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Circulating miRNAs as biomarkers for SARS-CoV-2 and long COVID: Mechanisms and prognostic potential

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SUMMARY MicroRNAs (miRNAs) are small non-coding RNA molecules that modulate versatile epigenetics of crucial host and viral factors. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) manipulates the miRNA crosstalk between the host and virus to impair host immunity and amplify its virulence. Despite significant efforts to develop miRNA-based therapeutics for the constantly evolving SARS-CoV-2, there has been limited research that focused on the application of miRNAs as biomarkers specifically for SARS-CoV-2 infections. Circulating exosome-associated miRNAs, such as miRNA-21, have shown to have atypical miRNA profiles for both short-term SARS-CoV-2, and long-term cognitive dysfunction of Long COVID. This article will aim to elucidate the link between miRNAs and biomarkers by first 1) exploring the potential mechanisms of action of circulating miRNAs on the host factors during COVID infection, with a focus on miRNA-21, and 2) looking at the prognostic potential of miRNAs for acute SARS-CoV-2 and Long COVID. Through mechanistic understanding of the shifts in miRNAs profile from baseline levels, there is possibility for the development of a clinical prognosis method. This new molecular diagnostic could predict the severity of acute SARS-CoV-2 infection to distinguish critical patients from the overall infected population and provide further insights towards the cognitive dysfunction of Long COVID. This applicability could be extended towards other viral infections that cause dysregulation of circulating miRNAs levels. This article will also consider the implications of developing this prognosis method by emphasizing the practicality and current limitations of miRNAs as biomarkers. The progression from fundamental understanding of miRNA to potential applications could result in a significant impact on clinical diagnostics for SARS-CoV-2 and other viral pathogens.

INTRODUCTION

The largest outbreak in recent memory, Coronavirus disease 2019 (COVID-19) has unprecedently impacted the world the last few years and is predicted to continue to plague the world for the foreseeable future (1). The COVID-19 causative agent, severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) is a member of the subfamily *Coronavirinae*, containing a single-stranded positive-sense RNA genome encoding at least 29 proteins (2). SARS-CoV-2 genome is prone to mutations, and new variants of concerns (VOCs) continue to emerge, bringing new implications on the pathogenicity of the disease and therapeutics interventions (3). Additionally, SARS-CoV-2 can cause multi-organ dysfunction and long-term sequelae referred to as Long COVID, which further adds to the complexity of the disease. (4, 5). This multiorgan tropism of SARS-CoV-2 results in heterogeneous assortment of clinical symptoms of varying severity that disallow effective allocation of resources and personalized therapeutics in the clinical setting. Therefore, effective biomarkers that can differentiate VOCs and stratify the severity and symptoms of disease progression are needed (6).

Before discussing biomarker potential, an introduction to microRNAs (miRNAs), the candidate biomarkers proposed in this paper, is required. miRNAs are master regulators that affect epigenetics of host and viral factors. Canonically, miRNA function initiates with the formation of the miRNA-RISC complex that binds to RNA molecules, most commonly messenger RNA (mRNA) (7). This binding would post-transcriptionally regulate the gene

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severity, with 3'UTR region inhibiting, and 5'UTR region activating messenger RNA (mRNA) translation (8). MicroRNAs have been demonstrated to be essential in diverse biological processes such as cell differentiation, signalling, and development (7). Some miRNAs can be secreted and spread throughout the biological fluids of the body such as urine, blood, and plasma (8). There are varied mechanisms of secretion out of the cell (10), but of particular interest are those of exosome-associated miRNAs. The exosome is an extracellular vesicle that is found in almost all biological fluids and is responsible for intercellular communication while being relatively stable (11). These exosome-associated circulating miRNAs can be implicated in host-virus crosstalk and have been of great interest within the scientific community (12).

