



Commentary

Important Considerations for Studies of Circulating MicroRNAs in Clinical Samples



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MicroRNAs (miRNAs) are small non-coding RNA molecules that suppress gene expression at the post-transcriptional level (Bartel, 2009). It is now well appreciated that miRNAs, of which there are over 1000 encoded in the human genome, are involved in the regulation of a wide array of biological processes. Dysregulation of miRNA expression and activity have been implicated in a broad spectrum of human pathologies, including but not limited to developmental disorders, cancers, psychiatric disorders, chronic inflammatory diseases, cardiovascular diseases, and metabolic conditions such as diabetes. Given the critical role of miRNAs in human health and disease, they have emerged as compelling candidate therapeutic targets (Janssen et al., 2013), though some genuine challenges remain in order to realize this potential. MiRNAs are also remarkably stable in circulation (Mitchell et al., 2008) as they are transported by a variety of carriers, including lipoproteins and extracellular vesicles (Boon and Vickers, 2013). In part for their stability, as well as the fact that miRNAs can be quantified by relatively straight-forward, highly sensitive, and cost-effective methods, circulating miRNAs have been proposed as potential non-invasive diagnostic and prognostic markers of human diseases.

A recent article published in *EBioMedicine* leveraged the stability of miRNAs in circulation to define non-invasive markers of treatment-induced clearance of hepatitis C virus (HCV) (Hyrina et al., 2017). The authors carried out a longitudinal analysis of plasma miRNAs in clinical samples over the course of treatment for chronic hepatitis C (CHC). Almost all patients received the same treatment and were stratified into three groups: those who cleared the virus by the end of the therapy (termed “sustained virologic responders” or “SVR”), individuals who cleared the virus at some point but relapsed (termed “relapser”), and patients who did not clear the virus and therefore were non-responsive (NR) to therapy. A primary goal of the study was to determine whether the levels of specific miRNAs in circulation are indicative of treatment efficacy. One of the major findings according to the authors is that two miRNAs, miR-24 and miR-223, which have been implicated previously in the control of cholesterol and lipid homeostasis, are significantly increased in circulation of SVRs after treatment and highly correlated with one another. The latter result in particular is suggestive of shared upstream regulation of miR-24 and miR-223, which warrants further

investigation. In addition to this, the study merits follow-up discussion on at least three major points:

Firstly, the study is comprised of a cohort of almost 100 individuals, with samples collected from most patients before, during, and after the course of therapy. The sample size and the relative concordance across patient groups in terms of treatment modality as well as other parameters including HCV genotype are important features of the study, given that interpretation of miRNA data in clinical studies is often stymied by lack of statistical power, small effect sizes, and other confounding factors (e.g., variables that can influence miRNA abundance in circulation independent of the disease or treatment under study). However, the low sample size in the relapser group, as well as the lack of post-therapy measurements of plasma miRNAs among the non-responders, mitigates the specificity of the findings.

Secondly, the study sheds light on the potential relevance of miRNAs beyond the liver-enriched miR-122 in CHC. Since the discovery of the importance of miR-122 in HCV replication, a tremendous amount of effort has been applied to uncover the molecular mechanisms by which HCV hijacks miR-122. Indeed, an inhibitor of miR-122 was developed as a candidate therapeutic (miravirsin) and it has achieved promising results in a Phase IIa clinical trial. More recent work has implicated other liver miRNAs in CHC as well. This study adds to the growing literature in support of the idea that miRNAs likely provide mechanistic links between HCV infection and cholesterol/lipid dysregulation. However, the study would have been greatly strengthened by screening a larger set of candidate miRNAs. The evidence for miRNAs as potential links between HCV and dyslipidemia would be bolstered by the inclusion of certain important miRNAs, notably miR-27, which has stronger reported connections than either miR-24 or miR-223 to lipid regulation in the context of HCV infection (Shirasaki et al., 2013).

