SARS-CoV-2 might manipulate against its host the immunity RNAi/Dicer/Ago system

Does mitochondria collapse upon COVID-19 infection?

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Abstract

The role of the RNAi/Dicer/Ago system to degrade RNA viruses has been elusive, which prompt authors to think that interferon (IFN) synthesis is essential, relegating the dsRNAs as accessory function. We investigate SARS-CoV-2 genome responsible of the new deadly COVID-19 pandemic for the theoretical possibilities to engage intra pairing within the viral RNA and also hybrid pairing with human transcriptome. Segmental pieces of RNAs that originate from SARS-CoV-2 were computationally searched as a potential source of one strand, the complementary strand being from the host transcriptome. We therefore considered perfect complementarity of host RNA with any piece of SARS-CoV-2 RNA as a collection of theoretical siRNAs potentially Dicer substrates. Few human genes seems targeted by SARS-CoV-2 RNA, among them mitochondrial deubiquitinase USP30 and a subunit of ubiquitin protein ligase complex FBXO21 could explain premature death of infected cell by the collapse of mitochondria.

Introduction

Two large-scale pandemics occurred already in the recent past caused by two coronaviruses: the severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) [1,2]. The third recent new outbreak causing large-scale pandemics through the world and provoked by a new coronavirus, named SARS-CoV-2, exhibits terrifying spread out of control and qualifies for one of the largest scale pandemic since one century. More frightening, many people that are infected don't present clinical signs, which helps its spread, making it likely endemic for a long time. A minority of people (about 15% of infected) endures degraded conditions requiring

intensive care unit for treatment. The virus presently spreads throughout the world and could end up with the death rate of 2-3% of infected population. Metagenomic RNA sequencing along with the phylogenetic analysis of the complete viral genome from a sample of broncho-alveolar lavage fluid of a patient infected by so called 'Wuham coronavirus' (now referred to as SARS-CoV-2) have been performed [1,2]. These analyses have shown that this strain is very close to a group of SARS like coronaviruses previously found in bats in China [1,2]. The high homology of the predicted protein of the RBD domain of the spike protein with the precedent SARS-CoV hints that the human angiotensin-converting enzyme 2 (ACE2) acts as a receptor for fixation and entry to human host cells [3,4]. The cell entry is a multi-step process ordered cascade of events that involves viral attachment to the cell surface, receptor binding, protease processing and endocytose. In the RBD domain of spike protein of SARS-CoV-2, a polybasic cleavage site and the three adjacent predicted O-linked glycans make this strain unique compared to the other family members [3,4]. A proline is also inserted at this site allowing likely specific binding properties to human host cells [3,4].

Coronaviruses belong to the family of positive-sense single-stranded RNA viruses that encodes a RNA dependent RNA polymerase for its replication in host membranous invaginations upon host cell infection. Usually viruses are rapidly detected in host cells by receptors that acts as sensors of foreign nucleic acids. In fungi, plants and invertebrates, the viral dsRNAs are trapped and cleaved by Dicer/ago machinerie into small interfering RNAs (siRNAs) that degrade the targeted viral messenger RNAs within the RNA-induced silencing complex (RISC) by a base pairing mechanism [5]. In mammals/humans the detection of virus-derived small RNAs has been mainly undetectable upon RNA virus infection [6]. Human unique Dicer processed long double-stranded RNA (dsRNA) and hairpin dsRNA into small interfering RNAs (siRNAs) and microRNAs respectively but its role as producer of antiviral RNAis is yet far to be admitted by many authors. However, mammalian viruses were successfully eliminated and replication inhibited in cultured host cells via synthetic small interfering RNAs (siRNAs) [6]. All these elements are in accordance with the fact that viral double-stranded RNA (dsRNA) induces a powerful and non-sequence-specific synthesis of interferon (IFN), which appears as the major molecular weapon to fight mammal RNA viruses [7]. This effector response might have overwhelmed the initial RNAi role, which is inferred by the accumulation of virus-derived small RNAs (vsRNAs) in mammal host cells for which any specified functions have been found [7]. The question whether RNA virus infection of mammalian

