Hepatoma Research

Review

Circulating tumor DNAs and non-coding RNAs as potential biomarkers for hepatocellular carcinoma diagnosis, prognosis and response to therapy

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Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide and despite improvement in therapeutic approaches, prognosis remains poor. This can be partly attributed to the fact that the majority of HCCs are diagnosed at intermediate or advanced stages. Availability of circulating biomarkers able to detect HCC at early stages could improve patients' prognosis. At present, however, alpha fetoprotein or des-γ-carboxyprothrombin are unable to reliably detect HCC at early stages and better circulating biomarkers are needed. Circulating tumor DNA (ctDNA) and non-coding RNAs (ncRNAs) are emerging as promising biomarkers to achieve the goal. Genetic and epigenetic alterations in ctDNA allow to pinpoint tumor-specific biomarkers, reveal tumor heterogeneity, help monitor tumor evolution over time and assess therapy efficacy. It remains to be fully evaluated the possibility of detecting these biomarkers at early tumor stages. Circulating ncRNAs are quantitative biomarkers with potential use in diagnostic, prognostic and predictive clinical settings. They may help to reveal HCC at early stages. However, because of heterogeneous and sometimes conflicting reported results, they still require validation and standardization of pre-analytical and analytical approaches before clinical applications could be envisaged.

Keywords: Liquid biopsy, hepatocellular carcinoma, circulating tumor DNA, non-coding RNA, diagnosis, prognosis, therapy response

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and second leading cause of cancer-related deaths worldwide. Despite new therapeutic approaches, prognosis remains poor. According to the Barcelona Clinic Liver Cancer (BCLC) system[1], treatment options rely on cancer staging. Patients with an early stage HCC (BCLC stage A) can take advantage of curative treatments, such as tumor resection, ablation and liver transplantation. Although termed curative, risk of recurrence in post-surgical resection is about 70% at 5 years[2]. Patients who presents an intermediate (Stage B) or advanced (Stage C) HCC, about 70% of the total, can only benefit from palliative treatments, chemoembolization or sorafenib respectively, with survival in fewer than 10% of patients at 3 years[3-5].

Poor prognosis of HCC can be partly attributed to the fact that the majority of neoplasms are diagnosed at intermediate or advanced stages. Availability of blood biomarkers would be extremely important to improve early diagnosis in individual at risk or for a better management of prognosis and response to therapy in HCC patients. Among biomarkers presently in use, alpha fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are the most commonly employed. AFP was the most widely used serum biomarker in HCC[6], but due to suboptimal sensitivity (55%-65%)[7-9], it is now employed to the monitoring of therapy effectiveness in HCC patients, together with ultrasound examination. DCP is a second biomarker utilized in HCC; although initially indicated as superior to AFP with a sensitivity of 92% and specificity of 93%[10], other studies showed suboptimal sensitivity (48%-62%)[11,12]. These studies signify that more effective biomarkers are needed for better management of HCC patients at various clinical phases.

Since 80%-90% of HCCs develop in a cirrhotic liver, a distinction between regenerative nodules and early HCC can be a challenge[13]. Albeit DCP exhibits the potential capability of differentiating HCC from non-malignant liver diseases[14], tissue biopsy remains the most dependable option for diagnostic purposes as well as for recognizing the molecular changes that characterize the tumor. However, tissue biopsy is invasive and associated with potential risks for the patients, it cannot be repeated and cannot be performed on patients with unresectable advanced HCC.

In recent years, liquid biopsy has become a valid alternative to overcome the above mentioned limitations. It is only modestly or not invasive at all and it offers the possibility of carrying out repeated tests over time. Moreover, it can be used for an early detection of tumors, for monitoring its growth dynamics, for evaluating the efficacy of treatments and for spotting tumor genetic heterogeneity and identifying mutations responsible for acquired resistance, becoming a highly promising approach for the clinical management of cancer patients. Liquid biopsy is the sampling and analysis of biological samples, such as blood, urine, saliva or stool, where nucleic acids originating from all or part of body districts can be found. In the presence of cancer, its derived materials, such as circulating tumor cells, cell-free tumor DNA (ctDNA) and microvesicles containing mRNAs, microRNAs (miRNAs) and proteins, are present in peripheral blood or other body fluids and can be measured through the use of specific tests [Figure 1]. This review is focused on ctDNA and circulating non coding RNAs, like miRNAs and long non-coding RNAs (lncRNAs), as potential biomarkers of HCC for early diagnosis, monitoring patients' follow-up and assessing response to treatments.

ctDNA

The presence of free circulating DNA in serum/plasma has been used to reveal tumor-associated biomarkers, such as the increased abundance of cell-free DNA (cfDNA) in cancer patients or the presence of specific genetic or epigenetic alterations, which have been discovered in numerous types of cancer including HCC. Several studies have indeed proposed the cfDNA as a source of HCC biomarkers in diagnostic, prognostic or predictive clinical settings [Table 1].

cfDNA as biomarkers in plasma or serum in HCC

The level of plasma cfDNA was found significantly increased in patients affected by HCC, compared to individuals with liver fibrosis. A model that included this parameter together with patient age and AFP levels

