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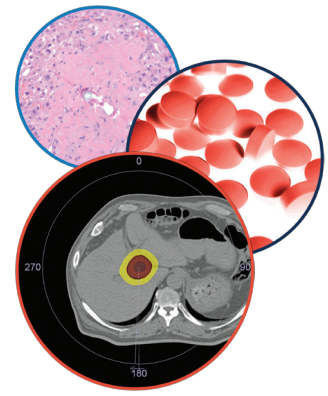

Hepatic Oncology

miRNAs: new tools for molecular classification, diagnosis and prognosis of hepatocellular carcinoma

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Practice points

- Circulating miRNAs are very stable in plasma and serum, and both of them are acceptable types of specimen for miRNA analysis.
- Within a study, it is recommended to use the same type of specimen, given that a global comparison of miRNA expression between plasma and serum has not been established.
- Both plasma and serum contain a very high concentration of protein; extraction of RNA therefore requires scaling up of denaturing solution (e.g., Trizol).
- Considering the small amount of RNAs in plasma and serum, quantitation by traditional spectrophotometry might be inaccurate. The spike-in of synthetic nonhuman miRNAs is often used as an internal reference for technical variations in sample processing.
- The strategy for normalizing biological variation is not well developed. Efforts are still underway to search for a specific miRNA or set of miRNAs as suitable endogenous controls.
- Absolute quantification of miRNAs can be performed by generation of a standard curve using a synthetic microRNA target, providing absolute copy number of the target miRNA in biological samples.

SUMMARY: Hepatocellular carcinoma (HCC) remains one of the most common malignancies worldwide, ranking as the third leading cause of cancer-related death. With recent advances in understanding HCC biology, progress has been made in early detection and management of HCC; however, its prognosis remains dismal. Novel biomarkers for HCC that are acceptable for clinical utility are urgently in need. Recently, miRNA has emerged as an important class of gene regulator that controls various cellular processes including cancer development. In HCC, miRNAs are frequently dysregulated, and studies have shown great promises of miRNAs as biomarkers for tumor classification, diagnosis and prognosis. Given miRNAs are highly stable in blood plasma and serum, they are suggested as a new class of noninvasive biomarker for detection of HCC. In this article, we provide an up-to-date review of the recent findings of the use of miRNAs in molecular classification of HCC tumors, diagnosis and prognosis.

KEYWORDS
• biomarker • diagnosis
• hepatocellular carcinoma
• miRNA • prognosis

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, with more than 500,000 cases diagnosed each year [1]. The prognosis of HCC is poor, with a 5-year survival rate of only approximately 5–9% [2]. However, in patients with small size (<2 cm) tumor, the 5-year survival rate can be improved to 69% when treated with surgical resection [3,4]. Therefore, one of the unremitting concerns is to diagnose HCC at early stages. Major risk factors for HCC include hepatitis B virus (HBV), which accounts for approximately 50%

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of HCC cases, followed by hepatitis C virus (HCV), alcoholic liver disease and nonalcoholic fatty liver disease [5]. These risk factors could lead to the development and progression of cirrhosis, which is present in 80–90% of patients with HCC. Therefore, unlike other solid malignancies, the coexistence of chronic hepatitis and cirrhosis posted the early diagnosis and prognostic assessment of HCC a major challenge.

Over the last decade, a number of new technologies such as microarray technologies and deep-sequencing have emerged [6], providing important insights into underlying HCC pathogenic mechanisms. With these technologies, it is now relatively easy to examine whole-genome variations of tumor (gene copy number variations, single nucleotide polymorphisms and gene mutations, among others), mRNA and miRNA expression [7,8]. To date, there are various techniques readily available for miRNA quantification, among which microarrays and quantitative real-time PCR (qRT-PCR) are the most commonly adopted techniques.

miRNAs are a class of small noncoding RNAs that regulate gene expression and have important regulatory roles in numerous important pathways, including cancer development and progression [9]. The control of gene expression by miRNAs is mainly mediated by miRNA–mRNA base pairing. The 3′ untranslated region of mRNAs has specific miRNA recognition elements, and the base pairing between miRNAs with miRNA recognition elements leads to degradation of mRNA transcripts, or inhibition of translation [10]. Since each miRNA could have numerous mRNA targets, a large number of genes are under regulation of miRNAs.

