

# Molecular Advances in Severe Acute Respiratory Syndrome-associated Coronavirus (SARS-CoV)

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The sudden outbreak of severe acute respiratory syndrome (SARS) in 2002 prompted the establishment of a global scientific network subsuming most of the traditional rivalries in the competitive field of virology. Within months of the SARS outbreak, collaborative work revealed the identity of the disastrous pathogen as SARS-associated coronavirus (SARS-CoV). However, although the rapid identification of the agent represented an important breakthrough, our understanding of the deadly virus remains limited. Detailed biological knowledge is crucial for the development of effective countermeasures, diagnostic tests, vaccines and antiviral drugs against the SARS-CoV. This article reviews the present state of molecular knowledge about SARS-CoV, from the aspects of comparative genomics, molecular biology of viral genes, evolution, and epidemiology, and describes the diagnostic tests and the anti-viral drugs derived so far based on the available molecular information.

**Key words:** severe acute respiratory syndrome, SARS-CoV, genome, phylogenetics, human leukocyte antigen (HLA) system, molecular epidemiology

## Introduction

The first SARS case was reported in late 2002 in China's Guangdong Province (1). The disease was contagious and spreaded rapidly, resulting in a SARS outbreak in Hong Kong in mid-February 2003, and other outbreaks elsewhere in the world. At the end of March 2003, a virus of the *Coronaviridae* family was identified as the causative agent of the disease (2-4). This identification has been confirmed by the World Health Organization, and the virus concerned has been designated as the SARS-associated coronavirus (SARS-CoV). During the SARS outbreaks in 2002 and 2003, SARS cases were identified in 19 countries, and in total 8,605 individuals became infected, of whom 774 died ([http://www.who.int/csr/sars/country/table2003\\_09\\_23/en/](http://www.who.int/csr/sars/country/table2003_09_23/en/)).

In addition to its cost in human lives, the SARS outbreak also had a great impact on the health care system and economy of Hong Kong and other infected regions. In Hong Kong, the estimated economic loss was about HK\$46 billion (US\$5.9 billion; ref. 5). The possibility that SARS-CoV transmission can occur between human beings without reinforcement from the

animal reservoir (5) and the capability of the virus to infect multiple cell types (6) and animals (7) further increased the epidemiological burden of the SARS pandemic. Although the spread of the virus had seemed to be confined by July 2003 through rigorous quarantine measures ([http://www.who.int/csr/sars/country/table2003\\_09\\_23/en/](http://www.who.int/csr/sars/country/table2003_09_23/en/)), it may still be circulating in the animal reservoir and it is impossible to say that it will not return (8-10). Because of this possibility, better monitoring of SARS outbreaks through accurate diagnostic tests and the development of effective anti-viral therapies are urgently required. These in turn depend on better molecular knowledge about the SARS-CoV. Such research is therefore of vital importance if the community is to be properly prepared for a possible recurrence of the SARS pandemic.

## Molecular Biology of SARS-CoV

### Molecular characterization of the SARS-CoV genome

The etiological entity of a viral infection relies on both molecular and traditional virological methods includ-

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ing serological techniques, virus isolation by cell culture, and electron microscopy (2, 10). Both molecular approaches and conventional approaches were employed for the initial characterization of the SARS pathogen (2). Peiris *et al* (2) firstly isolated the virus from *in vitro* tissue culture and subsequently yielded a 646-bp genomic fragment by RT-PCR using degenerate primers, which showed more than 50% homology to the RNA polymerase gene of bovine coronavirus (BCV) and murine hepatitis virus (MHV). The use of gene chip further confirmed the coronavirus as a possible cause of SARS (11).

Soon after the identification of the SARS-CoV, laboratories started to investigate the phylogenetic relationship between the virus and the other members of the same family through extensive comparison of their genome sequences. In mid-April 2003, the British Columbia Cancer Agency (BCCA) Genome Science Center in Canada (12), the Center of Disease Control in the United States (13) and the University of Hong Kong (14) announced at nearly the same time that the complete genome sequence of the SARS-CoV had

been isolated in the corresponding areas (15). The results of independent sequencing of the SARS-CoV genome all indicated that it was a polyadenylated genomic RNA of 29.7 Kb in length. Comparative analysis of the genome with other coronaviruses suggested that the virus genome was very similar to previously characterized coronaviruses, with the order (starting from the N-terminal): replicase (R), spike (S), envelope (E), membrane (M) and nucleocapsid (N) gene, where there are few accessory genes or motifs spanning between the structural genes and at the 3' UTR (untranslated region), which may not be necessary for viral replication (12). The replicase gene, with two open reading frames (ORF) 1a and 1b, covering more than two thirds of the genome, is predicted to encode only two proteinases (12-14) that regulate both the replication of the positive-stranded genomic RNA and the subsequent transcription of a nested set of eight subgenomic (sg) mRNAs (Table 1; ref. 16), which is a common transcription strategy adopted by coronavirus members (17-21).

**Table 1 Features of SARS-CoV Genome Sequence and Subgenomic Transcripts**

g/sg mRNA	ORF				Start-End	No. of a.a.	No. of Bases	Frame
	Thiel <i>et al.</i>	Zeng <i>et al.</i>	Marra <i>et al.</i>	Rota <i>et al.</i>				
mRNA 1	ORF 1a	ORF 1a	ORF 1a	ORF 1a	265-13,398	4,382	13,149	+1
mRNA 1	ORF 1b	ORF 1b	ORF 1b	ORF 1b	13,398-21,485	2,628	7,887	+3
mRNA 2	S protein	S protein	S protein	S protein	21,492-25,259	1,255	3,768	+3
mRNA 3	ORF 3a	X1	ORF 3	X1	25,268-26,092	274	825	+2
mRNA 3	ORF 3b	N/R	ORF 4	X2	25,689-26,153	154	465	+3
mRNA 4	E protein	N/R	E protein	E protein	26,117-26,347	76	231	+2
mRNA 5	M protein	M protein	M protein	M protein	26,398-27,063	221	666	+1
mRNA 6	ORF 6	N/R	ORF 7	X3	27,074-27,265	63	192	+2
mRNA 7	ORF 7a	X2	ORF 8	X4	27,273-27,641	122	369	+3
mRNA 7	ORF 7b	N/R	ORF 9	N/R	27,638-27,772	44	135	+2
mRNA 8	ORF 8a	X3	ORF 10	N/R	27,779-27,898	39	120	+2
mRNA 8	ORF 8b	N/R	ORF 11	X5	27,864-28,118	84	255	+3
mRNA 9	N protein	N protein	N protein	N protein	28,120-29,388	422	1,269	+1
mRNA 9	ORF 9b	N/R	ORF 13	N/R	28,130-28,426	98	297	+2