Table 1. Cell-free DNA as biomarker of hepatocellular carcinoma

Target	Serum/ plasma	Technology	Experimental setting	Clinical setting	Cohort comparison	Ref.
DNA abundance	Plasma	Fluorimeter	cfDNA levels	Diagnosis	HCC *vs.* HBV-related LF	[15]
DNA abundance	Serum	Branched DNA	cfDNA levels	Diagnosis	HCC *vs.* HL	[114]
DNA abundance	Plasma	Spectrophotometer	cfDNA levels	Prediction of radiotherapy response	HCC	[19]
DNA abundance	Plasma	Fluorimeter	cfDNA levels	Prognosis	HCC	[115]
GSTP1	Serum	Real-time PCR	cfDNA levels	Diagnosis	HCC *vs.* HCV carriers	[16]
GSTP1	Serum	Real-time PCR	cfDNA levels	Prognosis	HCC	[17]
hTERT	Plasma	Real-time PCR	cfDNA levels	Diagnosis	HCC *vs.* CHC + LC	[18]
hTERT	Plasma	Real-time PCR	cfDNA levels	Prognosis	HCC	[18]
APC, FHIT, p15, p16, E-cadherin	Plasma	MSP	Methylation	Diagnosis	HCC[1]	[116]
APC, GSTP1, RASSF1A, SFRP1	Plasma	MSRE-qPCR	Methylation	Diagnosis	HCC *vs.* HL	[21]
APC, GSTP1, RASSF1A, SFRP1	Plasma	MSRE-qPCR	Methylation	Diagnosis	HCC *vs.* BLD	[21]
APC, GSTP1, RASSF1A, SFRP1	Plasma	MSRE-qPCR	Methylation	Prognosis	HCC	[21]
Gene panel (12 genes)	Plasma	NGS	Methylation	Diagnosis	HCC *vs.* CLD + HL	[25]
p15, p16	Plasma	MSP	Methylation	Diagnosis/ prognosis	HCC *vs.* CLD + HL	[23]
SEPT9	Plasma	MSP	Methylation	Diagnosis	HCC *vs.* LC	[117]
SOCS3	Plasma	MSP	Methylation	Diagnosis/ prognosis	HCC *vs.* LC, BLD + HL	[24]
UBE2Q1	Serum	MSP	Methylation	Diagnosis	HCC *vs.* LC + CHB	[118]
Gene panel	Plasma	NGS	Mutations	Guiding therapy choice	HCC	[31]
Gene panel (574 genes)	Plasma	NGS	Mutations	Tumor heterogeneity and prognosis	HCC[1]	[32]
Gene panel (58 genes)	Plasma/serum	NGS	Mutations	Patient monitoring	HCC[1]	[27]
KRAS, NRAS	Plasma	BEAMing	Mutations	Guiding therapy choice	HCC	[31]
TERT	Plasma	ddPCR	Mutations	Diagnosis	HCC *vs.* LC	[119]
TERT	Plasma	ddPCR	Mutations	Prognosis	HCC	[119]
TERT, CTNNB1, TP53	Plasma	NGS	Mutations	Prognosis	HCC[1]	[120]
TP53	Plasma	ddPCR	Mutations	Diagnosis	HCC *vs.* CLD + HL	[26]
TP53	Plasma	COLD-PCR	Mutations	Patient monitoring	HCC	[121]
TP53, CTNNB1, TERT	Plasma	ddPCR	Mutations	Diagnosis	HCC[1]	[28]
Genome-wide	Plasma	NGS	CNV	Diagnosis	HCC *vs.* CH + LC	[122]
Genome-wide	Plasma	NGS	SNV, CNV	Diagnosis	HCC *vs.* HL	[123]
Genome-wide	Plasma	NGS	SNV, CNV	Patient monitoring	HCC	[123]
Gene panel (54-70 genes)	Plasma	NGS	SNV, CNV, fusions	Guiding therapy choice	HCC	[29]

[1]Tumor DNA *vs.* matched cfDNA. *GSTP1*: glutathione S-transferase p1; *hTERT*: telomerase reverse transcriptase; *APC*: adenomatous polyposis coli; *FHIT*: fragile histidine triad; *p15*: cyclin dependent kinase inhibitor 2B; *p16*: cyclin dependent kinase inhibitor 2A; *RASSF1A*: ras association domain family member 1; *SFRP1*: secreted frizzled related protein 1; *SEPT9*: septin 9; *SOCS3*: suppressor of cytokine signaling 3; *UBE2Q1*: ubiquitin conjugating enzyme E2 Q1; *KRAS*: KRAS proto-oncogene; *NRAS*: NRAS proto-oncogene; *CTNNB1*: beta catenin 1; TP53: tumor protein p53; MSP: methylation-specific PCR; MSRE-qPCR: methylation-sensitive restriction enzymes qPCR; NGS: next generation sequencing; BEAMing: beads, emulsion, amplification, and magnetics PCR; ddPCR: droplet digital PCR; CNV: copy number variations; SNV: single nucleotide variations; cfDNA: cell-free DNA; BLD: benign liver disease; CH: chronic hepatitis; LF: liver fibrosis; CHB: chronic hepatitis B; CHC: chronic hepatitis C; CLD: chronic liver disease; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HL: healthy liver; LC: liver cirrhosis; HCV: hepatitis C virus