cells can trigger an effective and powerful antiviral RNAi weapon remains unknown and little documented as no siRNAs of viral origin were experimentally found in mammal infected cells in contrast with in plants and invertebrates [8]. The absence of virus specific siRNAs in mammal cells remains presently a puzzling observation for which an explanation could be the scarcity of intra RNA pairing offered by the virus genomes. In light of their evolutionary success to replicate in mammal host through genetic innovation, RNA viruses have developed mechanisms of avoidance of host immunity/defense by masking their own RNA sequences or sheltering away from the cytosol and/or receptor sensors in order to escape the battlefield leading to their own degradation. However if the hallmark of RNAi action was not observed in virus infected mammalian cells, recent studies have demonstrated that virus-infected mammal embryonic cells present potent RNAi suppressor activity through viral proteins [9]. Thus, this illustrates the evolutionary co-adaptation host/virus that engineers molecular weapons on both sides over million years.

In the past weeks we urgently proceeded to a computational search of the SARS-CoV-2 full RNA sequence to explore whether short readings presenting perfect match in reverse complementary strand with RNA candidates from the full human transcriptome might exist. We also examined the possibilities of intra or inter pairing within the full SARS-CoV-2 RNA. Comparative studies were conducted with the SARS-CoV, the MERS-CoV along with two non-virulent coronaviruses (HCoV-229E and HCoV-OC43). The purpose of these searching was guided by the hypothesis that hybrid duplex RNA (one strand from human transcriptome, the other from SARS-CoV-2) could be theoretically formed and consequently might lead to degradation of RNA targets not only in SARS-CoV-2 but more importantly, the other way around, in the human transcriptome. Regarding the length of pairing that qualifies substrates for Dicer, the known biochemistry data documents that a 25- or 30-base pairing length is not necessary for loading onto Dicer and subsequent activation of Ago2 in the RISC complex (although some conflicting reports exist about the structural basis and activity of Dicer) [10-12]. Dicer can trap dsRNAs pairing over a 19-base length with at least a 2 nt overhang at the 3’ end and can then directly transfer the modified/unmodified siRNA to the Ago2 site. This has been demonstrated with fluorometric dsRNA [13]. Some paired 19-mers with a dTdT overhang at the 3’ end bind Dicer with high affinity; these sequences are not cleaved by Dicer, are fully transferred to Ago and trigger high-efficiency gene silencing activity [10,11]. Dicer also binds single RNAs at a specific site with high affinity, the function of which is to accelerate hybridization with a partner from the “soup” of RNA metabolism [14]. The newly in situ-formed dsRNA, which

is asymmetric, slides into the endonuclease site for cleavage, and the processed RNAi product is transferred successfully into RISC complex. Authors have shown that the pairing length can be as short as 16 nt, with a several-base overhang at the 3' end to fully bind to Dicer and consecutively activate Ago in the RISC context [10-12]. In this study, a pairing length for computational searching was set minimally to 20 bases, which leads to 24 mers considering the 2nt overhang in both 3' extremities. Rare candidates were found above 20 bases length considering perfect match. The introduction of one mismatch open to larger catalogue as expected. Characteristics of Dicer reside in some features like to be able of generating siRNAs from a mixture of asymmetric RNA duplexes as long RNAs that anneal discontinuously with short pieces of complementary RNA. RISC/Ago might be fueled by diverse source of RNAi, byproducts of the action of Dicer, originated from hybrid and asymmetric RNA duplex, one strand coming from host and the other from the virus. Our conclusions reside in the fact that i) intra or inter matching within the 5 coronaviruses are rare, ii) the list of hybrid duplex was significantly more important in SARS-CoV-2 than in the other strands, iii) regarding SARS-CoV-2, amazingly, hybrid duplexing sequences were found in three important human genes: mitochondrial ubiquitin specific peptidase 30 (USP30), a subunit of ubiquitin ligase complex (FBXO21) and finally ubiquitin specific peptidase 31 (USP31). USP 30 is a mitochondrial deubiquitinase involved in mitochondria homeostasis functions, one of which being to counter the *Parkin* mediated mitophagy [15]. USP 31 is known to increase the activity of the transcription factor NF kappa B (NF-kB), strongly involved in interferon (IFN) synthesis [16]. Interestingly, mitochondria shelter MAVS (mitochondrial antiviral signaling proteins), known to stimulate an interferon-dependent signaling pathway which orchestrate antiviral response in mammals [17]. SARS-CoV-2 might affect mitochondria via ubiquitination alteration, which could be a plausible pathway of interference to explain the dramatic deteriorating conditions of some patients affected by this virus.