Convincing research has shown that the expression of circulating miRNAs is dysregulated in human cancers, exacerbating cancer progression and malignancy. Certain miRNAs function as oncogenes or tumour suppressors, being labelled as oncomiRs that allow for tumour classification, diagnostic and prognostic (13). Interestingly, miRNA-21 (miR-21) has been recognized to be one of the cancer-promoting oncomiRs and is one of the most abundantly expressed miRNAs in cells performing regulatory roles in disease development (14). Extending this towards viral pathogens, the interactions between host miRNAs, like miR-21, and viruses appear to influence the etiology of the virus and host response, reflecting the interplay found between SARS-CoV-2 lifecycle and host immune response (15, 16). This interplay is substantiated by how some miRNAs have been associated with specific severity and symptom outcomes (17). These all lend preliminary evidence that the crosstalk between SARS-CoV-2 and host is at least partially due to miRNAs, thereby rendering certain microRNAs as potential biomarkers.

PROPOSED RESEARCH QUESTIONS

Given the compelling evidence behind the interface of SARS-CoV-2 with the host via miRNAs, it is becoming increasingly important to discover the mechanism of action behind this virus-host crosstalk. The encompassing nature of exosome-associated circulating miRNAs in reaching their target, including across the blood brain barrier, could justify them as important master regulators that are heavily contributing to both short-term Covid-19, and Long COVID symptoms (12, 18). It is a natural progression to utilize these circulating microRNAs that are being dysregulated from baseline status as effective biomarkers for COVID-19 infection. Therefore, this article will **1)** aim to elucidate the crosstalk of SARS-COV-2 and the host by exploring potential mechanisms of action of circulating miRNAs, utilizing miRNA-21 as an example. Then the article will **2)** explore the feasibility of these circulating miRNAs as a prognostics tool for SARS-CoV-2 and Long COVID.

PROPOSED PROJECT NARRATIVE

Research Question 1: What are the potential mechanisms of action of circulating miRNAs during SARS-CoV-2 infection with a focus on miRNA-21?

Understanding the potential mechanisms of action of SARS-CoV-2 on circulating miRNAs is critical for interpreting the host miRNA dysregulation that occurs during COVID-19 infection. A deeper knowledge of the mechanisms of SARS-CoV-2 interactions with host miRNA can provide potential avenues of therapeutics for patients suffering from short-term COVID-19 and Long COVID, and a clearer grasp of how the SARS-CoV-2 might be increasing its virulence while impairing host immunity (7). This paper will explore two main mechanisms of action by contextualizing it through miR-21: miRNA sponge effect, and viral miRNAs inhibiting host RNA molecules (Figure 1).

One proposed mechanism of action is that the virus is acting as competing endogenous RNAs (ceRNA) in what is termed to be miRNA sponge effect. These are RNAs that contain multiple binding sites for miRNAs that "soak up" the host miRNAs due to having a higher binding affinity than the original target of the host miRNA (19). A study conducted by Chow et. al bioinformatically identified 128 human miRNAs that could bind to the genomic RNA of SARS-CoV-2 (20). This suggests that the RNA transcripts and direct genomic RNA of SARS-COV-2 could act as ceRNAs to regulate the activity of endogenous miRNAs. These