displayed 87.0% sensitivity and 100% specificity as diagnostic performance[15]. As a surrogate of abundance of circulating DNA, some studies evaluated the amount of cfDNA by quantifying specific circulating gene fragments. Iizuka *et al.*[16] found a significant increase in serum levels of the *GSTP1* gene in HCC patients and found correlations with tumor grade and size. An increased *GSTP1* gene in cfDNA was also associated with a shorter overall survival (OS) and metastasis occurrence[17]. Similarly, higher than normal levels of *hTERT* gene in plasma of HCC patients correlated with presence of advanced disease and shorter survival[18]. Quantification of cfDNA revealed its potential usefulness also for assessing therapy response. Reduction of plasma cfDNA after radiotherapy correlated with a better tumor response[19].
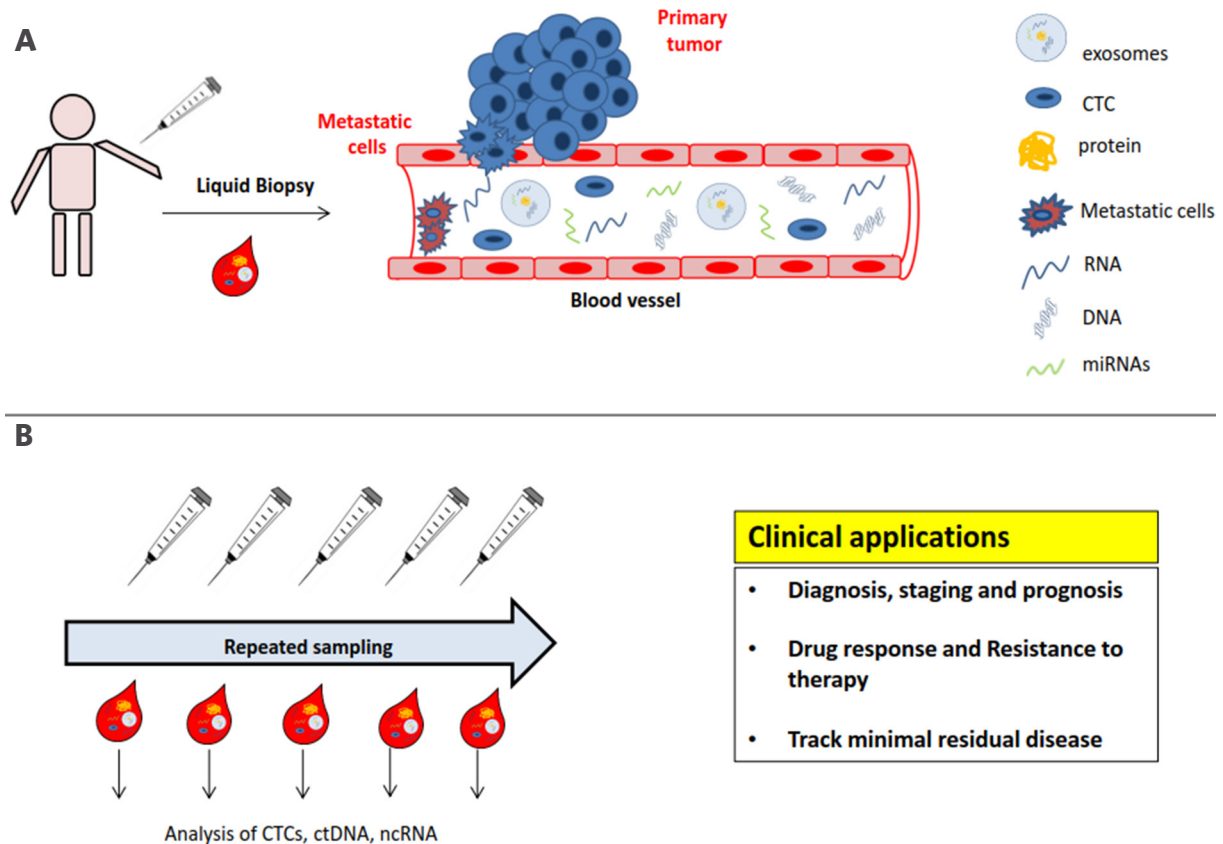
Figure 1. Liquid biopsies and their clinical applications. A: Liquid biopsy is an approach for detecting and analyzing DNA and RNA in biological fluids, such as serum, plasma, urine and saliva. Being a minimally invasive procedure, it offers the possibility of performing repeated sampling over time, thus providing a practical method for patient surveillance. Blood plasma or serum from patients contain cancer derived material, such as CTCs, ctDNA, miRNAs and other RNAs; B: analysis of such DNA/RNA content can provide evidences on the presence of HCC at an early stage, assessment of prognosis and patients' monitoring during and after therapy, thus helping the clinical management of patients at different phases of disease. CTC: circulating tumor cell; ctDNA: cell-free tumor DNA; ncRNA: non-coding RNA

Quantification of circulating DNA is very easy to perform and inexpensive. However, this approach lacks specificity for type of cancer; in addition, levels of cfDNA can also increase in inflammatory conditions unrelated to cancer or in some physiological conditions, such as pregnancy. Furthermore, this analysis does not provide information about tumor genetic landscape and cannot reveal actionable targets. For these reasons, most investigations moved toward the detection of more specific genetic or epigenetic alterations in blood, as biomarkers for HCC.

Aberrant methylation of cfDNA in plasma or serum in HCC
Promoter methylation is a well-known mechanism for gene transcriptional repression. Aberrant methylation in promoters of cancer genes represents a tumor-specific event and its detection is potentially useful for the prediction or diagnosis of HCC. Concordance between aberrant methylations in tumor tissues and plasma is generally good, indicating that plasma could represent a tumor surrogate when tissue is not available. The field has been widely investigated and a meta-analysis of these studies has been published[20]. In the diagnostic setting, from the analysis of 150 plasma samples from patients with HCC, benign liver disease (including cirrhosis and chronic inactive hepatitis) and normal controls, Huang and co-workers found that the combined aberrant methylation of four genes (*APC*, *GSTP1*, *RASSF1A* and *SFRP1*) has a significant diagnostic value for HCC[21], confirming the results obtained in tumor tissues[22]. In particular, the combination analysis of plasma methylation levels of these genes allowed to discriminate HCC from both benign or normal controls, with a sensitivity of 84.7% (in both cases) and a specificity of 81.1% and 87.8% respectively[21].