Results

Analysis of short segments of SARS-CoV-2 genome that are potential sources of siRNA by hybridization with human RNAs

Considering perfect base pairing over a length of at least 20 bases, we computationally identified the transcripts of 7 known coding human genes that are theoretically complementary to a segment

originating from SARS-CoV-2 RNA. Results are summarized in Table 1 and the full list of transcripts shown in supplemental Table S1.

Pos. in virus		Human sequence forming hybrid duplex RNA	len	Human target	Organisms
start	ORF				
3778	orf1a/b	AGACAGCTAAGTAGACATTT	20	2(1) mRNAs of DNAJC13	Tetrapoda
4395	orf1a/b	TTCTGCATGTGCAAGCATTT	20	3 mRNAs of ELP4	Simiiformes
7803	orf1a/b	AGAGAGAGAATGTCTTTCAT	20	2(3) mRNAs of FBXO21	Euteleostomi
10971	orf1a/b	TGTTCTTTTCACTGCACTTT	20	1 ncRNA of LOC105377468	Homo Sapiens
11755	orf1a/b	CATCTATGCTATTCTTGGGT	20	4 mRNAs of FLRT2	Homininae
16896	orf1a/b	GCACAAAATAATCACCAACA	20	1(4) mRNAs of USP31	Boreoeutheria
21820	S	AGCAAAATAAACACCATCATTA	22	1 ncRNA of LOC105372614	Homo Sapiens
23034	S	GAAACCATATGATTGTAAAG	20	1 mRNA of ZNF443	Euarchontoglires
25698	orf3a	AAAGATAGAGAAAAGGGGCT	20	2(13) mRNAs of USP30	Homo Sapiens

Table 1: list of hybrid duplex RNA potentially formed with one strand from human transcriptome and the other from SARS-CoV-2. The table shows the position of the sequence in the RNA virus (start position and ORF name), the aminoacids making up the sequence in human RNA, the corresponding features in human and the organisms sharing the exact same sequence. The numbers in the column 'Human target' are specified using the format x(y) where x and y are the numbers of known and predicted features respectively.

Among the human genes, we noticed the presence of DNAJC13 (which regulates endosomal membrane trafficking [18]), FBXO21 (a F-box protein that is one of the four subunits of ubiquitin protein ligase complex [19]), FLRT2 (which encodes a fibronectin leucine rich transmembrane (FLRT) acting as cell adhesion molecule), ELP4 (which encodes an histone acetyltransferase, a subunit associated with RNA polymerase type II), USP31 (an ubiquitine specific peptidase that has been described to activate transcription factor NF-kappaB that stimulates interferon synthesis [16]) and USP30 (a mitochondrial ubiquitin specific peptidase [15]). The segment of pairing in USP30 was found exclusively in humans, which highlights its unexpected and intriguing presence in SARS-CoV-2. Moreover the pairing segment is observed in all the isoforms (2 known and 13 predicted) of the transcripts of USP30. The pairing are identified at the junction between exons 17 and 18 of DNAJC13, into exon 10 of ELP4, exon 12 of FBXO21, exon 2 of FLRT2, exon 13 of USP30, exon 16 of USP31 and exon 4 of ZNF443.

In SARS-CoV-2, the sequences are originating from 3 putative open reading frames (orf): orf1a/b, Spike and orf3b. In a recent study, Chan et al., show that there are no remarkable differences

between orf1a/b in SARS-CoV-2 with the one in SARS-CoV [20], the major distinction being located in orf3b, Spike and orf8. The authors note that orf3a encodes a completely novel short protein that may have a role in viral pathogenicity. Orf3a also include the 20-base sequence that potentially targets ubiquitin specific peptidase 30 (USP30). The sequence of 20 bases, originating from orf3a of SARS-CoV-2 has the potential to target the sequence AAAGATAGAGAAAAGGGGCT which is shared by 15 predicted and/or found transcripts of USP30. In parallel the SARS-CoV-2 fragments that match with USP31, FLRT2, FBXO21 were found in only 5, 4 and 5 isoforms (predicted or experimentally found) respectively. The level of all these human proteins could be affected by this RNA duplexing. Regarding the USP30, the data gathered by the "1000 genomes project" shows that this 20 bases sequence is present in all Homo Sapiens with the exception of 0,49 % of Han Chinese in Bejing that have a mutation A/T in 2nd position within this matching sequence [21]. On the other side, this sequence in USP30 was not found in other mammal species (those for which the genome is available).

