BRIEF REPORT

ceRNA analysis of SARS-CoV-2

Walter Arancio[1,2,3]

Abstract

Viral RNAs can perturb the miRNA regulatory network, competing with host RNAs as part of their infective process. An *in silico* competing endogenous RNA (ceRNA) analysis has been carried on SARS-CoV-2. The results suggest that, in humans, the decrease of microRNA activity caused by viral RNAs can lead to a perturbation of vesicle trafficking and the inflammatory response, in particular by enhancing KLF10 activity. The results suggest also that, during the study of the mechanics of viral infections, it could be of general interest to investigate the competition of viral RNA with cellular transcripts for shared microRNAs.

The global outbreak of a novel betacoronavirus named SARS-CoV-2 (causing COVID-19) has posed a global health emergency. Although SARS-CoV-2 usually causes mild symptoms, some patients develop fatal complications including severe pneumonia and acute respiratory distress syndrome. At present, there are few therapeutic procedures (the majority of them are being used on a trial basis) and no preventive vaccines for COVID-19. Due to its novelty, the detailed pathophysiological mechanisms of COVID-19 is largely unknown, and our knowledge is mainly inferred from previous studies on other coronaviruses, which, however, cannot give a full explanation of the inflammatory response that can lead in turn to severe pneumonia and death [1].

Viral infection is a multilayered event that can be simplified in a step-by-step process beginning with attachment to a specific membrane receptor of the target cell and cell penetration, uncoating of the viral particle, hijacking of the cellular program for viral replication, assembly of viral particles, and release of mature infectious forms of the virus. Each of these steps is dissected by researchers in order to understand the mechanics of viral infection and to design potential therapies interfering with key viral mechanisms.

An often-overlooked aspect of viral infection regards the non-coding role of viral RNAs within infected cells, more so if considering the significant abundance of viral transcripts during an ongoing infection. Competing endogenous RNA (or ceRNA) analysis allows predictions to be made about RNAs that can regulate other RNA transcripts by competing for shared microRNAs (miRNAs), which are key players of cellular homeostasis in eukaryotes with recognized regulatory roles that are often used in diagnosis, prognosis, monitoring, and therapy [2–4]. ceRNA analyses have been able to give plausible explanations of several biological phenomena that would otherwise be difficult to interpret, although caution is always recommended in understanding the true meaning of the analyses, and strict validation procedures are always required [5–8]. The high variability of the symptoms and prognosis of COVID-19 patients suggests that there are many aspects of SARS-CoV-2 infection that still need to be clarified. Therefore a ceRNA analysis on how SARS-CoV-2 infection can perturb the regulatory network of interactions between miRNAs and messenger RNAs was carried out.

In order to perform an *in silico* ceRNA analysis to identify host gene transcripts that might be perturbed by SARS-CoV-2 RNAs, the NCBI Reference Sequence NC_045512.2 of SARS-CoV-2 was analyzed using miRDB (https://mirdb.org), an online database for miRNA target prediction and functional annotation [9, 10]. By custom target prediction,