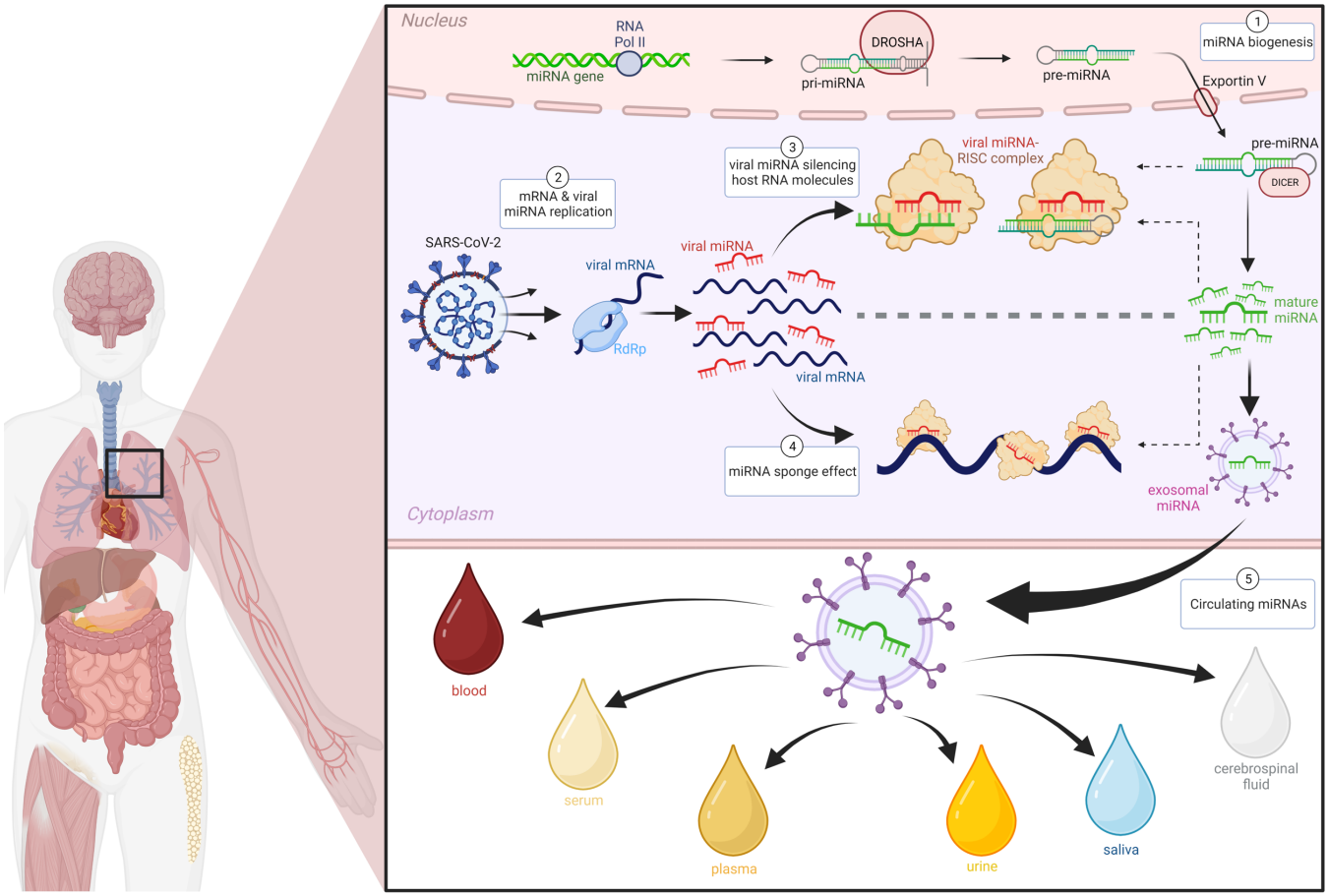

FIG. 1 Proposed mechanisms of action of circulating exosome-associated miRNAs during SARS-CoV-2 infection. The host miRNAs are synthesized in the nucleus and the pre-miRNA are exported to the cytoplasm (1). In the cytoplasm of a SARS-CoV-2 infected cell, the virus would have replicated its genome, synthesizing copies of its mRNA and viral miRNA (2). One proposed mechanism of action between circulating miRNAs and SARS-CoV-2 could be viral miRNAs silencing host miRNA molecules in the cytoplasm, by inhibiting the translation of host pre-miRNA or host miRNA directly (3). The second proposed mechanism of action is where the viral mRNA acts as a sponge for miRNAs due to having a higher binding affinity than the original target of host miRNA (4). The interactions of both proposed mechanisms would therefore lower the quantity of host miRNA that are encapsulated in exosomes and secreted for circulation in biological fluids of the host (5). All processes depicted in the figure are largely simplified. The figure was created with BioRender.com.

miRNAs appear to play a role in various biological processes involved in COVID-19 pathogenesis, like excessive reactive oxygen species levels, MAPK signalling and oxidative stress-induced neuron death. This suggests that sponging host miRNA by viral ceRNAs is significant to the pathophysiological progression of COVID-19 (19, 21). Focusing on miR-21, it was recently shown to have high binding affinity for the nucleocapsid sequence of SARS-CoV-2 genome (22), but still contains at least 27 binding sites on other sites on SARS-CoV-2 genome (16). This is proposed to sequester miR-21 in the cells, and prevent exosome secretion into the biological fluids, thereby reducing miR-21 in circulation in the body.