Thirdly, the study provides evidence in support of a role for proprotein convertase subtilisin/kexin type-9 (PCSK9) in HCV infection. This intriguing link was also suggested by a previous report in which it was shown that PCSK9 regulates HCV infectivity in human cells through modulation of low-density lipoprotein receptor (LDLR) (Labonte et al., 2009). Two anti-PCSK9 drugs, evolocumab and alirocumab, are already FDA-approved for treatment of hypercholesterolemia, and many new therapies targeting PCSK9 are in development. The impact of these medications on HCV infection and CHC risk has not yet been evaluated. While the PCSK9 finding is interesting on its own, its relationship to the miRNAs included in the study remains unclear. The authors correctly

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note that miR-24 has several predicted target sites in the PCSK9 3' untranslated region, but the relevance of this putative regulatory interaction is ambiguous at best because *both* miR-24 and PCSK9 are elevated in circulation among SVRs. The emphasis in the article on the potential “interplay” among the miRNAs and PCSK9 raises the question of whether hepatic miRNAs directly target PCSK9, and if so, whether this can be exploited for therapeutic purposes. One recent study suggested that miR-224, a miRNA that is highly up-regulated in the liver in CHC (Selitsky et al., 2015), may target PCSK9 (Bai et al., 2017); unfortunately, this miRNA was not included in this study.

More researchers than ever before are aiming to leverage circulating miRNAs for better understanding disease etiology and progression as well as treatment efficacy. Below we provide five important considerations for any translational study involving extracellular miRNAs:

- (1) **Method of detection.** As stated previously, a compelling feature of miRNAs is that they are highly stable in circulation and relatively straightforward to measure. MiR-24 and miR-223 are highly abundant in circulation and therefore are readily detectable. However, some miRNAs are present in circulation at very low levels and therefore are challenging to detect by standard PCR approaches and sometimes even by sequencing. Such cases may motivate the use of highly-sensitive methods such as digital droplet PCR (ddPCR) (Hindson et al., 2013). Unfortunately, unlike sequencing, this strategy will not effectively discriminate between miRNAs and their isomiRs, which can differ by only one or two nucleotide shifts at the 5'-end. Generally, it is advisable to apply more than one detection approach in order to gain confidence in the result.
- (2) **Other classes of small RNAs.** Another benefit of sequencing is that it facilitates the discovery of other classes of small RNAs that are also present in circulation. For example, small RNA fragments from parent tRNA molecules are often abundant in plasma fractions. Also, Y-RNA derived RNAs (ydRNAs) are thought to be enriched in extracellular vesicles, and have been proposed as candidate markers in cancer for determining the most appropriate therapeutic strategy (Dhahbi et al., 2014). At this time, very little is understood about the biological functions of these new classes of small RNAs; however, they certainly merit further investigation in studies of circulating RNAs from clinical samples.
- (3) **Sources of circulating miRNAs.** Most miRNAs are expressed, albeit to varying degrees, in a wide-array of tissues and cell types. Therefore, for the vast majority of miRNAs in circulation, the cellular sources are difficult to predict. For example, given that both miR-24 and miR-223 are expressed in a plethora of different cell types (hepatocytes, macrophages, T cells, platelets, adipose, etc) that could in theory secrete miRNAs, the primary cellular source for the rise in circulation among SVRs cannot be assumed to be the liver. This inherent ambiguity should be carefully considered before developing hypotheses about the physiological implications of changes in circulating miRNAs.
- (4) **Specificity of biomarkers.** A growing number of studies have reported changes in circulating miRNAs under different conditions. However, very few of these have demonstrated that the results are specific to a particular condition, which greatly mitigates the potential clinical utility of the findings. Given the broad expression patterns and functional roles of most miRNAs, it is unsurprising to find that sometimes the same circulating miRNA is associated with rather disparate conditions. For example, although in this study miR-223 is proposed as a marker of treatment efficacy in CHC, it has also been reported as a candidate marker of acute liver failure (Schueller et al., 2017). In order to improve specificity, it may be useful to define miRNA signatures rather than one or two miRNA markers (Leidinger et al., 2013).
- (5) **Functional implications.** The potential functional relevance of circulating miRNAs remains an open and active area of research.

Are extracellular miRNAs present in enough copies and delivered to recipient cells with functional integrity? Several recent studies suggest this is indeed possible in certain situations. For example, Thomou et al. reported that circulating miRNAs secreted by adipose are delivered to the liver to regulate gene expression for the purpose of maintaining glucose tolerance and energy homeostasis (Thomou et al., 2017). These findings support the growing idea that miRNAs represent novel messengers in inter-organ communication; however, it remains to be seen whether this is a widespread and prevalent mechanism in health and disease. Moreover, it remains to be determined if miRNA-based intercellular communication utilizes canonical miRNA targeting mechanisms in recipient cells or if other processes are involved.

This study adds to the growing interest in the potential translational relevance of circulating miRNAs. Although extracellular RNAs are promising candidates as diagnostic and prognostic tools, more work remains to be done to improve methods of detection, expand beyond miRNAs, define the primary cellular sources under different conditions, establish specificity, and decipher mechanisms of inter-organ transport and function.

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