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•LETTER TO THE EDITOR•

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SARS-CoV-2-encoded nucleocapsid protein acts as a viral suppressor of RNA interference in cells

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Dear Editor,

Coronaviruses (CoVs) are large enveloped non-segmented positive-strand RNA viruses that broadly distribute among humans and other animal species, including bats, mice and birds. SARS-CoV-2 infections can cause diseases, named the 2019 novel coronavirus disease (COVID-19). The symptoms of COVID-19 range from mild symptoms to severe respiratory syndromes, including pneumonia, and even death (Chen et al., 2020b; Jiang and Shi, 2020; Xia et al., 2020). So far, the COVID-19 outbreak has been reported to cause more than 1,353,000 confirmed cases, and has been declared by the WHO as a global public health emergency.

RNAi is a post-transcriptional gene silencing mechanism that is evolutionarily conserved in all eukaryotes and has been recognized as a cell-intrinsic antiviral immune defense mechanism in diverse eukaryotes including mammals (Ding et al., 2018). In antiviral RNAi, viral infection and replication generates virus-derived dsRNA (vi-dsRNA), which could be recognized and cleaved by the host endoribonuclease Dicer into virus-derived siRNAs (vsiRNAs). These vsiRNAs are integrated into the Argonaute protein within the RNA-induced silencing complex (RISC) to direct the destruction of cognate viral RNAs in infected cells in a sequence-specific manner. As a countermeasure, viruses encode viral suppressors of RNAi (VSRs) to antagonize the RNAi pathway at different steps. For example, Influenza A virus (IAV) NS1, human enterovirus A71 (EV-A71) 3A and Dengue virus 2 (DENV2) NS2A have been identified to act as VSRs to inhibit vsiRNA production and antiviral RNAi response in the context of viral infections (Qiu et al., 2020; Ding et al., 2018). Thus, the understanding of how SARS-CoV-2 interacts with antiviral RNAi is important for the better knowledge of this novel coronavirus, and may contribute to the efforts of controlling the spread of infections and developing effective therapies.

Previous study has reported that severe acute respiratory syndrome coronavirus (SARS-CoV) nucleocapsid (N) protein displayed a VSR activity in mammalian cells via a cellular reversal-of-silencing assay (Cui et al., 2015). Given the high homology (94%) of the amino acid sequences among coronavirus N proteins (Figure S1 in Supporting In-

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formation), it is intriguing to examine whether SARS-CoV-2 N also has the VSR activity. Therefore, in this study, we evaluated the role of SARS-CoV-2 N in the suppression of RNAi in cultured human cells. We first examined whether SARS-CoV-2 N possessed VSR activity via a classic reversal-of-silencing assay, in which enhanced green fluorescent protein (EGFP)-specific shRNA was transfected into EGFP-expressing 293T cells, together with a plasmid encoding SARS-CoV-2 N protein with Flag tag. At 48 h post-transfection (hpt), EGFP protein levels were examined via fluorescent microscopy and Western blotting. EGFP-specific shRNA expression resulted in low EGFP protein levels (Figure 1A, panel "Vec"; Figure 1B, lane 2), confirming the efficiency of shRNA in this RNAi system. Our data showed that expression of SARS-CoV-2 N markedly restored the protein level of EGFP (Figure 1A, panel "N"; Figure 1B, lane 3), indicating that SARS-CoV-2 N displays the VSR activity in cells. Of note, the ectopic expression of Ebola virus (EBOV) VP35, a well-characterized VSR, suppressed the shRNA-induced RNAi as expected (Figure 1B, lane 4).

Because RNAi directly results in the cleavage and degradation of target mRNAs, we further examined the VSR activity of SARS-CoV-2 N using the reversal-of-silencing system via Northern blotting with a digoxin (DIG)-labeled RNA probe targeting EGFP ORF 1–400 nt. Our results showed that SARS-CoV-2 N markedly restored the EGFP mRNA levels in 293T cells (Figure 1C). Together, our data show that SARS-CoV-2 N protein has the VSR activity in cultured human cells.