## SARS-CoV protein products

### 5' and 3' UTR

The 5' UTR of the SARS-CoV genome was characterized by 5' Rapid Amplification of cDNA Ends (5' RACE; ref. 14) and Northern blot assay (13, 16, 22). These procedures elucidated the leader sequence and the transcription regulatory sequence (TRS). The leader sequence found in the viral sg mRNA tran-

scripts is at least 72 nucleotides long. Through the alignment of the leader sequence at the 5' end of the eight sg mRNAs, there is a minimal consensus TRS, namely, 5'-ACGAAC-3', which participates in the discontinuous synthesis of sg mRNAs as a signaling sequence. The degree of sequence variance flanking the TRS showed no clear relationship with the abundance of the sg mRNAs (22). A highly conserved s2m motif with 32 nucleotides was also identified in the 3' region

of the genome, which had also been described in avian infectious bronchitis virus (AIBV; ref. 12-14).

### **Replicase Gene**

The replicase gene of the SARS-CoV encodes for at least two proteins as a consequence of the proteolytic processing of the large polyprotein (ORF 1a and 1b; ref. 16). The translation of segment 1b of such polyprotein is interrupted by the -1 ribosomal frame shifting by a putative "slippery" sequence and a putative pseudoknot structure (16). Two functional domains—papain-like cysteine proteinase (PL2<sup>PRO</sup>) and 3C-like cysteine proteinase (3CL<sup>PRO</sup>), were identified experimentally and were responsible for the proteolytic processing of the polyprotein into 16 subunits (16, 22, 23). A 375-a.a. SARS-CoV unique domain was identified upstream of the PL2<sup>PRO</sup> domain, which is unparalleled in any other known coronaviruses (16). In addition, seven more putative regions encoding RNA processing enzymes were identified, namely, RNA-dependent RNA polymerase (RDRP), RNA helicase (HEL) poly (U)-specific endoribonuclease (XendoU), 30-to-50 exonuclease (ExoN), S-adenosylmethionine-dependent ribose 20-O-methyltransferase (20-O-MT), adenosine diphosphate-ribose 100-phosphatase (ADRP), and a cyclic phosphodiesterase (CPD; ref. 16).

The translation of two polyproteins from ORF 1a and 1b starts the genome expression. The two proteinases, PCL2<sup>PRO</sup> and 3CL<sup>PRO</sup>, are then coupled with the proteolytic processing of the two polyproteins into 16 units. PCL2<sup>PRO</sup> is responsible for the N-proximal cleavage and 3CL<sup>PRO</sup> is responsible for the C-proximal cleavage. The helicase is then released. ATPase activity and DNA duplex-unwinding activity were demonstrated by purified helicase, indicating that the protein has RNA polymerase activity (16, 24).

### **S Gene**

Together with the M protein, the spike protein is believed to be incorporated into the viral envelope before the mature virion is released (17). Initial analysis of the 1255-a.a. peplomer protein of the virus reveals the possible existence of a signal peptide that would likely be cleaved between residues 13 and 14 (12). The whole structure is predicted to contain a receptor-binding unit (S1) in the N-terminus (14, 25-27) and a transmembrane unit (S2) in the C-terminus (13, 14, 25, 27). Molecular modeling of the S1 and

S2 subunits of the spike glycoprotein (26, 28) suggested that the former unit is consisted of mainly anti-parallel  $\beta$ -sheets with dispersed  $\alpha$  and  $\beta$  regions, in addition to the three domains identified in the S2 unit. The confidence level of the predicted molecular models was strengthened by the good correlation between predicted accessibility and hydrophathy profiles and by the correct locations of the N/O-glycosylation sites and most of the disulfide bridges. Whether the experimentally determined N-glycosylated sites from purified spike protein treated by tryptic digest together with PNGase followed by time-of-flight (TOF) mass spectrometry (29) are correctly located in the proposed model remains to be clarified. In the aspect of biological activities, receptors for the binding of the SARS-CoV remain mysterious, as comparative genomics did not point out any significant similarity with the S1 domain of other human coronaviruses, implying that these viruses are using different receptors for cell entry (12). Subsequently, angiotensin-converting enzyme 2 (ACE2) was demonstrated to be a functional receptor for the SARS-CoV *in vitro*. Syncytia was observed in cell culture expressing ACE2 and the SARS-CoV S1 domain, which could be inhibited by anti-ACE2 antibody (30). Fine mapping on the N-terminal unit of the spike protein indicates that the receptor-binding domain is probably located between the residues 303 and 537 (31).

### **ORF 3a**

The sequence of the gene product from ORF 3a shows no homology to any known proteins (12, 14). Signal peptide or a cleaved site is likely to be present in the protein except three predicted transmembrane domains (12). The exact function of the protein is yet to be determined, though the C-terminal of the protein may be involved in ATP-binding properties (12).

### **E Gene**

The envelope protein of the SARS-CoV is thought to be the component of the virus envelope. Topology prediction suggested that the E protein is a type II membrane protein with the C-terminus hydrophilic domain exposed on the virion surface. Comparative protein sequence analysis suggested the SARS-CoV E protein resembles the protein connected with MHV (12, 32, 33).

## *M Gene*

The matrix glycoprotein is not likely to be cleaved (12) and contains three putative transmembrane domains (12-14). Its hydrophilic domain is believed to interact with the nucleocapsid protein and is located inside the virus particle (12). Linear epitope mapping of the M protein using synthetic peptides revealed that amino acid residues 2,137-2,158 interacted with SARS patient sera by ELISA assay, implying the potential capability of the M protein to induce immune response (34, 35).