For prognostic assessment, Wong *et al.*[23] evaluated the methylation status of p15 and p16 in tumor tissues, plasma, serum and buffy coat samples from HCC patients, non-HCC controls and healthy individuals and found promoter methylation in plasma or serum of 40%-50% HCC patients. Most of patients associated with gene methylation exhibited a poorer prognosis in comparison with patients negative for aberrant methylation[23]. The detection of methylation of *APC* and *RASSF1A* promoters was also associated with shorter OS in HCC patients and *RASSF1A* methylation was demonstrated to be an independent prognostic factor[21]. Very recently, another gene whose aberrant methylation detected in plasma was associated with patients' poorer prognosis was *SOCS3*[24].

The analysis of panels of aberrantly methylated genes through the use of next generation sequencing (NGS) is expected to further improve sensitivity and specificity of aberrant methylation biomarkers. For example, targeted deep-sequencing of plasma DNA after bisulfite treatment could be used to simultaneously assess the methylation status of several targets. While Holmila *et al.*[25] identified two genes (*VIM* and *FBLN1*) whose promoters were differentially methylated in HCC using this approach, these areas of study have not been thoroughly investigated.

Compared to cfDNA abundance in plasma or serum, the detection of aberrant DNA methylations in cfDNA provides a more specific tumor biomarker, especially if a combination of multiple genes is employed. However, it still contains a limitation. Considering that aberrant methylation generally affects tumor suppressor genes, the analysis cannot reveal alterations in oncogenes potentially targets of specific therapies. With the development of more sophisticated approaches, the identification of tumor-specific genetic alterations has become feasible and has been applied to HCC.

Cancer gene mutations in plasma or serum cfDNA

An analysis of cancer gene mutations in serum or plasma of HCC patients was investigated in the diagnostic, prognostic and predictive settings.

In the diagnostic setting, R249S mutation of the *TP53* gene is hallmark of aflatoxin B1 exposure, one of the major causes of HCC in certain geographic areas. Using droplet digital PCR the authors identified a higher prevalence of this mutation in plasma cfDNAs of HCC in Cameroonian and Central African patients in comparison with control subjects with and without liver disease (almost 25% of patients with HCC and 3%-9% of non-HCC subjects were R249S carriers), suggesting a potential use of this biomarker as an early risk factor for HCC in individuals exposed to aflatoxin B1[26]. Targeted deep sequencing was used to investigate several cancer genes involved in HCC. For example, the ultra-deep sequencing analysis of 58 cancer genes performed in 8 HCC tissues and paired plasma/serum samples revealed that 15 of the 21 somatic tumor mutations (71%) could also be detected in plasma/serum cfDNA[27], thus indicating the translational potential of this approach for HCC diagnosis. In another recent study, cancer alterations in four hot-spot regions of *TP53, CTNNB1* and *TERT* genes were investigated in plasma cfDNA and corresponding tumor DNA from 48 HCC patients. Interestingly, the authors found that many gene alterations found in plasma DNA were different from those found in tumor tissues, an evidence of tumor heterogeneity[28]. Confirming tumor heterogeneity in HCC, a recent study analysed plasma cfDNA from 26 HCC patients for the presence of mutations in a large set of genes. Authors found tumor heterogeneity and evolution over time by tracking circulating mutation pattern in a patient who developed progression after capecitabine treatment[29].

The identification of gene mutations offers the possibility of identifying potential actionable alterations, useful for guiding treatment choice. It has been demonstrated that HCC harbouring mutant *RAS* exhibited a better clinical response to refametinib plus sorafenib, compared to wild-type *RAS* tumors[30]. Notably, in the course of the study aimed at detecting *KRAS* or *NRAS* mutations in plasma cfDNA of a large cohort of HCC patients, authors found other actionable mutations in *EGFR, JAK2, BRAF, FLT3, PIK3CA*, and *cKIT*, suggesting that available target therapies could potentially be effective in defined, albeit small, subsets of

HCC patients[31]. These results highlight the usefulness of cfDNA analysis for identifying actionable targets and for stratifying patients according to the potentially most appropriate therapeutic approach.

The approach has also been employed to monitor therapy efficacy over time. The mutation analysis of 574 cancer genes applied to plasma cfDNA and matched HCC from four patients, demonstrated that 97% of tumor alterations were present in the blood and that it was possible to assess tumor progression, to track the possible sites of recurrence and understand tumor clonal dynamics in relation to sequential therapies. The possibility to track tumor dynamics from plasma analysis provides a valuable strategy for monitoring therapy efficacy and infer clinical outcomes[32], helping clinician modulate therapeutic approaches in a more rational and proper direction.

MIRNAS

miRNAs are 20-24 nucleotides long RNAs. By interacting with homologous target mRNAs, they act by fine-tuning gene expression through a post-transcriptional mechanism. Each tissue exhibits a unique profile of miRNAs, which is altered in pathological conditions. In tumor tissues, miRNAs are aberrantly regulated and it has been demonstrated that some deregulated miRNAs can act as oncogenes and others as tumor suppressors[33]. The interest in circulating miRNAs as non-invasive tumor biomarkers surfaced when their presence was reported as stable molecules in serum or plasma of healthy individuals and cancer patients[34,35].

Approaches for detection and quantification of circulating miRNAs

The most common technologies employed to measure miRNA expression in biological samples include microarray, NGS, quantitative real-time PCR (RT-qPCR) and droplet digital PCR (ddPCR)[36-38]. Microarray and NGS technologies are suitable for screening and discovery purposes, qPCR and ddPCR remain the choices for validation and clinical tests development. Both microarrays and NGS provide high throughput analysis of miRNA expression profiles. Microarrays can quantify all the known miRNAs. NGS can also identify new miRNA species and differentiate closely related sequences. NGS can also detect miRNA length variation (isoforms of miRNA)[38]. qPCR and ddPCR are not high throughput technologies, but technology is relatively inexpensive, available in most laboratories and can offer higher sensitivity by exploiting amplification steps.