Start pos. in virus	Human sequence forming hybrid duplex RNA	len	Human target	Organisms
15256	AGAGGCCATTATCCTAAGCA	20	1 lncRNA of NUTM2B-AS1	Homo Sapiens
19102	AAACAAACAAACACCATCAG	20	0(3) ncRNAs of LOC101927871	Bilateria
20547	TCAACATGTCCATCCTTACA	20	1 lncRNA of LRP1-AS	Homininae
20792	CAGAGCCAGCACCAAAGTGA	20	0(2) mRNAs of LOC102724965	Catarrhini
27270	AAGAGAATAATTTTCATGTT	20	0(5) lncRNAs of LINC02272	Homo Sapiens

Table 2: list of hybrid duplex RNA potentially formed with one strand from human transcriptome and the other from SARS-CoV. The table shows the start position of the sequence in the RNA virus, the aminoacids making up the sequence in human RNA, the corresponding features in human and the organisms sharing the exact same sequence. The numbers in the column 'Human target' are specified using the format x(y) where x and y are the numbers of known and predicted features respectively.

Analysis of short viral segmental RNAs that are potential sources of siRNA by hybridization with human RNAs: comparison with other Coronaviridae

Four more coronaviruses were investigated for comparison with SARS-CoV-2; SARS-CoV (having caused the precedent China pandemic), MERS-CoV and two no virulent strains (HCoV- 229E and HCoV-OC43). The same methodology for hybrid RNA duplex search was applied. Results are shown in Tables 2-5 and supplemental Table S1. We notice that each strain presents a unique set of

sequences of 20 bases length matching in reverse complement with human transcripts. These unique sets of matches present no overlapping sequences between each other. Remarkably, SARS-CoV mostly targets ncRNAs (Table 2 and supplemental Table S1).MERS targets an histone methyltransferase (KMT2C) and a miscRNA included in this gene; a small GTPase of the Rho-subfamily that controls cellular functions like cell morphology, migration and endocytosis (CDC42); an ubiquitin protein ligase (ARH1); a calcium activated chloride channels (ANO9); a voltage and calcium-sensitive potassium channel (KCNMA1) and finally an adhesion glycoprotein that mediates cell-to-cell interactions (THBS3) (Table 3 and supplemental Table S1).

Start pos. in virus	Human sequence forming hybrid duplex RNA	len	Human target	Organisms
9467	CAGCAGCAACAACAGCAATT	20	1(27) mRNAs, 0(1) miscRNA of KMT2C	Eukaryota
15455	TGCAGTGGTGGCATCTCCGCT	21	2(0) mRNAs of CDC42	Homo Sapiens
21230	TTAATAGTACCCAAGTAATT	20	1 mRNA of ARIH1	Homo Sapiens
22581	TCAACACCTTCAGCCTGTTC	21	2(5) mRNAs, 0(10) miscRNAs of ANO9	Boreoeutheria
23098	AAGCCAGCCACCACCTTCAA	20	6(12) mRNAs of KCNMA1	Simiiformes
25583	TTTGAGGATGCAGTGACCAA	20	0(2) mRNAs, 1 ncRNA of THBS3	Catarrhini

Table 3: list of hybrid duplex RNA potentially formed with one strand from human transcriptome and the other from MERS-CoV. The table shows the start position of the sequence in the RNA virus, the aminoacids making up the sequence in human RNA, the corresponding features in human and the organisms sharing the exact same sequence. The numbers in the column 'Human target' are specified using the format x(y) where x and y are the numbers of known and predicted features respectively.