## *ORF 7a and 8a*

Like ORF 3a, sequence homology search yielded no significant result for any existing proteins, but the existence of a cleavage site (between residues 15 and 16) and a transmembrane helix were predicted. For ORF 7a, it is a putative type I membrane protein (12).

## *N gene*

The N gene sequence showed high homology with the nucleocapsid protein of other coronaviruses. A putative short lysine-rich nuclear localization signal (KTF-PPTPEPKKDKKKKTDEAQ) was identified (12). A potential and well-conserved RNA interaction domain was also identified at the middle region of the gene, in which its basic nature may assist its role (12, 14). The N protein was reported to activate the AP-1 signal transduction pathway, indicating that the protein may play a role in the regulation of the host cell cycle (36). Apart from the possible role in pathogenicity, N gene was also believed to be the most abundant antigen in the host during the course of infection, making it an excellent candidate for diagnostic purposes. The linear epitopes of the protein have been mapped (35, 37, 38), and the possibility of using these antigenic peptides or recombinant proteins in the diagnosis was discussed.

## **Phylogenetic analysis of the SARS-CoV**

### *Protein sequence based on individual ORFs*

The phylogenetic relationship by the comparison of the deduced amino acid sequences of the replicase gene and four structural genes (S, E, M, N) with other coronaviruses was described (12-14). The conclusions drawn by the different research groups were similar, with the observation that SARS-CoV itself forms a distinct cluster—the fourth group of *Coronaviridae*, a

notion supported by the high bootstrap values (above 90%). As a result, it has been concluded that the SARS-CoV is phylogenetically equidistant from all other known coronaviruses. Moreover, no detectable recombination event was concluded in the similarity plot on the whole genome alignment with other coronaviruses (14). The above findings suggest that the SARS-CoV is neither a mutant nor a recombinant of existing coronaviruses, and that the possibility of such a virus emerging as a product of genetic engineering can be excluded, as it is unlikely to generate an infectious coronavirus with 50% of its genome different from the existing coronaviruses (9).

### *Protein sequence based on functional domain of the replicase gene*

Snijder *et al* (22) conducted an extensive phylogenetic analysis concerning the replicase gene of the SARS-CoV by using torovirus as an outgroup. These authors criticized the phylogram construction based on different SARS-CoV proteins as unconvincing, and suggested the possibility that the SARS-CoV can be clustered into an existing group. As the structural and other accessory genes can either be gained or lost throughout the evolutionary process and in view of their low level of conservation, the author decided to target the replicase gene to perform the phylogenetic analysis. For this reason, the phylogenetic relationship was reconstructed through a rooted tree. The construction of the phylogram was done with the fused replicase gene with manual adjustment and exclusion of poorly conserved region. The resulting tree reveals that the gene was mostly related to group 2 coronaviruses and was assigned as a subgroup 2b. The author further pointed out that the SARS-CoV contains homologues of domains that are unique for group 2 coronaviruses, in the region of nsp1 and nsp3 (PL2<sup>PRO</sup>), in addition to the differences in the sequence and arrangements of the 3'-located ORFs, and the lack of antigenic cross-reactivity do not contradict their conclusion, as such a phenomenon was also observed in group 1 coronaviruses.

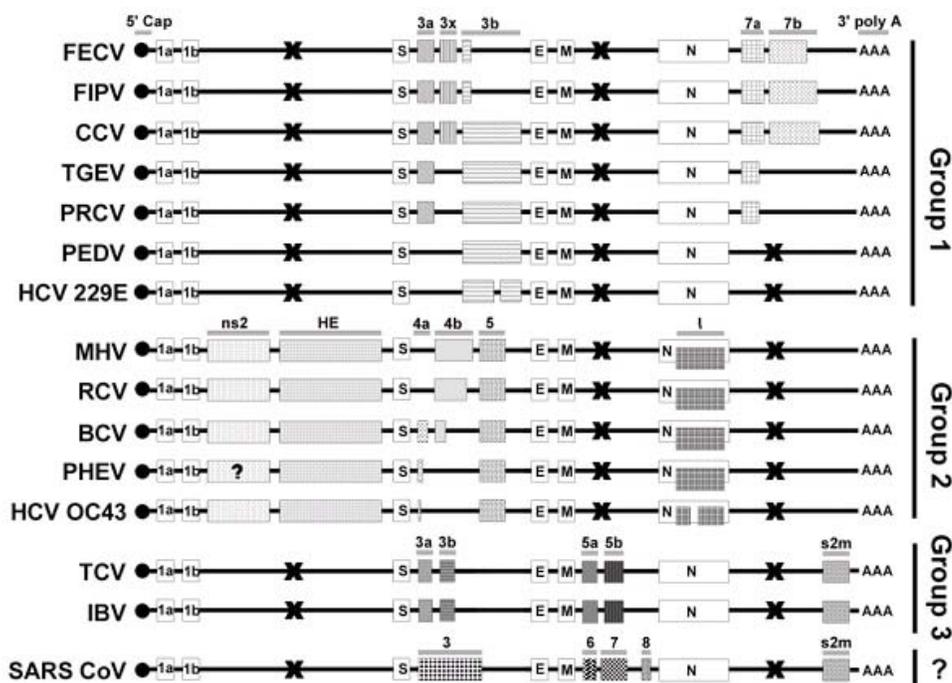
Using Bayesian phylogenetic inference approach, a recombination break point within the SARS-CoV RDRP was identified at protein sequence level (39). Phylogenetic analysis on the 5' end of the domain indicated that it might originate from the common ancestor of all existing coronaviruses, while the same analysis on the 3' end gave another tree topology that

suggests a sister relationship with group 3 avian coronaviruses. These results suggested that a recombination event occurred between the common ancestor of the SARS-CoV and that of other coronaviruses, or alternatively that the 5' fragment of the SARS-CoV diverged before the one between or within other known coronaviruses and the 3' fragment diverged more recently (39).