Among quantitative PCR approaches, ddPCR was shown to be superior to conventional real time qPCR for quantifying circulating miRNAs, as it allowed an easier absolute quantification of circulating RNAs without requiring an internal standard for normalization. Furthermore, ddPCR proved to be more tolerant than real time qPCR to the presence of inhibitors[39]. Finally, ddPCR generally exhibits a higher precision and reproducibility than real time qPCR, thus allowing an easier discrimination between cases and controls[37,40,41].

Circulating miRNAs for HCC diagnosis

Circulating miRNAs have been tested for their ability of discriminating HCC patients from control individuals [Table 2]. As shown in Table 2, however, it is evident that published studies are heterogeneous as they often differ for technical characteristics and experimental design. This heterogeneity makes it difficult to compare results and limits their transferability into applications of clinical interest.

A first source of heterogeneity is given by the use of serum or plasma for measuring circulating miRNA levels. Albeit early studies reported that composition and levels of miRNAs in serum and plasma are similar[35], there are several examples that subsequently contradicted this idea. Heegaard *et al.*[42] tested miRNA levels in paired serum and plasma samples of lung cancer patients and they concluded that these apparently similar sources of circulating miRNAs exhibit very different miRNA levels. Supporting this conclusion in liver cancer patients, miR-223-3p was found consistently low in plasma of HCC patients[43,44] but the same miRNA was high in the serum[45,46]; miR-21 was found low in serum of HCC patients in

Table 2. Circulating miRNAs as diagnostic biomarkers of hepatocellular carcinoma

miRNA	Expression	Body fluid	Experimental setting	Clinical setting	Cohorts comparison	Sample size	Sensitivity (%)	Specificity (%)	AUC	Ref.
miR-130b	Up	Serum	Single	Diagnosis	HCC *vs.* HL+ CHB	57 *vs.* 30 + 29	87.7	81.4	0.91	[124]
miR-29a-3p	Up	Serum	Single	Diagnosis	HCC *vs.* HL + LC (T)	74 *vs.* 60 + 43	N/A	N/A	0.71	[125]
Let-7f, miR-16, miR-21	Down	Serum	Multiple	Diagnosis	HCC *vs.* HL	90 *vs.* 60	N/A	N/A	N/A	[47]
miR-101	Up	Serum	Single	Diagnosis	HCC *vs.* HL	25 *vs.* 20	N/A	N/A	N/A	[126]
miR-101	Down	Serum	Single	Diagnosis	HCC *vs.* HL	67 *vs.* 30	76.1	70.0	0.79	[53]
miR-101	Down	Serum	Single	Diagnosis	HCC *vs.* LC	67 *vs.* 61	95.5	90.2	0.98	[53]
miR-101	Down	Serum	Single	Diagnosis	HCC *vs.* HL + CH	52 *vs.* 43 + 42	54.9	76.9	0.62	[52]
miR-101	Down	Serum	Single + AFP	Diagnosis	HCC *vs.* HL + CH	52 *vs.* 43 + 42	N/A	N/A	0.85	[52]
miR-101, miR-106b, miR-122, miR-195	Down	Exosomes	Multiple	Diagnosis	HCC *vs.* CHB	20 *vs.* 20	N/A	N/A	N/A	[127]
miR-101-3p, miR-106b-3p		Serum	Multiple	Diagnosis	HCC *vs.* LC	24 *vs.* 14	84.6	94.1	0.96	[51]
miR-101-3p, miR-1246, miR-106b-3p	Up	Plasma	Multiple	Diagnosis	HCC *vs.* HL	22 *vs.* 11	100	100	1	[51]
miR-101-3p, miR-1246, miR-106b-3p	Up	Plasma	Multiple	Diagnosis	HCC *vs.* HL + LC	7 *vs.* 14 + 21	N/A	N/A	N/A	[51]
miR-101-3p, miR-1246, miR-106b-3p	Up	Plasma	Multiple	Diagnosis	HCC *vs.* LC	9 *vs.* 6	100	92.9	0.99	[51]
miR-122	Up	Serum	Single	Diagnosis	CH *vs.* HL	30 *vs.* 10	N/A	N/A	N/A	[56]
miR-122	Up	Serum	Single	Diagnosis	HCC *vs.* HL	30 *vs.* 10	N/A	N/A	N/A	[56]
miR-122	Down	Serum	Single	Diagnosis	LC *vs.* HL	30 *vs.* 10	N/A	N/A	N/A	[56]
miR-122	Up		Single	Diagnosis	HCC *vs.* HL + CHB	3423 *vs.* 1887 + 2403	68.0	73.3	0.77	[128]
miR-122	Up	Serum	Single	Diagnosis	HCC *vs.* CHB	71 *vs.* 45	77.6	57.8	0.63	[45]
miR-122	Up	Serum	Single	Diagnosis	HCC *vs.* HL	71 *vs.* 34	81.6	83.3	0.87	[45]
miR-122	Down	Serum	Single	Diagnosis	CH *vs.* HL	48 *vs.* 89	80.0	91.2	0.93	[46]
miR-122	Down	Serum	Single	Diagnosis	HCC *vs.* CHB	101 *vs.* 89	N/A	N/A	N/A	[46]
miR-122	Up	Serum	Single	Diagnosis	HCC *vs.* HL	101 *vs.* 89	70.7	69.1	0.79	[46]
miR-122, let-7b	Up	Serum	Multiple	Diagnosis	HCC *vs.* HBV-related DN	30 *vs.* 47	84.8	50.0	0.65	[129]
miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801		Plasma	Multiple	Diagnosis	HCC *vs.* HL (V)	196 *vs.* 66	83.2	93.9	0.94	[49]
miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-802		Plasma	Multiple	Diagnosis	HCC *vs.* CHB (V)	196 *vs.* 72	79.1	76.4	0.84	[49]
miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-803		Plasma	Multiple	Diagnosis	HCC *vs.* LC (V)	196 *vs.* 56	75.0	91.1	0.88	[49]
miR-122, miR-885-5p, miR-221, miR-181b		Serum	Multiple + AFP	Diagnosis	HCC *vs.* LC	192 *vs.* 96	N/A	N/A	0.98	[60]
miR-122, miR-885-5p, miR-221, miR-181b		Serum	Multiple	Diagnosis	HCC *vs.* LC	192 *vs.* 96	N/A	N/A	0.84	[60]
miR-122, miR-885-5p, miR-29b		Serum	Multiple + AFP	Diagnosis	HCC *vs.* HL	192 *vs.* 96	N/A	N/A	1	[60]
miR-122, miR-885-5p, miR-29b		Serum	Multiple	Diagnosis	HCC *vs.* HL	192 *vs.* 96	N/A	N/A	0.89	[60]
miR-122-5p	Up	Plasma	Single	Diagnosis	CHB *vs.* HL	20 *vs.* 24	N/A	N/A	N/A	[43]
miR-122-5p	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	20 *vs.* 28	N/A	N/A	N/A	[43]