miRNAs have shown great promise as biomarkers for cancer [11]. The expressions of miRNAs are often dysregulated in cancers [12]; the expression signature of miRNAs could differentiate cancer types according to their developmental origin, whereas the profile of mRNAs does not accurately classify the tumors [13]. Another important aspect of miRNAs is their high stability in blood plasma and serum, which is of particular interest, because of the easily operable and noninvasive procedures of specimen collection. Here, we provide an up-to-date review of the miRNA biomarkers that have potential use for classifying tumor subtype, diagnosis and prognosis.

miRNAs in molecular classification of HCC tumors

The aberrant expression of miRNAs in HCC has been shown to be tissue specific (i.e., tumor vs adjacent nontumor, primary HCC vs liver metastases of other origins). Recent studies have also revealed that unique miRNA expression profiles could be identified in various subtypes of HCC based on histology (benign vs malignant) [14], etiology (HBV vs HVC infection) [15] and genetic alteration (underlying molecular pathways) [16].

Ladeiro *et al.* used qRT-PCR to analyze 18 benign HCC tumor samples (13 hepatocellular adenoma and five focal nodular hyperplasia), 28 malignant HCC tumor samples and four normal liver samples [14]. Compared with normal liver samples, both benign and malignant tumor samples showed upregulation of miR-224 and downregulation of miR-422b and miR-122a. More importantly, a six-miRNA signature was identified to successfully distinguish benign tumors from malignant HCC. Among them, miR-200c and miR-203 were downregulated in benign tumor samples, while miR-21, miR-10b and miR-222 were significantly upregulated in HCC samples, and miR-224, which was elevated in all tumor samples, exhibited differential expression levels between benign and malignant HCC tumors.

HBV and HCV infections are major risk factors of HCC development. HCC with HBV infection and HCV infection have very different virological features, but cannot be distinguished by histological examination and clinical manifestations. Studies have revealed unique miRNA expression profiles for HBV or HCV infection. Ura *et al.* used qRT-PCR to profile 12 HBV-related HCC and 14 HCV-related HCC samples [15]. A 19-miRNA signature was reported to clearly differentiate HBV-related HCC from HCV-related HCC. Six of them were significantly downregulated in HBV–HCC, and their target genes are involved in cell death, DNA damage and recombination, and transcription signal. Thirteen of them were significantly downregulated in HCV–HCC, and the target genes are mostly implicated in immune response, antigen presentation, cell cycle, proteasome and lipid metabolism. Most of them are not only involved in viral replication, but also in HCC development, providing insights to the mechanism of HCC with viral etiology.

In another study, Varnholt *et al.* examined the miRNA expression patterns of 52 primary liver tumor samples exclusively infected with HCV, including premalignant dysplastic liver nodules and HCCs [17]. Notably, this is the first study that validated the use of formalin-fixed and paraffin-embedded liver tissues by qRT-PCR. By comparing with normal liver samples, liver tumor samples showed a unique signature of 29 miRNAs including ten of them overexpressed and 19 underexpressed. Among them, miR-10 and miR-100 were most significantly upregulated and miR-198 and miR-145 were most significantly downregulated. Interestingly, miR-122 and miR-100 were also significantly upregulated in this cohort of HCC samples, despite previous reports on the downregulation of miR-122 in HCC [18–20]. The difference could be attributed to the different viral etiologies of HCC samples. This is supported by the observation that HCV replication is modulated by miR-122 [21]. miR-122 could be potentially implicated in the viral etiology of HCC, in particular, those with HCV infection.

miRNA expression profiles have been associated with specific molecular pathways underlying different subtypes of HCC. Toffanin *et al.* performed comprehensive genomic analysis on 89 HCV-related HCC samples collected from three HCC genomic consortiums: New York, Italy and Spain [22]. By integrating miRNA data with gene-expression analysis, copy number changes, immunohistochemistry assessment of cellular pathway and mutation analysis, they identified a miRNA signature, which successfully classified the samples into three groups based on the molecular pathway: activation of β-catenin pathway, upregulation of interferon-response-related genes, and activation of insulin-like growth factor and mTOR/Akt/PI3K pathway. Unsurprisingly, the miRNA signature was also previously reported to be associated with gene mutations involved in these molecular pathways [16,23,24].