### Genome organization

Based on the antigenic cross reactivity and genome characteristics, existing coronaviruses are generally classified into three subgroups (40). All coronaviruses share a very similar organization in their functional and structural genes, but the arrangement of the so-called non-essential genes is remarkably different

among the subgroups. Group 1 coronaviruses are mainly characterized by the presence of ORFs following the N gene. Group 2 coronaviruses have two additional ORFs, non-structural protein 2 (ns2) and HE gene, located between ORF 1b and the S gene. Only group 3 species have ORFs located between the M and N gene, and a conserved stem-loop motif s2m at their 3' UTR (Figure 1). Accessory ORFs are found between the S and E genes in all of the subgroups. However, these accessory ORFs within the S-E intergenic region do not seem to be homologous between the subgroups, though they are conserved within subgroups. The rate of evolution of these accessory genes is obviously higher than that of the essential genes, which provides an alternative to access the phylogeny of the coronavirus family.



**Fig. 1** Comparison of accessory genes among all known coronaviruses. The open boxes represent essential ORFs (not drawn to scale) while the shaded boxes represent accessory ORFs/motifs. Homologous ORFs are shaded with the same pattern. The names of the group-specific accessory ORFs were unified and denoted on the top of the corresponding subgroup ORFs. The X (black cross) represents the absence of ORFs within the region. Genome organization and accessory ORFs of these CoVs were confirmed except for the ns2s of PHEV. All the accessory genes are group-specific and highly diverged within subgroups, particular within the S–E intergenic region. SARS-CoV has a very similar genome structure with group 3 CoVs, with two ORFs located between M and N gene, and a conserved stem-loop motif s2m at their 3' UTR. Although the ORF 5a/5b of group 3 CoVs and ORF 5/6 of SARS-CoV are in homologous location, they do not have any significant sequence homology. FECV: feline enteric coronavirus (41-45); FIPV: feline infectious peritonitis virus (41-45); CCV: canine coronavirus (43, 46); TGEV: transmissible gastroenteritis virus (41, 47, 48); PRCV: porcine respiratory coronavirus (41, 47, 48); PEDV: porcine epidemic diarrhea virus (49, 50); HCV 229E: human coronavirus 229E (49, 51); MHV: murine hepatitis virus (52, 53); RCV: rat coronavirus (54); BCV: bovine coronavirus (55); PHEV: porcine hemagglutinating encephalomyelitis virus (56); HCV OC43: human coronavirus OC43 (57, 58); TCV: turkey coronavirus (59-61); IBV: infectious bronchitis virus (62-64).

Based on the confirmed ORFs of the SARS-CoV described above, a comparison of all homologous accessory and essential ORFs of known coronaviruses with the novel SARS-CoV is shown in Figure 1. From the results, it does not seem that the coding regions are a consequence of a newly occurring recombination event between any of the existing known coronaviruses, similar to the conclusion made by Holmes (9). Interestingly, the SARS-CoV genome has a very similar organization to that of group 3 avian coronaviruses (IBV and TCV), with the presence of three ORFs within the M-N intergenic region, two ORFs spanning between the S and E genes (65), and a stem-loop motif s2m in 3' UTR. The presence of s2m and the finding that the 3' fragment of SARS-CoV RDRP clustered into group 3 in the phylogenetic analysis (39) suggest that the avian coronaviruses and the SARS-CoV might share a common ancestor which gained the s2m from a single RNA horizontal transfer event from a non-related virus family, as the astroviruses did (39, 66). Another possibility, that a common coronavirus ancestor had once gained the motif but subsequently lost it, except the group 3 and SARS-CoV, cannot of course be excluded. Pairwise sequence homology search among the accessory ORFs at the S-E intergenic region of the SARS-CoV and all other coronaviruses shows no significant sequence homology (12-14) but they are homologous within subgroups. The ORF 5a/5b of group 3 coronaviruses and ORFs 6-8 of the SARS-CoV are in a homologous location, but they do not have any significant sequence homology. The above results imply that, although the SARS-CoV and group 3 coronaviruses have a very similar genome organization, they might have acquired these accessory genes from several RNA recombination events with different hosts or viral sources. It is observed that the accessory ORFs are group-specific but are usually truncated to a different extent within a subgroup (Figure 1). Another interesting observation is the genetic diversity at the S-E intergenic region. Usually two or three group-specific ORFs are found within this region of each subgroup, but only one confirmed ORF (ORF 3) is found in this region of the SARS-CoV genome (12-14, 16, 22). The diversity (mainly due to truncation and deletion) of these S-E intergenic ORFs within the subgroups is higher than that of other accessory ORFs. Their sequence divergence implies their common ancestors might have acquired these ORFs by RNA recombination, which is a common phenomenon in large RNA viruses (67, 68), rather than evolved

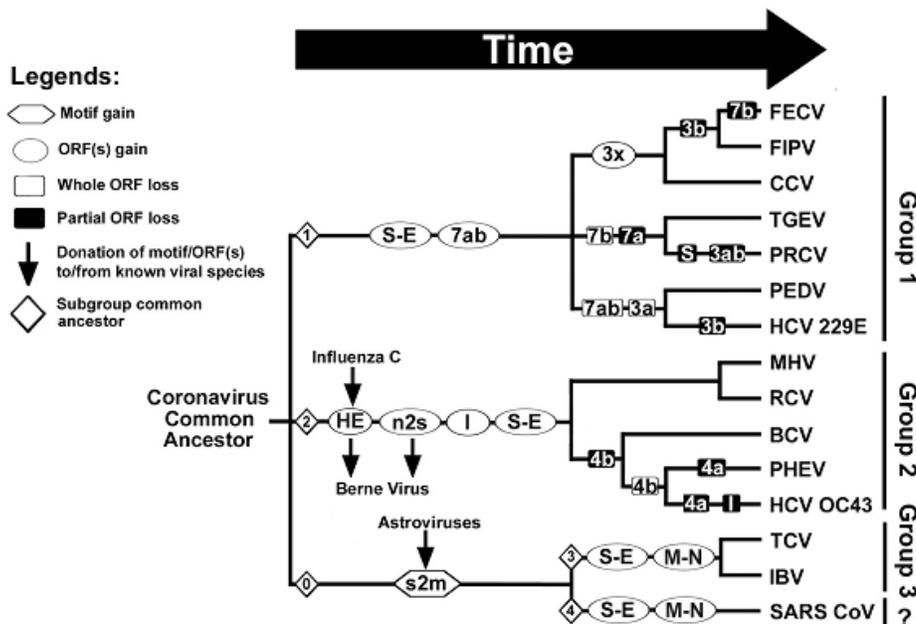
from mutations of a single ancestral RNA sequence segment (9). Typical examples are the acquirement of the HE gene from Influenza C (69) and recombination events with Berne virus at the HE-ns2 region (52).