miRNA		Sample		Diagnosis	Comparison	N	Sens	Spec	AUC	Ref
miR-122-5p	Up	Plasma	Single	Diagnosis	LC *vs.* HL	20 *vs.* 22	N/A	N/A	N/A	[43]
miR-122-5p	Up	Plasma	Single	Diagnosis	HCC *vs.* HL + LC	7 *vs.* 14 + 21	N/A	N/A	N/A	[51]
miR-122-5p	Up	Serum	Single	Diagnosis	HCC *vs.* LC	24 *vs.* 14	N/A	N/A	N/A	[51]
miR-122a	Down	Serum	Single	Diagnosis	HCC *vs.* HL	85 *vs.* 85	70.6	67.1	0.71	[130]
miR-122a	Down	Serum	Single + AFP	Diagnosis	HCC *vs.* HL	85 *vs.* 85	87.1	98.8	0.94	[130]
miR-1247-3p	Up	Serum	Single	Diagnosis	HCC *vs.* HL	110 *vs.* 25	N/A	N/A	N/A	[131]
miR-125a-5p	Down	Serum	Single	Diagnosis	HCC *vs.* HL + CHB	120 *vs.* 164 + 91	N/A	N/A	N/A	[76]
miR-125b	Down	Plasma	Single	Diagnosis	HCC *vs.* CHB	64 *vs.* 63	93.8	85.7	0.96	[58]
miR-125b	Down	Plasma	Single	Diagnosis	HCC *vs.* HL	64 *vs.* 56	85.9	78.6	0.89	[58]
miR-125b	Down	Plasma	Single	Diagnosis	HCC *vs.* LC	64 *vs.* 59	89.1	88.1	0.96	[58]
miR-125b, miR-223, miR-27a, and miR-26a	Down	Serum	Multiple + AFP	Diagnosis	HCC *vs.* HL	90 *vs.* 60	N/A	N/A	0.87	[50]
miR-125b, miR-27a	Down	Serum	Multiple + AFP	Diagnosis	HCC *vs.* HL	90 *vs.* 60	80.0	87.2	N/A	[50]
miR-125b-5p	Up	Plasma	Single	Diagnosis	CHB *vs.* HL	20 *vs.* 24	N/A	N/A	N/A	[43]
miR-125b-5p	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	20 *vs.* 28	N/A	N/A	N/A	[43]
miR-125b-5p	Up	Plasma	Single	Diagnosis	LC *vs.* HL	20 *vs.* 22	N/A	N/A	N/A	[43]
miR-143	Down	Serum	Single	Diagnosis	HCC *vs.* HL	131 *vs.* 122	80.3	82.4	0.83	[132]
miR-145	Down	Serum	Single	Diagnosis	HCC *vs.* CHB	85 *vs.* 50	88.2	78.0	0.85	[133]
miR-148a	Down	Serum	Single	Diagnosis	HCC *vs.* HL + BLD	76 *vs.* 55 + 62	67.7	59.2	0.67	[71]
miR-148a, miR-148b, miR-152	Down	Serum	Multiple	Diagnosis	HCC *vs.* BLD	76 *vs.* 62	96.1	91.9	0.94	[71]
miR-15b	Up	Serum	Single	Diagnosis	HCC *vs.* HL + CHB	57 *vs.* 30 + 29	98.3	15.3	0.49	[124]
miR-15b, miR-130b	Up	Serum	Multiple	Diagnosis	HCC *vs.* HL + CHB	57 *vs.* 30 + 29	98.3	91.5	0.98	[124]
miR-16	Down	Serum	Single + AFP	Diagnosis	HCC *vs.* HL + CLD	105 *vs.* 71 + 107	92.4	78.5	N/A	[134]
miR-16, miR-195, miR-199a		Serum	Multiple	Diagnosis	HCC *vs.* HL + CLD	105 *vs.* 71 + 107	N/A	N/A	N/A	[134]
miR-18, miR-221, miR-222, miR-224	Up	Exosomes	Single	Diagnosis	HCC *vs.* LC	20 *vs.* 20	N/A	N/A	N/A	[127]
miR-18, miR-221, miR-222, miR-224	Up	Exosomes	Single	Diagnosis	HCC *vs.* CHB + LC	20 *vs.* 20 + 20	N/A	N/A	N/A	[127]
miR-182	Up	Serum	Single + AFP	Diagnosis	HCC *vs.* HL + BLD	103 *vs.* 40 + 95	82.5	94.7		[135]
miR-182	Up	Serum	Single	Diagnosis	HCC *vs.* HL + BLD	103 *vs.* 40 + 95	78.6	91.6	0.91	[135]
miR-182, miR-331-3p		Serum	Multiple + AFP	Diagnosis	HCC *vs.* HL + BLD	103 *vs.* 40 + 95	93.2	95.8	N/A	[135]
miR-192-5p	Up	Serum	Single	Diagnosis	HCC *vs.* HL + LC (T)	74 *vs.* 60 + 43	N/A	N/A	0.69	[125]
miR-192-5p and miR-29a-3p	Up	Serum	Single	Diagnosis	HCC *vs.* HL (T)	50 *vs.* 50	N/A	N/A	N/A	[125]
miR-192-5p and miR-29a-3p	Up	Serum	Single	Diagnosis	HCC *vs.* HL (V)	100 *vs.* 70	N/A	N/A	N/A	[125]
miR-199a	Down	Serum	Single	Diagnosis	HCC *vs.* CH	23 *vs.* 17	54.5	100	0.85	[136]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* CH	23 *vs.* 17	100	82.1	0.94	[136]
miR-21	Up	Serum	Single	Diagnosis	CHC *vs.* HL	62 *vs.* 19	87.1	73.7	0.83	[137]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* HL	29 *vs.* 19	N/A	N/A	N/A	[137]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* LC (V)	175 *vs.* 78	80.8	72.9	0.81	[59]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* CHB (V)	175 *vs.* 64	76.9	85.7	0.79	[59]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* HL (T)	40 *vs.* 40				[59]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* HL + CHB + LC (V)	175 *vs.* 136 + 64 + 78	82.1	83.9	0.85	[59]
miR-21	Up		Single	Diagnosis	HCC *vs.* HL + CHB	3423 *vs.* 1887 + 2403	86.6	79.5	0.88	[128]
miR-21	Down	Serum	Single	Diagnosis	HCC *vs.* HL	70 *vs.* 34	N/A	N/A	N/A	[45]
miR-21	Up	Plasma	Single + AFP	Diagnosis	HCC *vs.* HL + CH	127 *vs.* 50 + 30	90.0	92.9	0.82	[48]
miR-21	Up	Plasma	Single	Diagnosis	HCC *vs.* HL + CH	126 *vs.* 50 + 30	61.1	83.3	0.77	[48]