Having established that SARS-CoV-2 N contains VSR activity, we sought to examine the mechanism of how SARS-CoV-2 N antagonizes RNAi. During the dsRNA/shRNA-induced RNAi, dsRNA/shRNA is initially recognized and cleaved by Dicer into siRNA. Thus, we examined whether SARS-CoV-2 N can sequestrate dsRNA via the RNA-IP assay. In brief, 293T cells expressing Flag-tagged N or empty vector, together with EGFP-specific dsRNA (EGFP ORF 1–200 nt) were lysed and immunoprecipitated with anti-Flag or mouse IgG antibodies, respectively. The RNAs extracted from the RNA-IP precipitates were then examined via Northern blotting using RNA probes targeting the 1–200 nt dsRNA of EGFP. Our results showed that SARS-CoV-2 N does associate with dsRNA in 293T cells (Figure 1D), implying that the mechanism by which SARS-CoV-2 N suppresses RNAi is to sequestrate dsRNA in cells, which probably prevents the recognition and cleavage of viral dsRNA by Dicer.

In the process of RNAi, Dicer-cleaved siRNAs are required to assemble siRNA-incorporated RISC to direct the degradation of cognate RNAs, which is the effector step of RNAi (Ding et al., 2018). After establishing that SARS-CoV-2 N can associate with dsRNA, we further examined whether SARS-CoV-2 N could also suppress siRNA-induced RNAi. To this end, we co-transfected SARS-CoV-2 N expression vector and chemically synthesized EGFP-specific siRNA into 293T cells expressing EGFP. The effects of RNAi were determined via fluorescent microscopy (Figure 1E), Western blotting to detect EGFP protein expression (Figure 1F), or via Northern blotting to detect EGFP mRNA level (Figure 1G). EGFP-specific siRNA reduced the protein and mRNA levels of EGFP, while the ectopic expression of SARS-CoV-2 N efficiently restored the expression of EGFP in both the protein and mRNA levels (Figure 1F and G). EBOV VP35 was used as a positive control (Figure 1F and G). Our findings indicate that SARS-CoV-2 N can suppress siRNA-induced RNAi in cells, implying that SARS-CoV-2 N antagonizes RNAi in the effector step, either. The methods and the primers (Table S1) used in this study are shown in Supporting Information.

The emergence of SARS-CoV-2 outbreak has caused a serious threat to human health and tremendous economic loss in China and across the globe, which pushes us to obtain the knowledge about all aspects of characteristics of this novel coronavirus as quickly as possible. In this study, we found that the SARS-CoV-2-encoded structural protein N displayed VSR activity in cultured human cells. Our findings showed that SARS-CoV-2 N can antagonize RNAi in both initiation (i.e., siRNA biogenesis) and effector (i.e., RISC assembly and target RNA cleavage) steps. Our findings showed that SARS-CoV-2 N can antagonize RNAi induced by either shRNA or synthetic siRNA. RNAi is initiated by shRNA after it is processed into siRNA by Dicer, whereas siRNA-induced RNAi requires the assembly of the synthetic duplex siRNA into mature RISC effector complex.

The finding that SARS-CoV-2 N suppresses RNAi in cells is consistent with the previous observation that SARS-CoV N also displayed VSR activity (Cui et al., 2015), implying that using N protein as the VSR is a common strategy for coronaviruses to antagonize antiviral RNAi. Moreover, the residues Lys 258 and Lys 262 that were shown to be critical for the VSR activity of SARS-CoV N were also conserved within N protein of SARS-CoV-2 (Figure S1 in Supporting Information). In addition to N protein, previous study has identified that SARS-CoV 7a could suppress RNAi in mammalian cells (Karjee et al., 2010), suggesting that coronaviruses may antagonize RNAi by encoding multiple VSRs. Considering the high homology of the amino acid sequences of 7a proteins between SARS-CoV-2 and SARS-CoV, it is possible that SARS-CoV-2 7a may also contain the VSR activity. Encoding multiple VSRs may offer these pathogenic viruses extra advantages for efficient inhibition of RNAi, highlighting the importance of antiviral RNAi for host cells in defending viral infection.