Based on the recombination and truncation events occurring within these intergenic regions, the phylogenetic relationship between the SARS-CoV and other group 3 coronaviruses has been reconstructed (Figure 2). At least four subgroup common ancestors ( $\diamond$  in Figure 2) have acquired their S-E intergenic ORFs and other group-specific ORFs from several independent RNA recombination events. Moreover, there is a tendency of deletions or truncations of these ORFs when crossing the species barriers within the subgroups, *e.g.* ORF 4a/b in group 2 (54-58); ORF 3a/b and ORF 7a/b in group 1 (41, 42, 47, 48, 50, 70-72). The deletions of these redundant accessory ORFs are likely to be the result rather than the cause of crossing the host barriers, as coronavirus host range specificity and tropism have been demonstrated, at least in four studies (7, 73-75), as determined by the receptor-binding domain of the spike glycoprotein.

Recombination within certain types of viruses is a common phenomenon in various virus families (67), particularly for large RNA viruses, as a means of shedding the deleterious effects of the errors accumulated during its genome replication (68). Recombination events within the coronavirus family (70, 76, 77) or with other non-related virus families (52, 66, 69) have been reported. Apparently, the diversity of the redundant accessory genes has been accompanied by extensive genome rearrangement by heterogeneous or homogenous RNA recombination events, providing useful information for the taxonomy of the coronaviruses. From this point of view, the SARS-CoV is definitely a new and unique member of the coronavirus family. The divergence of these redundant ORFs between the SARS-CoV and other known coronaviruses suggests that the SARS-CoV might have been circulating in other animal hosts long before its emergence, and somehow crossed into a human host several months ago either by a sudden bottleneck mutation event or a RNA recombination event with unknown sources.

### *Animal reservoir*

It has been demonstrated that the SARS-CoV possesses the ability to infect macaques, which display symptoms similar to the clinical signs of SARS patients (78), and to replicate in cats and ferrets (79).



**Fig. 2** Phylogenetic relationship of all known coronaviruses based on the putative RNA recombination events occurred at the accessory ORFs. There are at least four subgroup common ancestors ( $\diamond$  no.1-4) have acquired their redundant accessory ORFs from several independent RNA recombination events. Group 3 CoVs and SARS-CoV may have a common ancestor ( $\diamond$  no.0) which gained s2m from a single RNA horizontal transfer event from a non-related family of astroviruses (see text). There is a tendency of deletions or truncations of these accessory ORFs when crossing the species barriers within the subgroups. The abbreviations of the viral species are shown in the legend of Figure 1.

Together with the evidence implied by the phylogenetic studies, it is tempting to identify the possible animal reservoir of the coronavirus. Recent studies of domestic and wild animals in Guangdong, where the SARS epidemic was first reported, identified the existence of the SARS-CoV from several animals found in the livestock market, including Himalayan palm civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procyonoides*; ref. 80), in spite of the failure of another group to identify any SARS-CoV after the screening of more than 60 animal species (81). The genome sequences of the coronaviruses isolated from these animals are almost identical (99.8%) to that of the SARS-CoV, revealing the extremely close phylogenetic relationship between them. Another major finding from the sequence analysis highlighted a 29-bp deletion upstream the N gene, which was noted only in one Guangdong isolate available from the GenBank (GD01, accession number 278489). Such deletion leads to the fusion of the two ORFs identified in mRNA 8 into one ORF. Yet its biological significance remains to be elucidated (8). Comparison of the S gene nucleotide sequence of the animal and human SARS-CoV indicated 11 consistent nucleotide signature mutations that appeared to distinguish them.

The phylogenetic analysis of the S gene sequence between human and animal SARS-CoV likely ruled out the possibility that it is a consequence of human to animal transmission, implying the infected animals may have acquired the virus from a true animal source that has yet to be identified (80). This was also supported by the host-association analysis of coronaviruses based on the nucleocapsid gene (39), which pinpointed that host-shifts had played an important role in the evolution of the virus and the host. The occurrence of avian-mammal host-shift supports the hypothesis that the SARS-CoV emerged from an unknown animal coronavirus.

## Reverse genetics system

The reverse genetics system, a very useful tool in studying function of viral proteins and its mutations, was firstly described by Master's group (82) for MHV in *Coronaviridae*. In less than six months since the first identification of the SARS-CoV (2), Yount *et al.* (83) developed the reverse genetic systems for this coronavirus using the full-length cDNA clone of Urbani strain, by combining six component clones spanning through the entire genome. Following *in vitro*

transcription and the transfection of the resulting RNA transcripts, a rescued recombinant virus was found to be capable of replication in the same way as the wild type. Expected marker mutations introduced were also identified. The success of the experiment offers hope for the development of attenuated strains of live vaccine against the SARS-CoV (9).

## SARS and human leukocyte antigen (HLA) system

There is considerable scientific interest in the identification of the genetic agents responsible for the unusual susceptibility of the SARS-CoV in some ethnic groups. A molecular survey of the HLA system, a common method adopted to identify autoimmune disorders and emerging infectious diseases, was conducted in Taiwan during the SARS epidemic (84). Using PCR amplification plus sequence-specific oligonucleotide probing (PCR-SSOP), researchers identified the HLA genotype of SARS patients. Healthy, unrelated Taiwanese were used as controls, and the HLA genotype of SARS patients was compared with probable cases and with high-risk, uninfected health care workers. The results indicated that a higher frequency of HLA-B\*4601 allele was

found in severe SARS cases, which may explain the severity of SARS in these patients. Such genotype, as stated in the report, is common in Southern Han Chinese, Singaporeans and Vietnamese, but not in indigenous Taiwanese. There was no reported SARS case within the latter ethnic group. Such findings may explain the unusual SARS epidemic in South Asia.