miRNA	Reg	Source	Assay	Purpose	Comparison	n	Sens	Spec	AUC	Ref
miR-21	Up	Exosomes	Single	Diagnosis	HCC *vs.* HL + CHB	30 *vs.* 30 + 30	N/A	N/A	N/A	[78]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* HL	97 *vs.* 30	N/A	N/A	N/A	[79]
miR-21	Down	Serum	Single	Diagnosis	CH *vs.* HL	48 *vs.* 89	80.0	95.6	0.91	[46]
miR-21	Down	Serum	Single	Diagnosis	HCC *vs.* CHB	101 *vs.* 89				[46]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* HL	101 *vs.* 89	84.0	73.5	0.87	[46]
miR-21	Up	Serum	Single	Diagnosis	HCC *vs.* HL	56 *vs.* 43	67.4	55.8	0.62	[52]
miR-21, miR-122, miR-192	Up	Serum	Multiple + AFP	Diagnosis	HCC *vs.* CHB	118 *vs.* 100	N/A	N/A	0.94	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple	Diagnosis	HCC *vs.* CHB	118 *vs.* 100	N/A	N/A	0.90	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple + AFP	Diagnosis	HCC *vs.* CHB + LC	118 *vs.* 100 + 69	N/A	N/A	0.88	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple	Diagnosis	HCC *vs.* CHB + LC	118 *vs.* 100 + 69	N/A	N/A	0.81	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple	Diagnosis	HCC *vs.* HL + CHB + LC	118 *vs.* 119 + 100 + 69	N/A	N/A	0.85	[61]
miR-21, miR-122, miR-192	Up	Serum	Multiple + AFP	Diagnosis	HCC *vs.* LC	118 *vs.* 69	N/A	N/A	0.88	[61]
miR-21, miR-26a, miR-101		Serum	Multiple + AFP	Diagnosis	HCC *vs.* HL + CH	52 *vs.* 43 + 42	87.0	81.0	0.91	[52]
miR-218	Down	Serum	Single + AFP	Diagnosis	HCC *vs.* HL + BLD	156 *vs.* 64 + 98	N/A	N/A	0.91	[73]
miR-218	Down	Serum	Single	Diagnosis	HCC *vs.* HL + BLD	156 *vs.* 64 + 98	66.7	69.1	0.73	[73]
miR-22, miR-199a-3p		Serum	Multiple + AFP	Diagnosis	HCC *vs.* CHC	192 *vs.* 96	N/A	N/A	0.98	[60]
miR-22, miR-199a-3p		Serum	Multiple	Diagnosis	HCC *vs.* CHC	192 *vs.* 96	N/A	N/A	0.66	[60]
miR-221	Up	Serum	Single	Diagnosis	CH *vs.* HL	30 *vs.* 10	N/A	N/A	N/A	[56]
miR-221	Down	Serum	Single	Diagnosis	HCC *vs.* HL	30 *vs.* 10	N/A	N/A	N/A	[56]
miR-221	Up	Serum	Single	Diagnosis	LC *vs.* HL	30 *vs.* 10	N/A	N/A	N/A	[56]
miR-221	Up	Serum	Single	Diagnosis	HCC *vs.* HL	46 *vs.* 50	N/A	N/A	N/A	[72]
miR-221	Up	Serum	Single	Diagnosis	HCC *vs.* HL	45 *vs.* 45	93.3	77.8	0.94	[138]
miR-221	Up	Serum	Single + AFP	Diagnosis	HCC *vs.* HL	45 *vs.* 45	96.5	88.0		[138]
miR-223	Down	Serum	Single	Diagnosis	HCC *vs.* HL + CLD	39 *vs.* 14 + 17	97.2	94.1	0.99	[139]
miR-223	Up	Serum	Single	Diagnosis	HCC *vs.* HL	73 *vs.* 34	N/A	N/A	N/A	[45]
miR-223	Up	Serum	Single	Diagnosis	CH *vs.* HL	48 *vs.* 89	80.0	75.0	0.88	[46]
miR-223	Up	Serum	Single	Diagnosis	HCC *vs.* HL	101 *vs.* 89	80.0	76.5	0.86	[46]
miR-223-3p	Down	Plasma	Single	Diagnosis	CHB *vs.* HL	20 *vs.* 24	N/A	N/A	N/A	[43]
miR-223-3p	Down	Plasma	Single	Diagnosis	HCC *vs.* HL	20 *vs.* 28	N/A	N/A	N/A	[43]
miR-223-3p	Down	Plasma	Single	Diagnosis	LC *vs.* HL	20 *vs.* 22	N/A	N/A	N/A	[43]
miR-223-3p	Down	Plasma	Single	Diagnosis	HCC *vs.* HL	8 *vs.* 28	N/A	N/A	N/A	[44]
miR-223-3p	Down	Plasma	Single	Diagnosis	LC *vs.* HL	30 *vs.* 28	N/A	N/A	N/A	[44]
miR-224	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	20 *vs.* 20	N/A	N/A	0.91	[140]
miR-224	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	87 *vs.* 55	93.1	80.0	0.91	[140]
miR-224	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	33 *vs.* 22	87.7	86.3	0.91	[140]
miR-224	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	54 *vs.* 33	87.7	86.3	0.91	[140]
miR-24-3p	Up	Serum	Single	Diagnosis	HCC *vs.* HL + CLD	84 *vs.* 46 + 31	N/A	N/A	0.63	[67]
miR-24-3p	Up	Serum	Single + AFP	Diagnosis	HCC *vs.* HL + CLD	84 *vs.* 46 + 31	N/A	N/A	0.83	[67]
miR-26a	Down	Serum	Single	Diagnosis	HCC *vs.* HL + CH	52 *vs.* 43 + 42	75.0	70.0	0.76	[52]
miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505		Serum	Multiple	Diagnosis	HCC *vs.* CHB + LC (T)	108 *vs.* 51 + 47	80.6	82.7	0.81	[141]
miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505		Serum	Multiple	Diagnosis	HCC *vs.* CHB + LC (V)	153 *vs.* 68 + 71	74.5	89.9	0.82	[141]
miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505		Serum	Multiple	Diagnosis	HCC *vs.* HL + CHB + LC (T)	108 *vs.* 51 + 51 + 47	80.6	85.5	0.82	[141]
miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505		Serum	Multiple	Diagnosis	HCC *vs.* HL + CHB + LC (V)	153 *vs.* 60 + 68 + 71	74.5	88.9	0.81	[141]