Cell population in cancer is heterogeneous, and one of the important cell groups is cancer stem cells (CSCs). CSCs are capable of self-renewal and differentiation; and often responsible for HCC relapse after surgery. Using a specific hepatic stem cell marker, EpCAM, and serum α-fetoprotein (AFP), Yamashita *et al.* defined two subtypes of HCC, $EpCAM^{+}AFP^{+}$ hepatic stem cell-like HCC (HpSC-HCC), and $EpCAM^{-}AFP^{-}$ mature hepatocyte-like HCC (MH-HCC) [25–27]. Ji *et al.*, from the same research group, examined the miRNA expression profiles of 53 HpSC-HCC and 95 MH-HCC samples by microarray. A 20-miRNA signature identified by this study was able to discriminate HpSC-HCC from MH-HCC with 78% overall accuracy [28]. Among this cluster of miRNAs, five members of miR-181 family were significantly upregulated in HpSC-HCC. This finding was further validated by qRT-PCR. Interestingly, this conserved family was also highly expressed in embryonic livers and isolated hepatic stem cells, suggesting its potential role as a marker for hepatic CSCs.

Luk *et al.* examined a cohort of 97 HCC patients associated with HBV infection, and reported that DLK1-DIO3 miRNA cluster on chromosome 14q32.2 defined a subgroup of patients [7]. Overexpression of this miRNA cluster was positively correlated with HCC stem cell markers including EpCAM, as well as serum AFP level. This subtype of HCC was associated with poor survival rate.

miRNAs in HCC diagnosis

Early diagnosis of HCC is important to improve survival rate. Currently, AFP is the most commonly used diagnostic marker for HCC, but its performance is only modest; many of the patients with early-stage HCC have a normal AFP level. The value of using AFP as surveillance remains controversial [29]. In the past decade, miRNA has been suggested as a powerful serological biomarker for cancer, based on a few lines of evidences. First, tumors could affect miRNA levels in the bloodstream [30], and that the miRNAs in blood plasma or serum are highly stable as they are well protected from RNases [30,31].

Serum miR-15b and miR-130b were identified as a classifier that detected HCC with a receiver-operating characteristic curve area of 0.98, with a sensitivity of 98.2% and specificity of 91.5% [32]. Importantly, the classifier could detect HCC at an early stage, despite patients who had low AFP levels. Similarly, serum miR-16 has higher sensitivity than AFP for detecting HCC [33]. The combination use of miR-16 and conventional markers – AFP, lens culinaris agglutinin-reactive AFP, and des-γ-carboxyprothrombin – could differentiate HCC from chronic liver diseases with a 92.4% sensitivity and a 78.5% specificity.

A panel of miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) has been identified to have high diagnostic accuracy for HCC; in addition, the panel could highly differentiate HCC cases from healthy liver tissue, chronic hepatits B and cirrhosis [34]. The study used an adequate amount of samples consisting of discovery phase (n = 137), training phase (n = 407) and validation phase (n = 290), providing considerable clinical value in early diagnosis of HCC (area under the curve: 0.864). Indeed, circulating miR-21 [33,35] and miR-223 [36,37] were found to be upregulated in blood serum of plasma of HCC patients with a HBV or HCV background in other studies as well. Circulating miR-21 yielded an area under the curve of 0.773 with 61.1 and 83.3% of sensitivity and specificity, respectively [35].

miRNAs in HCC prognosis & drug responses

Surgical resection of HCC tumor offers a potential curative option, but patients' outcomes are varied due to the intrinsic characteristics of tumors, and prediction of outcome after surgical resection remains very difficult. Metastatic potential of the tumor is one of the major factors that affect the patient outcome. Budhu *et al.* examined the miRNA expressions of 131 HCC patients who were clinically well defined as metastatic or nonmetastatic, and they identified a 20-miRNA signature that was significantly related to metastasis [38]. This cluster of miRNAs was able to predict primary HCC with venous metastases from those metastasis-free with 72% overall prediction accuracy in an independent cohort of 110 HCC patients. The predicted metastatic patients had significantly lower survival rate then the nonmetastatic patients. It was well correlated with disease-free and overall survival. Recently, Wong *et al.* also analyzed the miRNA-expression profiles of nontumorous livers, primary HCCs and venous metastases. The group did not observe significant differences in miRNA expression between primary HCCs and venous metastases, but rather a global reduction of miRNA expression levels in venous metastases, as compared with primary HCCs [39].