Regarding the no virulent coronavirus (HCoV-229E and HCoV-OC43), a unique pattern of few human genes are targeted by a 20 bases sequences of viral RNA. Among these genes, an half expresses no coding RNAs for which a perturbed role by viral aggression is difficult to assess. The few coding genes do not appear to qualify for GO annotation of cellular signaling susceptible to be altered by the virus infection. These coding genes are SSH1 (Protein tyrosine phosphatase involved in actin filament dynamics in cellular lamellipodia formation), PTP4A1 (Protein tyrosine phosphatase involved in progression G1-S mitosis), GCNA (protein with acidic domain involved in genome stability. Mutants accumulate DNA-protein crosslinks resulting in chromosome instability), COX11 (a component of the mitochondrial respiratory chain, that catalyzes the

electron transfer from reduced cytochrome c to oxygen) and finally SRGAP1 (GTPase activator involved in neuronal migration) (see NCBI database for information).

Start pos. in virus	Human sequence forming hybrid duplex RNA	len	Human target	Organisms
1888	GTTCAACACGTGAAGATTTA	21	2 ncRNAs of GTF2IP4	Homo Sapiens
9372	TATTATAGAAAAATTATGCA	21	1(6) mRNAs of GCNA	Hominoidea
16936	TTGGCATTACAGGCATGAGC	20	2 mRNAs of SRGAP1 0(2) mRNAs of COX11	Simiiformes
25074	AACTGAAGAATAACAATGAATA	22	0(1) ncRNA of LOC107986455	Homo Sapiens

Table 4: list of hybrid duplex RNA potentially formed with one strand from human transcriptome and the other from HCoV-229E. The table shows the start position of the sequence in the RNA virus, the aminoacids making up the sequence in human RNA, the corresponding features in human and the organisms sharing the exact same sequence. The numbers in the column 'Human target' are specified using the format x(y) where x and y are the numbers of known and predicted features respectively.

Identification of internal pairing

We investigate whether the structure of the 5 tested coronaviruses RNAs permits the formation of intermolecular double-stranded structures. This feature would qualify these RNA sequences as potential human Dicer substrates. We computed the potential internal pairing minimally over 20 bases length and identified only one candidate in SARS-CoV, involving a sequence of 22 bases starting at position 25961 and located in orf3a. This highlights that coronaviruses, under pressure of selection, have evolutionary succeeded in escaping the vigilance of RNAi host defense strategy by minimizing internal dsRNA structure.

Start pos. in virus	Human sequence forming hybrid duplex RNA	len	Human target	Organisms
849	TCTTCAACTTTAAAATCATA	20	1 lncRNA of LINC01829	Catarrhini
5404	CAAATCAACTTGACTAAACA	20	0(1) ncRNA of LOC107986018	Homo Sapiens
12038	CCTCATCAAGATTTTTCTTA	22	0(1) mRNAs of SSH1	Hominidae
13797	TTCAACAAAATCATACCAAT	20	0(1) ncRNA of LOC105373593	Eukaryota
21968	TTGATGGTGATTTTATAATT	20	1 mRNA of PTP4A1	Amniota
23025	CACCAGTGTCTTTATTAAAA	20	0(1) ncRNA of LOC107985904	Homo Sapiens

Table 5: list of hybrid duplex RNA potentially formed with one strand from human transcriptome and the other from HCoV-OC43. The table shows the start position of the sequence in the RNA virus, the aminoacids making up the sequence in human RNA, the corresponding features in human and the organisms sharing the exact same sequence. The numbers in the column 'Human target' are specified using the format x(y) where x and y are the numbers of known and predicted features respectively.

Method

Viral sequences

We obtained the sequences of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); previously provisionally named 2019-nCoV), Middle East respiratory syndrome-related coronavirus (MERS-CoV), SARS coronavirus (SARS-CoV), Human coronavirus 229E (HCoV-229E) and Human coronavirus OC43 (HCoV-OC43) from NCBI GeneBank (RefSeq accession: NC_045512.2, NC_019843, NC_004718.3, NC_002645.1 and NC_006213.1) [1,2, 22-25].

Human transcriptome

RNA products annotated on the human reference genome build 38 release 13 (GRCh38.p13) were downloaded from NCBI FTP site (RefSeq accession: GCF_000001405.39).

