzyme *(8)*. ACE2 has been found to be an important regulator of cardiac function *(9)*. Since the identification of ACE2 as the functional receptor for the SARS coronavirus *(6)*, efforts have been spent on characterizing its molecular interaction with the virus *(10, 11)*. On the other hand, studies on mouse hepatitis virus, a group 2 coronavirus *(12)*, demonstrated that allelic variants of viral receptor were associated with altered virus-binding activity, which mediated host susceptibility *(13)*. Hence, it is plausible that genetic variants of *ACE2* may moderate the effects of SARS coronavirus infection and, possibly, gender-specific effects.

For this study, we obtained institutional ethics approval. We identified 103 single-nucleotide polymorphisms (SNPs) in *ACE2*, using the University of Washington and Fred-Hutchinson Cancer Research Center Variant Discovery Resource, SeattleSNPs *(14)*. Two of the identified SNPs were located within the coding regions [dbSNP identification nos. rs4646116 and rs4646179 *(15)*], whereas the remainder were located within the introns of *ACE2*. SNP validation by direct sequencing of the 101 noncoding SNPs was conducted with use of buffy coat DNA obtained from 10 female Chinese volunteers. This validation strategy allows the detection of SNPs with minor allele frequencies of at least 10% at 90% power. The two coding SNP loci were verified on buffy coat DNA from 20 female Chinese. Forty-eight pairs of primers were designed to facilitate direct sequencing of *ACE2* regions spanning all of the SNPs. Buffy coat DNA was extracted according to the Blood and Body Fluid Spin protocol in manufacturer's instructions for the QIAamp DNA Blood Mini Kit (Qiagen). DNA sequencing was performed by the dideoxy dye terminator method on an automated DNA sequencer (3100 Genetic Analyzer; Applied Biosystems) based on capillary electrophoresis. Sequences were edited and aligned, and comparisons were made with the SeqScape software (Applied Biosystems).

SNP validation confirmed sequence variations at five sites (Table 1). All five SNP loci were noncoding. Both of the coding SNPs were shown to be nonpolymorphic among the 20 females. The positions and orientations of the five verified SNPs are illustrated in Fig. 1. A case–control study was conducted to compare the frequencies of the five polymorphisms among 168 SARS patients (81 males and 87 females, among whom 30 males and 16 females had poor outcomes) from the Prince of Wales Hospital, Hong Kong *(4)*, and 328 healthy volunteers (174 males and 154 females). All of the individuals studied were unrelated individuals of Chinese ethnicity. Genotype characterization was performed with TaqMan (Applied Biosystems) allelic discrimination assays on an ABI Prism 7900HT sequence detection system (Applied Biosystems). Each assay consisted of two allele-specific minor groove binding probes with different fluorescent labels, i.e., 6-carboxyfluorescein (FAM) or VIC™, designed for the discrimination of the two respective alleles at each SNP locus. The assays were set up according to the manufacturer's instructions (TaqMan Core PCR Kit; Applied Biosystems) in a reaction volume of 10 μL. The primers and fluorescent probes were used at concentrations of 900 and 200 nM, respectively. We used 10 ng of buffy coat DNA for amplification. The thermal profile consisted of an initial denaturation period for 10 min at 95 °C, followed by 40 cycles of denaturation at 92 °C for 15 s, and 1 min of combined annealing and extension at either 62 °C (SNPs rs2285666, rs4646142, and rs714205) or 65.5 °C (SNPs rs2106809 and rs2074192; Table 1). The genotypes were scored with the SDS2.1 software.

The allele frequencies for the SARS and control groups are listed in Table 1. When we used the allele frequencies obtained from the control group, the group sample size provided a power of at least 80% for the determination of a genetic factor that contributes 50% increased likelihood toward the development of SARS or poor outcome with 95% confidence. Statistical significance among groups was examined by the χ^2 test for each SNP locus (SigmaStat, Ver. 3.0; SPSS). Statistical significance was denoted by a two-tailed $P < 0.05$. No significant difference was observed in the allele distributions between the female and male controls (data not shown),

between the SARS cases and controls, between SARS cases with poor outcomes and controls, between the male SARS patients with poor outcome and the male controls, or between the female SARS patients and female controls (Table 1). The observed genotype distributions for each of the five loci among the female controls did not deviate significantly from those expected from the Hardy–Weinberg equilibrium. The genotype frequencies for each of the five SNP loci were not statistically significantly different between the female SARS patients and the female controls. Because males are hemizygous for *ACE2*, the genotype frequency is equivalent to the allele frequency.

We therefore conclude that although ACE2 serves functionally as the receptor for entry of the SARS coronavirus into human host cells, the evidence provided by this study does not support an association between its common genetic variants and SARS susceptibility or outcome. Despite its X-chromosome location, poor outcomes in male SARS patients do not appear to be related to genetic variants of *ACE2*.

The project team is supported by the Research Fund for the Control of Infectious Diseases (RFCID) from the Health, Welfare and Food Bureau of the Hong Kong SAR Government.

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DOI: 10.1373/clinchem.2004.035436

Comparison of Cystine Determination in Mixed Leukocytes vs Polymorphonuclear Leukocytes for Diagnosis of Cystinosis and Monitoring of Cysteamine Therapy,

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Cystinosis is a rare autosomal recessive disorder caused by mutations of cystinosis gene (*CTNS*; chromosome 17p13), which encodes the lysosomal cystine carrier. The continuous accumulation of cystine in the lysosomes leads to intracellular crystal formation throughout the body. Patients with the common infantile form of cystinosis develop renal Fanconi syndrome 3–6 months after birth and end-stage renal failure before the age of 10 years. Treatment with the aminothiol cysteamine depletes intralysosomal cystine via a disulfide exchange reaction with formation of cysteine-cysteamine mixed-disulfides and cysteine; these exit the lysosomes via lysosomal carriers for lysine and cysteine, respectively *(1)*. When started at an early age, cysteamine treatment prevents or postpones the deterioration of renal function and the occurrence of extrarenal complications such as hypothyroidism, diabetes mellitus, retinopathy, encephalopathy, and myopathy *(1)*.

Accurate measurement of intracellular cystine content is obligatory for the diagnosis of cystinosis as well as for the monitoring of treatment with cysteamine. Historically, cystine has been measured in mixed leukocyte (ML) preparations, despite the fact that it preferentially accumulates in polymorphonuclear leukocytes (PMN) and monocytes *(2)*. We therefore compared intracellular cystine content in ML preparations and in PMN cells of healthy controls, obligate heterozygotes, and patients at diagnosis and under cysteamine therapy. Because the isolation of PMN may pose practical problems in some laboratories, we also investigated whether preservation of whole blood at room temperature influenced intracellular cystine content. If the cystine concentration remains constant, it would allow the shipping of whole-blood samples.

MLs were isolated exactly as described by de Graaf-Hess et al. *(3)*. All solutions were kept at 4 °C. PMN cells were isolated from 10 mL of blood by addition of 2 mL of dextran solution (50 g/L dextran T500, 15 g/L EDTA, 7