Verification of SARS-CoV-2-encoded small RNAs and contribution to infection-associated lung inflammation

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To the Editor: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus causing coronavirus disease 2019 (COVID-19).[1] As a new evolutionary branch within coronaviruses, SARS-CoV-2 contains around 29.8 kilobase and shows 79.2% identity with SARS-CoV-1.[2] As the successor to SARS-CoV-1, SARS-CoV-2 shares a highly similar sequence and behavior pattern with SARS-CoV-1.[3] Recent study found that SARS-CoV-1 encoded 18 to 22 nt small viral RNAs (svRNAs), which were independent of RNase III, cell type, and host species but relies on the extent of viral replication.[3] Evidence has supported that svRNAs could be generated from cytoplasmic RNA viruses, relevant to viral pathogenicity.[3] As shown in SARS-CoV-1, inhibiting one of the svRNAs named svRNA-N reduced *in vivo* lung pathology and inflammation.[4] We verified the existence of two SARS-CoV-2-encoded small RNAs and investigated the pro-inflammatory effects during their maturation in human bronchial epithelial cell line (16HBE), which might provide new insight into the pathogenesis and possible treatment options for COVID-19.

The study was conducted with the approval of the Ethics Committee of Jiangsu Provincial Center for Disease Control and Prevention (No. JSJK2020-B003-01). On the basis of homology between SARS-CoV-1 and SARS-CoV-2, we compared the ten most abundant SARS-CoV-1-encoded svRNAs with SARS-CoV-2 genome and proposed six potential svRNAs encoded by SARS-CoV-2 listed in Supplementary Table 1, http://links.lww.com/CM9/A986. We verified real existence of two out of six svRNAs in five SARS-CoV-2-infected nasopharyngeal swabs obtained from the Jiangsu Provincial Center for Disease Control and Prevention by poly(A) polymerase tailing followed by reverse transcription polymerase chain reaction (RT-PCR) combined with pyrosequencing, two formalin-fixed paraffin-embedded (FFPE) lung tissues from lung transplantation of two SARS-CoV-2 positive patients provided by the Department of Forensic Medicine, School of Basic Medicine, Nanjing Medical University and 16HBE cells transfected with svRNAs precursor by SYBR Green based stem-loop RT-PCR combined with pyrosequencing shown in Figure 1A and 1B. Materials and methods are presented in [Supplementary Tables 2 and 3, http://links.lww.com/CM9/A986 and Supplementary Figure 1, http://links.lww.com/CM9/A986]. Three non-infected control lung tissues were from the Department of Pathology, the First Affiliated Hospital of Nanjing