## Diagnosis of the SARS-CoV

Work on developing a laboratory diagnosis of the SARS-CoV began immediately after the SARS outbreak, although an ideal diagnostic system is still being sought. Numerous protocols have been developed for the diagnosis of infectious viral diseases. Most of these protocols are PCR-based, and the remainder depends on measurable immune response. Several factors affect the choice of proper diagnosis techniques, including time, the availability of equipment and expertise, the biological nature of the available samples, and the requirement of data output format (Table 2; ref. 10). The presence of the virus can be detected by molecular testing such as PCR and virus isolation. Measurable immune responses basically rely on SARS-CoV specific antibodies by enzyme-linked immunosorbent assay (ELISA).

**Table 2 Summary of Properties of Different Diagnostic Methods**

Features/Methods	RT-PCR	Virus isolation	ELISA	IFA	Microarray
Specificity	High	High	Relatively lower	Relatively lower	Relatively lower
Sensitivity	Not very high	Low	High	High	Not very high
Valid duration of +ve result <sup>#</sup>	d1–d10	d1–d10	d21–d31	d1–d31	d1–d10
Valid duration of –ve result <sup>#</sup>	N/A	N/A	d21–d31	d21–d31	N/A
Convenience*	Not very high	Moderate	High	Not very high	Low
Speed	Relatively lower	Slow	High	High	High

<sup>#</sup> Result is defined to be valid after the onset of fever where d=day. \* Convenience means the requirement of expensive equipment and skilled labor.

## Molecular assays

Advances have been made in molecular diagnostic techniques in recent years, and such rapid and sensitive methods allow efficient monitoring of infectious viral diseases. For SARS, the first genetic fragment of the virus was generated by reverse transcriptase-polymerase chain reaction (RT-PCR; ref. 2). Two RT-PCR protocols were then developed by two WHO SARS network laboratories (<http://www.who.int/csr/sars/primers/en>). The sen-

sitivities of the assay were demonstrated to be at least 50%, with the highest percentage found in throat swab specimens (85). No false positive was found in these assays.

The first rapid real-time assay was developed based on the most conserved region of the ORF1b gene sequence (86, 87). A person will be confirmed to be infected by the SARS-CoV if viral RNA is detected by either the two PCR assays, two aliquots of specimen, or two sets of primers ([http://www.cdc.gov/ncidod/sars/specimen\\_collection\\_sars2.htm](http://www.cdc.gov/ncidod/sars/specimen_collection_sars2.htm)). The sec-

ond generation of this test protocol can detect the existence of the virus within 10 days after the onset of fever (87–89) and provides 80% sensitivity and 100% specificity in the testing of 50 NPA samples collected from SARS patients within three days after the onset of the disease (87). To further increase the sensitivity, one-step real-time RT-PCR has been recently developed (89). Specificity of the PCR can be enhanced by coupling it with the use of an additional amplification target using the virus N gene fragment (89), which is theoretically the most abundant subgenomic mRNA produced during transcription (13). The technique provides information on viral load during anti-viral treatments in real time, so that the efficacy of the therapy can be evaluated (10). However, although the PCR assays are powerful, their performance is also technically demanding and labor intensive (10).

The development of microarray technology for viral discovery was firstly described by Wang *et al* in 2002 (90). The capability of the rapid high throughput screening of unknown viral pathogen gives it great potential to be used as a diagnostic tool. In the identification of the SARS-CoV, Wang *et al* (11) employed the use of an improved microarray platform, which comprised conserved 70mers from each of the 1,000 viruses, to characterize the coronavirus genome. Four hybridizing oligonucleotides from *Astroviridae* which share the s2m motif and three from *Coronaviridae* sharing conserved ORF1ab fragment were firstly recognized in the experiment. The sequence recovered from the surface of the microarray further confirmed that it is a member of the coronavirus family. The identity of the SARS-CoV was confirmed within 24 hours, and this feat was followed by the partial sequencing of the novel virus a few days later. Such technique demonstrated a rapid and accurate means of unknown virus characterization through genetic data.

## Virus isolation

Virus isolation by cell culture is used extensively as a traditional technique in virology. Coronavirus presenting in the clinical specimens of SARS patients was detected by inoculating the clinical specimens in cell cultures to allow the infection and the subsequent isolation of the virus. Fetal rhesus kidney (FRhK-4; ref. 2) and vero cells (3) were found to be susceptible to SARS-CoV infection. After the isolation procedure, the pathogen was identified as the SARS-CoV by further tests, such as electron microscopy, RT-PCR, or

immunofluorescent viral antigen detection. Virus isolation is the only means to detect the existence of live virus from the tissue. The methodology is generally employed only for a preliminary identification of an unknown pathogen, as the procedure requires skillful technicians and is time consuming. The requirement of infectious viruses and that the duration of live virus existence varies add on further problems for conducting such assays, but they are nevertheless of very high specificity.

## Enzyme-linked immunosorbent assay (ELISA)

The N protein is usually chosen as the antigen for anti-coronavirus antibody detection assay (91, 92) as it is believed to be a predominant antigen of the SARS-CoV (35, 36). It is also the only viral protein recognized by acute and early convalescent sera from patients recovering from SARS (29). In addition to the N protein, the S protein in the SARS-CoV was also reported as an antigen eliciting antibodies in human body (29), but at a much lower titer than that of the N protein (35, 36).

The assay based on the presence of SARS-CoV antibodies is suggested to be valid only for specimens obtained more than three weeks after the onset of fever (88, 89), although some patients have detectable SARS-CoV antibodies within 14 days of the onset of illness. Nevertheless, the negative result, *i.e.* absence of SARS-CoV antibodies, within the first three weeks cannot conclude that the patient is free of the virus, though the ELISA method was still defined as a good standard for rapid diagnosis of SARS (85). Seroconversion from negative to positive or a four-fold rise in antibody titer from acute to convalescent serum indicates recent infection (<http://www.who.int/csr/sars/diagnostictests/en/>).

## Molecular Epidemiology and Evolution of SARS

The epidemiology of SARS has been extensively investigated since the outbreak of SARS in November 2002 in Guangdong (1). This traditional method was used to access the epidemiology of SARS initially. Molecular epidemiology can be used to trace the disease transmission by using phylogenetic analysis of viral nucleotide sequence, which can quickly identify and aid in monitoring the transmission (93).