The second proposed mechanism of action is on how virally encoded miRNAs are capable of directly or indirectly inhibiting host miRNAs, thereby reducing total amount present in circulation. SARS-CoV-2 was demonstrated to encode their own viral miRNAs that primarily target host defense mediated pathways to impair host immunity or modulate different viral lifecycle phases to maintain pathogenicity (15). Although these virally derived miRNAs regulate host gene promoters, there is potential for these viral miRNAs to target other miRNAs directly or indirectly (23). It is known that miRNA targeting is solely dependent on the seed sequence present near the 5' end of the miRNA. This guides the miRNAs to only

bind to targets that have complementary base pairing to the seed sequence (7). Although there is a lack of empirical evidence, miRNAs have the theoretical capability of binding directly to miRNAs to inhibit their function post-transcriptionally. However, a higher and more established possibility is that viral miRNAs can bind to host factors or precursors upstream of host miRNA biosynthesis pathway, which could inhibit its function (6). This could be posited to occur to miR-21 by viral miRNAs inhibiting pre-miRNA-21, the mRNA precursor to miR-21, or the miR-21 itself. This could prevent its functionality and ability to be encapsulated by the exosome thereby rendering lesser amounts of miR-21 in circulation around the body.

The mechanistic understanding of the interplay between SARS-CoV-2 and host through miRNAs could provide further therapeutic targets, and implications on the COVID-19 progression (5). The two proposed mechanisms of actions can affect, likely decreasing, the fraction of circulating miRNAs which could lead to regulatory dysfunction, causing short and long-term detrimental effects. An example is miR-21, which has been implicated in modulating inflammatory responses and neurological processes (24). Decreasing circulating miRNA-21 would result in less inhibition of pro-inflammatory and neuroinflammatory genes in the NK-κB pathway and therefore increase innate inflammatory response (25). This systemic inflammatory response can result in the weakening of the blood brain barrier, introducing complications that lead to to sequela associated with Long COVID. Furthermore, exosomes were demonstrated to bypass the blood brain barrier, of which some are capable of encapsulating and transporting miRNAs (18). Therefore, the decrease of circulating exosome-associated miR-21 could result in an increase of apoptosis and neurodegeneration of the hippocampus, implying cognitive dysfunction, a commonly reported symptom of Long COVID (26, 27).

Research Question 2: What is the feasibility of circulating miRNAs as prognostics for SARS-CoV-2 and Long COVID?

A prognostic tool is an apparatus that predicts the likely course and symptoms of a disease using a predictor(s). In this section, exosome-associated circulating miRNAs are being evaluated as biomarkers, and their feasibility in being used as prognostics for SARS-CoV-2 and Long COVID. The progression of SARS-CoV-2 is important as it could determine the allocation of resources assigned to the patient. Furthermore, Long COVID sequala appears to be presented as a variety of endotypes across the infected population (28), thereby making an accurate prognostic necessary.

Circulating microRNAs have been characteristically shown to be affected by SARS-CoV-2. Giannella et al. demonstrated that there are certain circulating miRNAs that were upregulated or downregulated depending on if the patients had a severe or moderate/mild case of COVID-19. There was also *in vitro* evidence of unique miRNAs having different expression levels depending on the location where the cell lineage was isolated from (17, 29). These results lend credence to how certain miRNAs can be associated with relevant host and viral pathways, respective symptoms, and body location. The holistic consideration of different miRNAs in circulation in biological fluids can therefore be used to generate a "profile" of expected phenotype that can be used to predict several factors like severity, and symptoms throughout the course of a COVID-19 infection. This could be extended and applicable towards the prognosis of Long COVID and its complicated ailments. Therefore, it appears that circulating miRNAs are ideal as biomarkers for use in prognostics.

The technology required to develop circulating microRNAs into a prognostic tool needs to be considered. There have been studies conducted to facilitate point of care technology using miRNAs. The point of care technology has three main requirements: sample processing, miRNA extraction, and nucleic acid sensing methods (30). These usually are separated based on whether they use chip technology which is the incorporation of multiple processes on a single chip of a small scale. The off-chip miRNA extraction and detection methods often lack reliability due to their reliance on commercial kits needed for the miRNA extraction procedure (31). The hybrid method with a combination of on-chip and off-chip methods is inconvenient to utilize and be developed into an effective prognostic tool due to having processes that are required to be conducted manually (30). The fully integrated on-chip technology can be easily incorporated into a clinical setting making it the most fitting. Ramshani et al. manufactured a full on-chip system that can detect extracellular-vesical

associated miRNAs in plasma within 30 minutes with 10% uncertainty (32). Also, Cheng et al. developed an on-chip microfluidics platform that can detect cardiovascular disease associated miRNAs from blood in five hours (33, 34). Therefore, a point of care technology utilizing circulating miRNAs seems to be feasible as evidenced by the current advancements in technology, dispelling any notion of it being mere fiction. However, incorporating all of these advancements into a holistic system of detecting and quantifying a large set of miRNAs on a single chip still requires further research.