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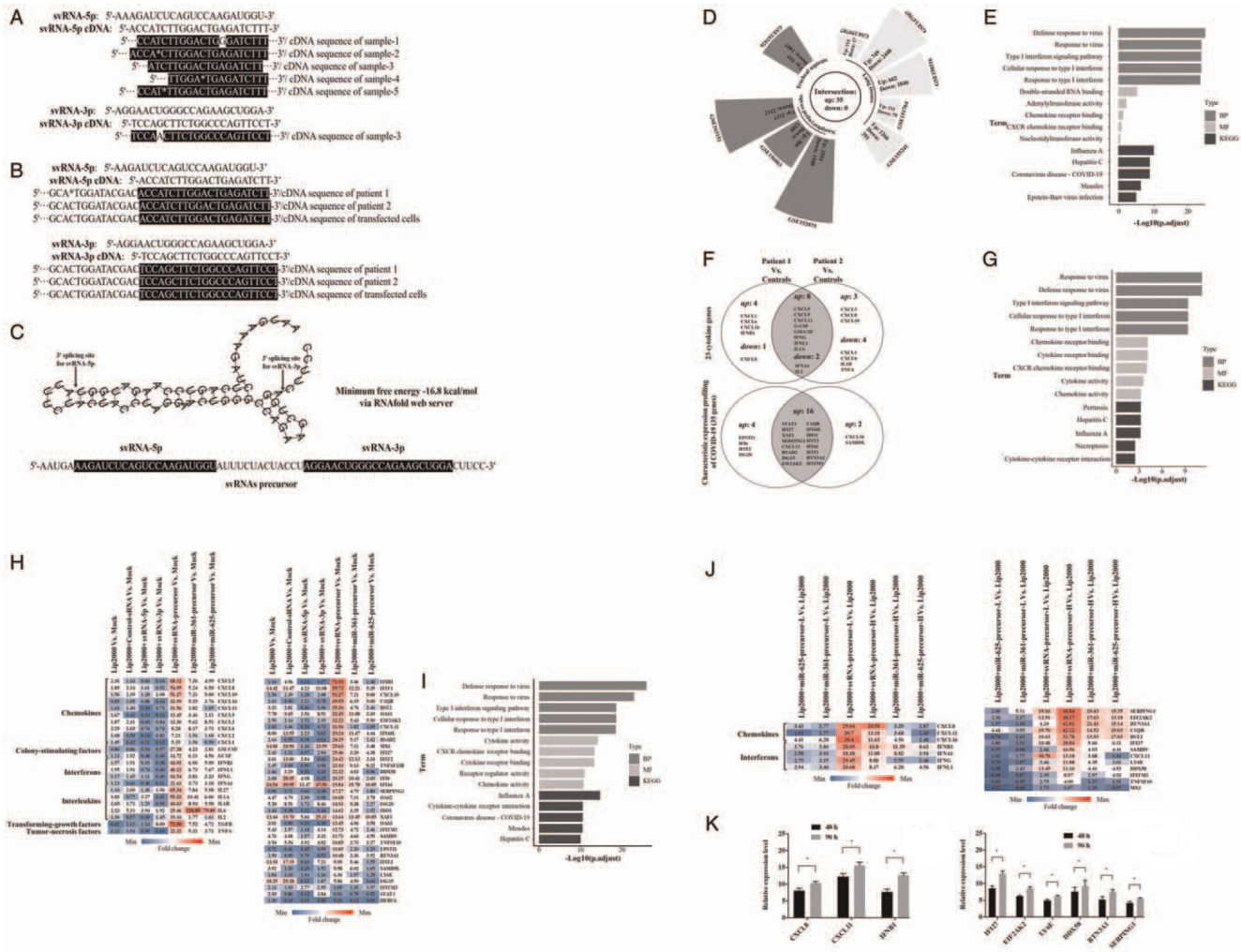

Figure 1: The pyrosequencing of the poly(A) RT-PCR and stem-loop RT-PCR products in five virus-infected pharyngeal swabs (A), two virus-infected explanted lungs, and 16HBE (human bronchial epithelial cell line) cells transfected with svRNAs precursor (B). The secondary structure of svRNA precursor with the length of 66 bp via RNAfold web server (C). The characteristic expression profiling of COVID-19 screened from database (D) and GO and KEGG functional enrichment analysis (E). RT-qPCR assay analysis of cytokine genes and characteristic expression profiling of COVID-19 (F) and GO and KEGG analysis (G). 16HBE cells transfected with 40 pmol control svRNA, control miR-361 precursor, control miR-625 precursor, svRNA-5p, svRNA-3p, and svRNAs precursor at 48 h post-infection (H). GO and KEGG functional enrichment analysis of differentially expressed genes (I). 16HBE cells transfected with control endogenous short RNA precursors and svRNA precursor at the high (H) and low (L) dosage of 40 and 80 pmol (J) and at 48 or 96 h post-infection (K). $n = 3$. $P < 0.05$. BP: Biological process; cDNA: Complementary DNA; COVID-19: Coronavirus disease 2019; CXCR: C-X-C chemokine receptor; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecular function; miR: MicroRNA; Poly (A) RT-PCR: Poly A polymerase tailing followed by reverse transcription polymerase chain reaction (RT-PCR); RT-qPCR: Reverse transcription quantitative polymerase chain reaction; svRNAs: Small viral RNAs.

Medical University. Notably, we found these two svRNAs (svRNA-5p and svRNA-3p) probably matured from the same precursor by exploring the secondary structure of svRNA precursor via RNAfold web server as shown in Figure 1C and Supplementary Figure 2, http://links.lww.com/CM9/A986. The potential sequences of svRNAs precursor were listed in Supplementary Table 4, http://links.lww.com/CM9/A986.