Sorafenib is a molecular-targeted therapy for advanced HCC. It is a small-molecule multikinase inhibitor that has antiproliferative and antiangiogenic properties. Randomized controlled clinical trials have shown that sorafenib is associated with an increased overall survival of 2–3 months in patients with advanced-stage HCC [40], suggesting only a small portion of patients benefit from this therapy. To date, there is no specific biomarker that can be used to guide the use of sorafenib in HCC in a clinical setting. However, Bai *et al.* found that miR-122 was able to sensitize HCC cells to sorafenib treatment [41], but this has only been shown in an *in vitro* study.

IFN-α is a multifunctional cytokine that has been suggested to prevent recurrence of HCC and improved overall survival after resection of tumors [42,43]. However, this treatment is only beneficial to a small portion of patients. In a recent study, 241 pairs of HCC tumor and adjacent nontumor samples were compared, Ji *et al.* found there was a subgroup of patients with downregulation of miR-26 [44]. The lower expression of miR-26 predicted poor overall survival, but better response to interferon therapy, suggesting miR-26 as a predictive marker for interferon treatment in patients with HCC. At present, a multicenter, randomized trial is ongoing to identify the efficiency of postoperative interferon treatment in low miR-26 expression patients with HCC [45].

ABC transporters are drug-efflux pumps responsible for drug resistance in HCC. Borel *et al.* studied 19 paired HCC samples, and found that the upregulation of five ABC transporters in HCC is associated with the downregulation of 13 cellular miRNAs [46]. This cluster of miRNAs might potentially be used to predict the drug resistance in HCC.

Conclusion & future perspective

HCC is a lethal malignancy affecting millions of people worldwide. With advances in understanding the underlying pathogenic mechanisms of HCC, different therapeutic approaches are emerging. It is clear that the disease can only be cured with early detection of tumors, as well as careful monitoring of the prognosis of patients receiving treatments.

Early studies have demonstrated that miRNAs could classify HCC tumor types and predict HCC patient outcome, whereas the circulating miRNAs provided a satisfactory specificity and sensitivity in detecting HCC (Table 1). These studies, however, are mostly retrospective studies. To translate them into clinical settings, conducting prospective and

Table 1. miRNA biomarkers in human hepatocellular carcinoma.

Types of biomarkers	miRNAs	Ref.
HCV tumor vs nontumor	29 miRNAs (miR-10, miR-100, miR-198 and miR-145, among others)	[17]
Benign vs malignant	6 miRNAs (miR-200c, miR-203, miR-21, miR-10b, miR-222 and miR-224, among others)	[14]
HpSC- vs MH-HCC	DLK1-DIO miRNA cluster on chromosome 14q32.2	[7]
	20 miRNAs (miR-181 family, and so on)	[28]
HBV- vs HCV-related HCC	19 miRNAs (miR-190, miR-134, miR-151, miR-193, miR-133b, miR-324-5p, miR-182*, miR-105, miR-211, miR-20, miR-191, miR-340, miR-194, miR-23a, miR-142-5p, miR-34c, miR-124b, let-7a and miR-27a)	[15]
Survival	DLK1-DIO3 miRNA cluster	[7]
Metastasis	20 miRNAs (miR-338, miR-219-1, miR-207, miR-185, miR-30c-1, miR-1-2, miR-34a, miR-19a, miR-148a, miR-124a-2, miR-9-2, miR-148b, miR-122a, miR-125b-2, miR-194, miR-30a, miR-126, let-7g, miR-15a and miR-30e)	[38]
Drug response/resistence	miR-26	[44]
	miR-122	[41]
Diagnosis	miR-15b and miR130b	[32]
	miR-16	[34]
	miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801	[35]
	miR-21	

HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HpSC: Hepatic stem cell-like; MH: Mature hepatocyte-like.

multicenter trials are important to validate their clinical significance. To date, many of the proposed miRNA biomarkers are still in research phase, and they are rarely confirmed by work from other groups. It is important to have a standardization of assay platforms, reagents, sample types and normalization methods. It should also be considered that the same specimen type (e.g., whole blood, plasma or serum) is used throughout the study, as different specimens may have differences in miRNA concentrations. Universal guidelines for collection, preparation and analysis of miRNA biomarkers are also important to minimize predictable variability.

Given the heterogeneous nature of HCC tumor – a variety of risk factors and multiple underlying pathogenic mechanisms – it is reasonable to utilize a combination of biomarkers to characterize HCC and monitor patients' responses toward treatments. The characterization will allow physicians to select appropriate therapeutic options for patients according to the molecular profile of the tumors, and shed light into the personalized treatment of HCC.

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