In coronavirus, variations in the spike protein can drastically affect viral entry, pathogenesis (94), antiviral immune response (29), virulence (95), cellular (6), or even species tropism (7). The S gene has been used as a target for genotyping most coronaviruses, like human coronaviruses (96) and IBV (97). Study of the N-terminal region of the SARS-CoV spike protein produced similar conclusions by conventional epidemiology methods (98). The investigation included the collection S1 gene sequences from SARS patients in Hong Kong and Guangdong during February-April 2003 mainly by direct sequencing of RT-PCR products derived from clinical specimens, and compared it phylogenetically to additional 27 other sequences available from GenBank. The majority of the Hong Kong viruses, including those from a large outbreak in a high-rise apartment block, Amoy Garden, clustered to a single index case that came from Guangdong to Hong Kong in late February (Figure 3). Most of the viruses derived from Hong Kong patients belong to the same lineage with viruses derived from the Hong Kong index case. Outbreaks in Canada, Singapore, Taiwan and Vietnam were also derived from the SARS-CoV of the same initial virus lineage as judged from the same phylogenetic analysis. A number of viruses derived from the early patients were excluded from the major lineage and formed distinct cluster, implying multiple introductions of the virus have occurred, although these viruses did not caused large-scale outbreaks. Viral sequences identified in Guangdong and Beijing are genetically more diverse (1, 98), implying that the SARS-CoV has been circulating there for a while before the introduction to Hong Kong. The Hong Kong index case that initiated the first super-spreading incident to affect 12 other patients might be simply a matter of chance or the viruses found in that patient were contagious to initiate super-spreading events, but these still need further investigations. Apart from findings that indicate the possible transmission routes, transitional isolates that possess both the characteristics of two lineages were also identified. Ruan *et al* (99) and Tsui *et al* (100) performed similar analysis based on the comparison of full genome sequences of different SARS-CoV isolates. They independently identified some of the variations, as Guan *et al* (98) did. Chiu *et al* (101) have recently identified the nucleotide substitution in the S gene that is unique to the Taiwan isolates and was linked to the Hong Kong index case. Sequence comparison of the Amoy Garden isolates revealed no significant variations within the S1 gene, or across the whole

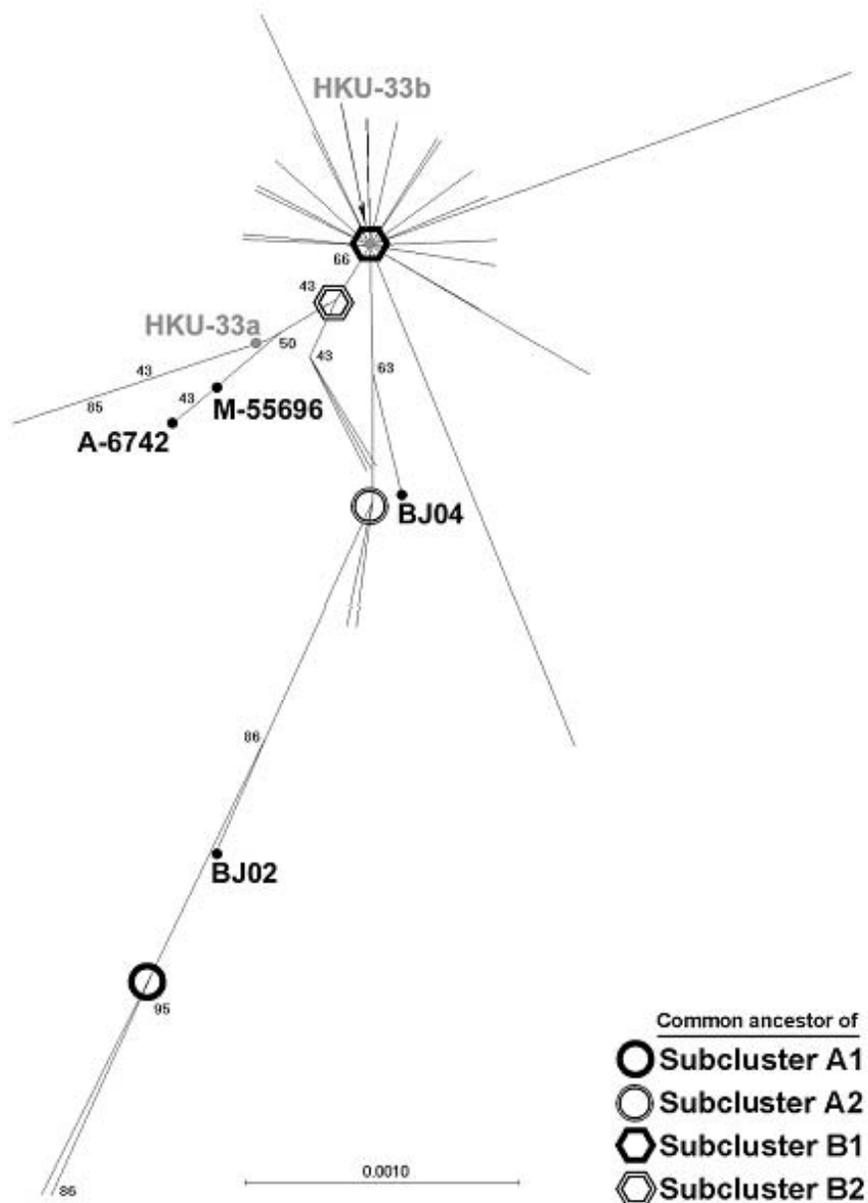
genome, implying that other non-viral factors may contribute to the abnormal transmission and clinical presentation of SARS in this cluster of high-rise apartments (98, 102). In summary, the transmission route of the SARS-CoV in different countries and areas correlates well with the traditional epidemiological findings, implying the successful application of molecular epidemiological techniques in tracing the virus transmission history.

Concerning viral evolution, Zeng *et al* (103) have performed a linear regression analysis and tried to estimate the last appearance of the SARS-CoV common ancestor. With such effort, which has been successfully applied in timing of the ancestral sequence of human immunodeficiency virus (HIV; ref. 104), the ancestral sequence is believed to have appeared last in late 2002. These preliminary findings provide important information for tracing the origin of the SARS-CoV and monitoring its spread.