To depict pulmonary inflammation feature of COVID-19 at transcriptional level, firstly, nine sequencing datasets were screened from Gene Expression Omnibus database [Figure 1D and Supplementary Table 5, http://links.lww.com/CM9/A986] and 35 genes were found significantly up-regulated in COVID-19 patients named as characteristic expression profiling of COVID-19 [Supplementary Table 6, http://links.lww.com/CM9/A986]. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis were shown in Figure 1E. Then 23 cytokine storm genes [Supplementary Table 7, http://links.lww.com/CM9/A986] and characteristic expression profiling of 35 genes, were included for RT-qPCR assay in the mentioned FFPE lung tissues. Sixteen out of thirty-five genes and eight from 23 cytokine genes related to chemokines (chemokine C-X-C motif ligand (CXCL) 5 [*CXCL5*], *CXCL9*, *CXCL11*), interferons (interferon [IFN] gamma [*IFNG*], interferon lambda-1 *[IFNL1])*, granulocyte colony-stimulating factor, granulocyte-macrophage colony stimulating factor, and interleukin 1 alpha, were commonly over-expressed in both two infected lung tissues shown in Figure 1F. All results were in Supplementary Tables 8 and 9, http://links.lww.com/CM9/A986. We also conducted GO and KEGG functional enrichment analysis [Figure 1G]. As shown in

Figure 1E and 1G, type I interferon biological process and chemokine molecular activation were obviously involved in SARS-CoV-2 induced inflammation.

To explore pro-inflammatory effects of svRNAs, 16HBE cells were transfected with 40 pmol immune-associated endogenous short RNA precursor controls (microRNA [miR]-361 precursor and miR-625 precursor), the svRNAs precursor, mature svRNAs and control at 48 h postinfection. Materials and methods were in [Supplementary Table 10, http://links.lww.com/CM9/A986]. Those cells transfected with svRNA precursor exhibited the most remarkable inflammatory reaction, while no distinguishing reaction in cells transfected with mature svRNAs was observed, as shown in Figure 1H. The GO and KEGG functional enrichment analysis was presented in Figure 1I.

To further estimate the pro-inflammatory effects of svRNA precursor, cells were transfected with exogenous and endogenous precursors at different dosages (40 and 80 pmol) and time courses (48 h post-infection and 96 h postinfection). No obvious dose dependence between svRNA precursor and inflammation reaction was observed. Seven cytokine genes (*CXCL8, CXCL11, CXCL16, IFNA1, IFNB1, IFNG,* and *IFNL1*) and thirteen genes within characteristic expression profiling of COVID-19 were over-expressed consistently at two dose levels as shown in Figure 1J and Supplementary Figure 3, http://links.lww.com/CM9/A986. Three of seven cytokine genes (*CXCL8, CXCL11*, and *IFNB1*) and six of thirteen genes (interferon alpha inducible protein 27 [*IFI27*], eukaryotic translation initiation factor 2 alpha kinase 2 [*EIF2AK2*], lymphocyte antigen 6 family member E [*LY6E*], DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 58 [DDX58], butyrophilin subfamily three member A1 *[BTN3A1*], and serpin family G member 1 *[SERPING1])* were commonly up-regulated in cells transfected with svRNA precursor at different time courses and the gene expression levels were higher in cells at 96 h post-transfection as shown in Figure 1K, which showed to be time dependent. Taken together, the svRNA precursor contributed to pulmonary inflammation, mainly via activation of *CXCL8, CXCL11,* and type I interferon signaling pathway.

It was worthy of note that these two svRNAs might mature from the same precursor and be located near the genome ends. This pattern was also found in other RNA viruses, suggesting that these svRNAs were not produced randomly or just degradation fragments. Exactly, the previous study had reported that the SARS-CoV-1-derived svRNAs were mainly generated independent of canonical cellular pathways, but dependent on alternative mechanisms which were still unknown.[5] Further research on the svRNAs biogenesis is needed and might be valuable to control the exacerbated host immune response caused by SARS-CoV-2.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients or their authorized representatives have given their consent for their information to be reported in the journal. The patients or their authorized representatives understand that their names and initials will not be published and due efforts will be made to conceal their identity but anonymity cannot be guaranteed.

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Conflicts of interest

None.

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