Coronavirus N protein contains nonspecific RNA-binding activity (Takeda et al., 2008). In this study, we also found that SARS-CoV-2 N could associate with dsRNA in cells. Our

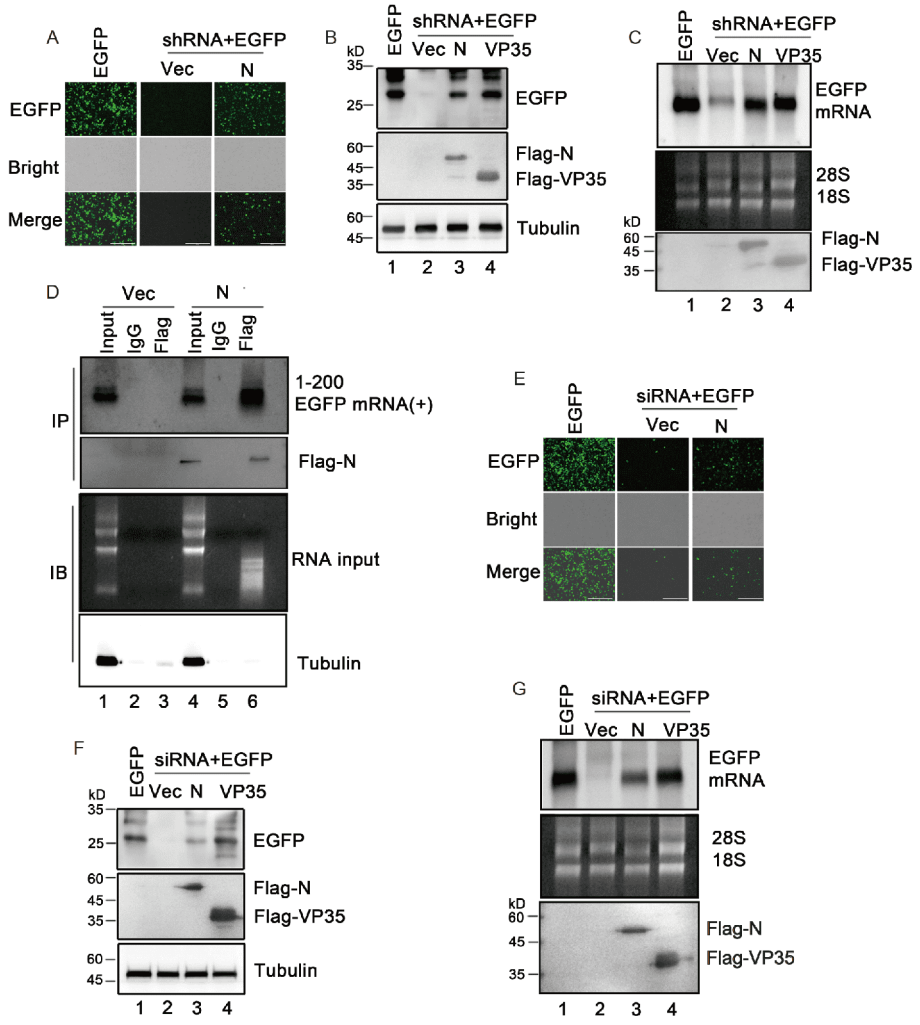

Figure 1 SARS-CoV-2 N suppressed RNAi in cultured human cells. HEK293T cells were co-transfected with plasmids encoding EGFP (0.1 μg) and EGFP-specific shRNA (0.3 μg), together with either empty vector or the plasmid encoding SARS-CoV-2 N or EBOV VP35 (2 μg each). A, At 48 hpt, cells were observed via fluorescent microscopy. Scale bar, 400 μm. B, Cell lysates were harvested and analyzed by Western blotting with anti-EGFP, anti-FLAG and anti-Tubulin antibodies. C, Total RNAs were extracted and EGFP mRNA levels were examined by Northern blotting with a DIG-labeled RNA probe targeting EGFP ORF 1–400 nt. 18S and 28S rRNAs were used as loading controls. D, HEK293T cells were transfected with the plasmid encoding SARS-CoV-2 N or empty vector, together with EGFP-specific dsRNA. At 24 hpt, the cell lysates were subjected to RNA-IP with anti-FLAG or anti-IgG antibodies. Input and precipitated RNAs and proteins were detected by Northern blotting and Western blotting, respectively. HEK293T cells were co-transfected with the plasmid encoding EGFP (0.1 μg) and EGFP-specific siRNA (0.3 μg), together with either empty vector or the plasmid encoding SARS-CoV-2 N or EBOV VP35 (2 μg each). E, At 48 hpt, cells were observed via fluorescent microscopy. Scale bar, 400 μm. F, Cell lysates were harvested and analyzed by Western blotting with anti-EGFP, anti-FLAG and anti-Tubulin antibodies. G, Total RNAs were extracted and EGFP mRNA levels were examined by Northern blotting. 18S and 28S rRNAs were used as loading controls.