Identification of potential double-stranded RNA fragments

Following the same approach as the one used by Pasquier et al. [26], each virus sequence have been cut up into short overlapping sequences of 15 bases. These 15-base sequences were extracted at intervals of 6 bases to ensure that any sequence of at least 20 bases belonging to the complete virus sequences includes at least one of the 15-base sequences. The potential pairing were computed by aligning short 15-base sequences on RNA sequences with the STAR RNA-seq aligner [27] and retaining only perfect matches for which the short sequence was reverse complemented.

We then extended the matches to obtain the maximum alignment length. We subsequently applied a second post-processing step to eliminate duplicate alignments and to remove sequences aligned over less than 20 bases. This procedure has been applied to compute the potential pairing between RNA viruses and human transcripts and also to identify the possible internal pairing within RNA virus sequences.

Conclusions

Our previous published analysis, along with other published works, confirmed that the catalogue of siRNAs is far more complex and extensive than previously thought and that it encompasses larger sets of the transcriptome [26,28,29]. A study has shown that an extensive presence of dsRNAs in the *Drosophila* and *C. elegans* by high-throughput sequencing involving many categories of RNA included mRNAs and in which miRNA and lncRNA populations appear as a minority component [30]. The extreme severity of COVID-19 regarding the clinical impact in human heath resides in the co-evolution virus/*homo sapiens*. Amazingly the absence of intra RNA pairing in SARS-CoV-2, the protection of viral genome in *de novo* synthesized pseudo organelles by host membrane invagination in which the dsRNA during replication by the RNA dependent RNA polymerase is sheltered from degrading host cellular process, make these viruses an evolutionary success to escape and evade RNAi/Dicer/Ago weaponry. Host cells are sensing RNA virus infection by internal receptors. The first pathway of innate immunity receptors is orchestrated by Toll like receptors (TLR3). The second pathway is related to retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Both pathways converge to produce interferons (IFN-α and -β) and pro-inflammatory cytokines via NF-kappa B and IRF-3 transcription factors [31-33]. Upon RNA viral infection, the RNA helicases (RIG-I and MDA-5) binds virus-derived RNAs. The complexes then translocate at the outer mitochondrial membrane to bind with MAVS (mitochondrial antiviral signaling). The bound MAVS acting as scaffold protein recruits downstream effectors to form a "MAVS signalosome" of which the major function resides in drastically inducing the stimulation of the NF-κB and IRF-3 factors [34]. Mitochondria appear to constitute an hub of communication/transmission in the line of cascade of antiviral defense events [34]. Regarding the humans genes targeted by SARS-CoV-2, we observe the presence of USP30 a deubiquitinase specific of mitochondria and FBXO21which is a subunit in an ubiquitin protein ligase complex. Remarkably FBXO21 ubiquitin ligase complex has been reported to be required for antiviral innate response [19]. The host ubiquitin system is known to be crucial in innate immunity, the host E3-ubiquitin

ligase having clearly antiviral functions [35]. In support of this, a viral protein (HBX) of hepatitis B (DNA virus) has been reported to interact with MAVS in inhibiting its action by promoting its ubiquitination [36]. Finally DNAJC13 is an endosome-related protein and believed to regulate endosomal membrane trafficking. Mutations provoke Parkinson disease and neurodegeneration [18]. Amazingly the found sequences in our computational search converge to major genes involved in endosome formation, mitochondria and ubiquitine system. The importance of ubiquitin system in innate viral immunity has been largely documented, which concurs to solidify the theoretically computational finding. Putatively dsRNAs, hybrid form with one strand coming from human transcriptome and the other viral RNA might have the unfortunate role to direct the Dicer/ago/RISC defense system against his own host. All way around SARS-CoV-2 might have "stolen" a piece of genetic information from host cells namely a reverse complement segment of an important gene, which *in fine* might use Dicer as self destruction or self suicide for infected cells. Q-PCR analysis will be required to confirm the hypothesis of down regulation of human transcripts via activation of RNAi pathway directed against the host and triggered by pieces of RNA information arising from SARS-CoV-2. Regarding the MERS virus, the collection of hybrid dsRNA seems more disparate with the targeting of 2 ionic channels and a GTPase of Rho subfamily. The no virulent viruses analysis did not reveal a targeting of essential genes.

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Conflict of interest

None declared

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Supplemental data

Supplemental Table S1

The table details hybrid duplexing RNA involving all the transcript isoforms for each targeted genes. The 5 tested coronaviruses were analyzed in depth.