✉ Walter Arancio
walter.arancio@gmail.com; walter.arancio@unipa.it

1 University of Palermo, Viale delle Scienze, 90127 Palermo, Italy

2 Scuola "Borgese XXVII Maggio", Piazza Contardo Ferrini, 13, 90146 Palermo, Italy

3 Advanced data analysis group, Fondazione Ri.MED, Via Bandiera 11, 90133 Palermo, Italy

Gene ID	shared miRNA	Full gene name
NUP50	13	nucleoporin 50
STXBP5	13	syntaxin binding protein 5
UNC80	13	unc-80 homolog, NALCN channel complex subunit
ZBTB44	13	zinc finger and BTB domain containing 44
KLF10	12	Kruppel like factor 10
NFAT5	12	nuclear factor of activated T cells 5
RORA	12	RAR related orphan receptor A
SAMD8	12	sterile alpha motif domain containing 8
SNX16	12	sorting nexin 16
TBL1XR1	12	TBL1X receptor 1
TNRC6B	12	Trinucleotide repeat containing adaptor 6B
ZBTB11	12	zinc finger and BTB domain containing 11
SEC24A	11	SEC24 homolog A, COPII coat complex component
STOX2	11	storkhead box 2
UBN2	11	ubinuclein 2
LATS1	9	large tumor suppressor kinase 1
C5orf24	8	chromosome 5 open reading frame 24
CDK6	8	cyclin dependent kinase 6
IKZF2	8	IKAROS family zinc finger 2
NEGR1	8	neuronal growth regulator 1
TMED7	8	transmembrane p24 trafficking protein 7
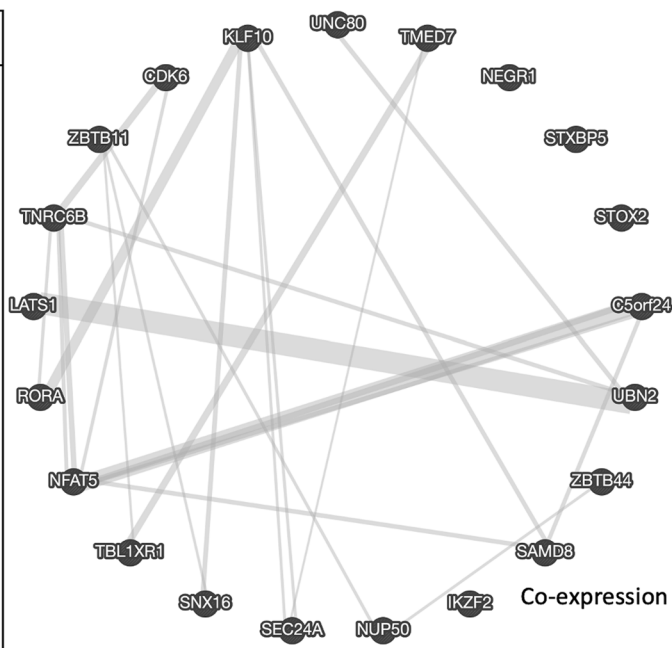
Fig. 1 Genes that code for competing endogenous RNAs for SARS-CoV-2 transcripts are usually co-expressed.

a panel of miRNA sequences able to recognize the SARS-CoV-2 sequence was collected. Only targets with a very high score (95-100) were retained for further analyses. Human gene targets for each miRNA isolated were predicted with very high stringency (including only functional miRNAs of the FuncMir collection with a target prediction score of 95 or above and excluding miRNAs with more than 5000 predicted targets in the genome). The genes were then ordered according to the number of miRNAs that recognized each gene transcript: the higher the number, the higher the probability that a particular gene can code for a competing endogenous RNA for SARS-CoV-2 RNAs. The rationale and a protocol were the same as in other studies [5, 6] with minor modifications. The detailed results of the analysis are reported in Supplementary material 1. Network analysis was performed using the Genemania tool (https://genemania.org) [11]. The 2-tailed Student's t-test was performed in Excel. A heat map was drawn using Heatmapper [12], with the average linkage clustering method and Manhattan distance measurement.

The ceRNA analysis was performed twice, on both the positive and negative strand of the SARS-CoV-2 sequences, but only the positive strand yielded significant results (as detailed in Supplementary material 1), suggesting that the positive strand might indeed be able to regulate endogenous RNAs competing for shared miRNA and that the results are

not just only a procedural artefact. The results were not weighted for the relative abundance of transcripts.

A total of 25 functional miRNAs satisfied all of the stringent requirements described previously, and each human gene transcript was recognized by no more than 13 miRNAs. Only 21 genes were recognized by eight or more miRNAs, and those genes were thus considered putative ceRNAs and retained for further network analysis. The selected genes were usually co-expressed, suggesting that they could be co-regulated by a miRNA network. The results are reported in Fig. 1.

Interestingly, the selected genes are involved in cell cycle regulation (*CDK6* and *LATS1*) and intracellular vesicle trafficking (*SEC24A, SNX16, STXBP5*, *TMED7*), which is essential for the viral life cycle, together with inflammatory and immune responses, which are key aspects of the pathological mechanisms of COVID-19 and the host response. In detail, *KLF10* is involved in the antiviral immune response [13], *NFAT5* limits infection-induced type I interferon responses [14], *RORA* is a negative regulator of the inflammatory response [15], and *IKZF2*, also known as *Helios*, codes for a hematopoietic-specific transcription factor that is essential for regulatory T-cell development [16].

Although the analysis was performed using very stringent criteria, several genes that are potentially involved in SARS-CoV-2 infection (vesicle trafficking) and the pathophysiological mechanisms of COVID-19 (inflammatory and immune

response) were identified. A number of studies have shown that the immunological and inflammatory response is crucial in the pathological mechanism of COVID-19 [17–19] and might be associated with pediatric multi-system inflammatory syndrome [20] and cytokine storm [21]. It has also been reported that the severity of COVID-19 might be influenced by host miRNAs in elderly patients [22], who tend to exhibit chronic inflammation (inflammaging) and represent the vast majority of COVID-19 victims [1].