The use of liquid biopsy for the detection of circulating miRNAs in biological fluids is a less invasive alternative to solid biopsy and can be easily used to obtain samples for a prognosis (35). The high stability of miRNA associated with exosomes in the biological fluids renders them amenable for experimental handling. There can be a unique miRNA profile associated with relevant levels of set condition, allowing the clinician to predict with high accuracy the course of the disease, whether differentiating severity and symptoms. The limitations include confounding variables such as comorbidities, age, and sex which can make it challenging to have a consensus profile of the miRNAs and their quantities that can be used for comparison of a specific infection outcome (17). Furthermore, SARS-CoV-2 is prone to mutate which affects its pathogenesis and escalates the difficulty in forming profiles due to requiring new comparison points to be generated (3). Clarifying the SARS-CoV-2 interaction with miRNAs would make this procedure more manageable.

POTENTIAL IMPACT/CONCLUSIONS

As the pandemic seems to be remaining with us for the near future, there will be an increasing number of individuals that will contract COVID-19 and experience the long-term complications of Long COVID. Therefore, resources will have to be sequestered and dedicated to pacifying this pandemic, leading to further burden on the languished healthcare system and economy (36). This highlights the need for therapeutics, diagnostics, and prognostics that are of a higher calibre. Circulating miRNAs are excellent potential prospects as they are master regulators that can target and possibly alter anywhere from 30-60% of human genes (37).

The mechanisms proposed in this paper will allow for a better understanding of the crosstalk between SARS-CoV-2 and host miRNAs (Figure 1). Elucidating these mechanisms can aid in developing therapeutics towards mitigating the pathogenesis of SARS-CoV-2 (38). There have been miRNA mimics that have been developed to target nodes and repress critical molecular pathways of cancer that influence tumour progression, angiogenesis, and metastasis (39). Focusing on miR-21, it was demonstrated in a study conducted by Feng et Tsao that miR-21 was upregulated in a variety of cancers and miR-21 mimics are currently in clinical trials, seeking FDA approval (40). As mentioned previously, miRNAs are multi-faceted in their regulations and therefore, there are research potential for repurposing previous miR-21 mimics to function successfully for COVID-19 infection.

Circulating miRNAs being utilized as biomarkers for prognosis can provide an accurate and extensive report on the condition of the patients. The optimal instrument would be a benchtop prognosis tool that can differentiate the specific causative agent of any infection, whether bacterial or viral, and rapidly provide a report on the prognosis that is accurate and timely. This would allow healthcare professionals to prescribe more personalized treatments which can improve patient outcomes and reduce costs associated with ineffective treatments or hospital admissions (3). This could potentially have a profound impact on the healthcare industry as a whole, enabling a greater allocation of resources towards therapeutics development, while alleviating hardships on disease identification and outcome predictions. Research in this area has shown promising progress, exemplified by the work of Tsalik et al. who developed a BIOFire Film Array diagnostic tool that could differentiate viral infections from bacterial with ~85% accuracy through detecting gene transcripts (41). Although this tool does not use miRNAs as the biomarkers and does not identify or provide prognosis of the specific infection, it displayed that the development of a benchtop prognostic tool might be a closer reality than initially perceived.

With the strong feasibility of circulating miRNAs being utilized as biomarkers after further understanding of the mechanisms of action gives validity to their applicability as a prognostic tool (42). Further research on this topic can be conducted using retrospective

analysis on samples available in biobanks for experimental procedures (43). Furthermore, next-generation sequencing might be utilized to continuously update, discover, and quantify circulating miRNA biomarkers in the population (44). Overall, the increasing interest on circulating miRNAs underscores the importance of continued research in this field for the benefit of patient health and outcomes.

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