results that SARS-CoV-2 N suppressed RNAi by sequestrating dsRNA are consistent with the previous findings that coronavirus N is directly involved in viral RNA replication (Almazán et al., 2004). Moreover, the RNA-binding of SARS-CoV N was shown to be critical for its antagonism of interferon induction (Lu et al., 2011). During viral life cycle, coronavirus N protein encapsulates viral genomic RNAs to protect the genome and co-enter the host cell with viral genomic RNAs, indicating that N is important for viral RNA replication, especially at the initiation step.

In summary, SARS-CoV-2 can act as a VSR in cells in both initiation and effector steps of RNAi, thereby probably representing a key immune evasion factor of SARS-CoV-2 and contributing to the pathogenicity of this novel coronavirus. Our study extends our knowledge about the interaction between antiviral RNAi immunity and SARS-CoV-2 in a timely manner and may be helpful in the efforts of controlling this dangerous virus.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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References

Almazán, F., Galán, C., and Enjuanes, L. (2004). The nucleoprotein is required for efficient coronavirus genome replication. J Virol 78, 12683–12688.

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qiu, Y., Wang, J., Liu, Y., Wei, Y., et al. (2020b). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in wuhan, china: A descriptive study. Lancet 395, 507–513.

Cui, L., Wang, H., Ji, Y., Yang, J., Xu, S., Huang, X., Wang, Z., Qin, L., Tien, P., Zhou, X., et al. (2015). The nucleocapsid protein of coronaviruses acts as a viral suppressor of RNA silencing in mammalian cells. J Virol 89, 9029–9043.

Ding, S.W., Han, Q., Wang, J., and Li, W.X. (2018). Antiviral RNA interference in mammals. Curr Opin Immunol 54, 109–114.

Jiang, S., and Shi, Z.L. (2020). The first disease x is caused by a highly transmissible acute respiratory syndrome coronavirus. Virol Sin https://doi.org/10.1007/s12250-020-00206-5.

Karjee, S., Minhas, A., Sood, V., Ponia, S.S., Banerjea, A.C., Chow, V.T.K., Mukherjee, S.K., and Lal, S.K. (2010). The 7a accessory protein of severe acute respiratory syndrome coronavirus acts as an RNA silencing suppressor. J Virol 84, 10395–10401.

Lu, X., Pan, J.'., Tao, J., and Guo, D. (2011). SARS-CoV nucleocapsid protein antagonizes IFN-β response by targeting initial step of IFN-β induction pathway, and its C-terminal region is critical for the antagonism. Virus Genes 42, 37–45.

Qiu, Y., Xu, Y.P., Wang, M., Miao, M., Zhou, H., Xu, J., Kong, J., Zheng, D., Li, R.T., Zhang, R.R., et al. (2020). Flavivirus induces and antagonizes antiviral RNA interference in both mammals and mosquitoes. Sci Adv 6, eaax7989.

Takeda, M., Chang, C., Ikeya, T., Güntert, P., Chang, Y., Hsu, Y., Huang, T., and Kainosho, M. (2008). Solution structure of the c-terminal dimerization domain of SARS coronavirus nucleocapsid protein solved by the SAIL-NMR method. J Mol Biol 380, 608–622.

Xia, S., Zhu, Y., Liu, M., Lan, Q., Xu, W., Wu, Y., Ying, T., Liu, S., Shi, Z., Jiang, S., et al. (2020). Fusion mechanism of 2019-ncov and fusion inhibitors targeting hr1 domain in spike protein. Cell Mol Immunol https://doi.org/10.1038/s41423-020-0374-2.

SUPPORTING INFORMATION

Supplementary Materials and Methods

Figure S1 Schematic diagram of N proteins of SARS-CoV-2 (GISAID, accession number: EPI_ISL_402124) and different SARS-CoV strains (NCBI, accession number: NC_004718, AY502924 and AY536760).

Table S1 The primers and oligonucleotides used in this study

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