In order to validate the analysis, datasets from differential expression analysis based on RNA sequencing by Blanco-Melo and colleagues were used [23]. An extract of the analysis regarding only the ceRNA genes examined here can be found in Supplementary material 2.

A comparison of epithelial lung cancer cell lines (Calu-3 [24] and A549 cells made permissive to the infection) infected with SARS-CoV-2 with uninfected cells has pointed to several putative ceRNA genes that are upregulated more than twofold after SARS-CoV-2 infection (*i.e.*, *KLF10, NFAT5, RORA, SAMD8, SNX16, SEC24A,* and *LATS1*), consistent with the original ceRNA model [7]. In particular, *KLF10* expression is upregulated more than sixfold after SARS-CoV-2 infection in Calu-3 cells and more than eightfold in A549 cells expressing the viral receptor ACE2. Interestingly, A549 cells, which naturally lack the receptor, showed only a modest increase in *KLF10* transcripts. Moreover, the increase in *KLF10* transcripts was not hampered by the addition of ruxolitinib, a JAK1 and JAK2 kinase inhibitor, suggesting that the increase in *KLF10* transcripts is independent of IFN-I and -III signaling [23].

However, when normal human bronchial epithelial cells were infected with SARS-CoV-2, no significant differential expression of ceRNA genes was observed in comparison with uninfected controls. When ferrets were used as an animal model of the disease, the results were highly variable. Transcription of *KLF10* increased at the beginning of the observation period and then progressively decreased in the infected upper respiratory cell populations in comparison with uninfected controls, while in the tracheas there was only a small increase caused by the infection. However, in the trachea, the vast majority of the other ceRNA genes are upregulated by SARS-CoV-2 infection. Finally, when comparing post-mortem lung tissues of COVID-19 patients and controls, a generalized downregulation of ceRNA genes can be observed, with the exception of the highly expressed NFAT5. This is probably due to the inflammatory infiltrate, and possibly other factors.

In order to test if the expression of the ceRNA genes are altered in immune cells upon SARS-CoV-2 infection, two independent panels of PBMCs from patients and controls were used (NCBI accession numbers GSE155106 and GSE152418). Interestingly, in those cells, the only gene that was both highly expressed and showed an increase in its expression upon infection was *KLF10*. In GSE155106, *KLF10* showed an almost threefold increase in expression in human PBMC-derived dendritic cells and macrophages upon SARS-CoV-2 infection, while in GSE152418, its expression increased slightly (1.2 times) in PBMCs of a group of 17 COVID-19 subjects in comparison with 17 healthy controls, but this increase was statistically significant ($p < 0,05$). An extract of the analysis is presented in Supplementary material 3, and the heat map of expression of the ceRNA genes present in GSE152418 is presented in Supplementary material 4.

Although *KLF10* is usually recognized as an anti-inflammatory gene, its role is far from straightforward [13, 25–27]. While *KLF10* is involved in the antiviral immune response [13], it has been suggested to play a role in pathological airway remodeling [25] and fibrosis in other organs [26], probably during the TGF beta response [27]. It is noteworthy that, although no commercial drugs are available that target *KLF10,* small molecule-inhibitors have been already identified [28].

The interpretation of these data is far from straightforward, but a possible explanation is that competing endogenous RNAs can play a role at the beginning of SARS-CoV-2 infection in specific tissues and under specific conditions and that their role is less crucial after tissue remodelling due to COVID-19 disease, highlighting the cell-intrinsic role of these ceRNAs. The significant increase in *KLF10* transcripts in immune cells of infected patients and in a subset of permissive cells upon infection suggest a possible role of this gene in the etiopathogenesis of COVID-19 upon SARS-Cov-2 infection that is worth further investigation.

It is possible that the abundant viral RNAs present in the cell during infection may specifically perturb the miRNA regulatory network, competing with host RNAs as part of their infectious process. This could plausibly be a widespread phenomenon that is not limited to SARS-CoV-2 infection. The results suggest that SARS-CoV-2 RNAs can lead to specific perturbations in vesicle trafficking and the inflammatory response, in particular by enhancing KLF10 activity.

Compliance with ethical standards

Conflict of interest The author has no competing interests to declare.

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