## Immunity, Vaccination and Antiviral Drug Design

Current knowledge on coronavirus immunity has mainly been acquired from research on animal coronaviruses. Clinical observations have shown that humoral and cell-mediated immune responses may be both necessary against SARS-CoV infection (105). It was reported that T cell (CD3+, CD4 and CD8+) depletion was observed in early infection, but that levels returned to normal as the disease was improved (106). IgG antibody could be detected at the 7th day after the onset of symptoms and kept at high titer at least three months (107). Another report indicated that the virus was still detectable in respiratory and stool specimens by RT-PCR diagnosis but could not be cultured more than 40 days after presentation (108), implying that the antibody could be stimulated rapidly and might restrict the virus infection. However it has also been reported in fowl and feline coronaviral diseases that low-level antibody may exacerbate diseases (109). It is therefore important to conduct further investigations into the immune response to SARS patients in the future so as to benefit the vaccine development and disease control.

Concerning the candidate target for vaccine development, the S1 unit of the spike proteins has been identified as the host protective antigen and used as a vaccine candidate in other coronaviruses (110). An extensive structural analysis of the corresponding pro-



**Fig. 3** Phylogenetic analysis of 169 SARS-CoV spike genes. Unrooted trees were constructed based on the optimal alignment by neighbor-joining method using MEGA 2. Number at the nodes indicates bootstrap values in percentage. The branch length shows the genetic distance with reference to the horizontal scale bar. All sample names were hidden for the convenience of display, except the index case isolate HKU-33 (gray) and subcluster transition isolates (dark). The locations of these isolates on the tree were pinpointed by dots besides their names. The hypothetical common ancestors of the subclusters were highlighted as described in the right bottom of the figure.

tein in SARS is thus desirable. With the identification of the SARS-CoV functional receptor (30) and the mapping of the receptor-binding domain on the spike protein (31), subunit vaccine targeting the receptor-binding domain and the preparation of killed or attenuated vaccine using ACE2 expression cell line may be promising (30).

Antiviral drugs represent an alternative anti-SARS strategy to vaccination. Inhibiting chemicals

targeting the SARS-CoV replication-related proteins were considered as anti-SARS-CoV drug candidates, *e.g.* inhibition the enzymatic activity of 3CL<sup>PRO</sup>. An extensive structural analysis of 3CL<sup>PRO</sup> encoded from nsp5 on ORF 1a was performed (28, 111). The 3CL<sup>PRO</sup> structure showed a considerable degree of conservation of the substrate-binding sites, with the evidence that it could retain its proteolytic activity upon TGEV (transmissible gastroenteritis virus)

main proteinase (111), though another group mentioned that the inactive property of the enzyme might exist *in vitro* (112). From this result, these authors suggested that the use of rhinovirus 3C<sup>PRO</sup> inhibitor might be useful in anti-SARS therapy. Two months later, a research group from the US conducted a study on the interaction of two chemicals (KZ7088 and the AVLQSGFR octapeptide) with 3CL<sup>PRO</sup> (113), further highlighting the importance of the main proteinase as a target for anti-viral drug design. Fan *et al* (114) provided valuable additional information, and concluded that only the dimeric form of the 3CL<sup>PRO</sup> is active and that the proteinase-substrate interaction can be speeded up if more beta-sheet-like structure is involved in the substrate. Recently the crystal structure of 3CL<sup>PRO</sup> was reported by Yang *et al* (115). The 3CL<sup>PRO</sup> crystal underwent conformational changes under different pH conditions while complexing with the specific inhibitor at the same time. A serine-protease fold with a Cys-His at the active site was recognized. On the other hand, the modeling of the structure of 20-O-MT domain located at nsp16 was proposed by von Grotthuss *et al* (116) using the 3D jury system with high reliability (3D jury score >100). The conservation of the unique tetrad residues K-D-K-E of the domain assigned a proposed mRNA cap methylation function of this domain, suggesting an alternative target for anti-viral drug design. In addition to main proteases, blocking the virus entry should be considered as well. Structural analysis of the S2 domain of the SARS-CoV S protein, which plays a role in fusion of the virus with host cell, revealed a conservation of sequence motifs with the well-studied gp41 protein of HIV-1 and other viruses with class I transmembrane domain (27). Such a structure may be another target for drug design.

## Conclusion

The collaborative efforts of the global scientific community have provided invaluable insights into the molecular biology of the SARS-CoV. The development of a rapid and accurate method of diagnosis based on the molecular findings has helped to identify SARS patients at an early stage of the disease, thereby providing valuable information for national authorities to monitor the spread of the disease and take effective quarantine measures, and contributing to the understanding of the clinical presentations of the syndrome. The elucidation of the molecular biology of

the SARS-CoV has provided a foundation for vaccine design and narrowed down the targets for large-scale high throughput drug screening program for anti-viral therapy. These advances helped the global community to contain the spread of SARS within four months since its first identification. However, much remains to be discovered about this novel coronavirus, and it may yet pose a serious threat. Unlike other recently identified viral diseases like Ebola and West Nile virus, it seems the transmission of SARS-CoV does not need a visible vector for spreading, and that a tiny, invisible, respiratory droplet is sufficient to infect another person (117). The nearly undetectable symptom presented by the recently confirmed SARS case in Singapore suggests that the virus may continue to circulate undetectably (65). The possibility that common domestic animals are also a virus reservoir for SARS further complicates the struggle to contain and ultimately eradicate this disease. In these aspects, sensitive, accurate and rapid diagnosis plays an extremely important role in limiting the disease spread, especially in the developing world and densely populated countries. Luckily, the aggressive quarantine measures imposed by the WHO proved to be effective in containing the outbreak, and the experience gained in the last SARS outbreak has prepared us to face another outbreak with some confidence. Nevertheless, nobody can predict exactly when an effective vaccine or anti-viral drug will be developed. All that can be said is that, based on our growing knowledge of the molecular epidemiology and evolution of the virus, the successful development of countermeasures to SARS is very possible.

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