miR-30e	Down	Serum	Single	Diagnosis	HCC *vs.* HL + CLD	39 *vs.* 14 + 17	91.7	70.5	0.93	[139]
miR-331-3p	Up	Serum	Single	Diagnosis	HCC *vs.* HL + BLD	103 *vs.* 40 + 95	79.6	92.6	0.89	[135]
miR-331-3p	Up	Serum	Single + AFP	Diagnosis	HCC *vs.* HL + BLD	103 *vs.* 40 + 95	91.2	92.6	N/A	[135]
miR-335	Down	Serum	Single	Diagnosis	HCC *vs.* HL + CH	125 *vs.* 125 + 125	N/A	N/A	N/A	[86]
miR-519d	Up	Exosomes	Single	Diagnosis	HCC *vs.* LC	87 *vs.* 31	N/A	N/A	0.82	[57]
miR-595	Up	Exosomes	Single	Diagnosis	HCC *vs.* LC	87 *vs.* 31	N/A	N/A	0.92	[57]
miR-638	Down	Exosomes	Single	Diagnosis	HCC *vs.* HL	126 *vs.* 21	N/A	N/A	N/A	[75]
miR-665	Up	Exosomes	Single	Diagnosis	HCC *vs.* HL	30 *vs.* 10	N/A	N/A	N/A	[74]
miR-885-5p	Up	Serum	Single	Diagnosis	HCC + CHB + LC *vs.* HL	46 + 23 + 26 *vs.* 24	90.5	79.2	0.94	[142]
miR-939	Up	Exosomes	Single	Diagnosis	HCC *vs.* LC	87 *vs.* 31	N/A	N/A	0.84	[57]
miR-96	Up	Serum	Single	Diagnosis	HCC *vs.* CHB	104 *vs.* 100	77.9	75.3	0.83	[143]
miR-96	Up	Serum	Single + AFP	Diagnosis	HCC *vs.* CHB	104 *vs.* 100	83.6	82.4	0.88	[143]
miR-96	Up	Serum	Single	Diagnosis	HCC *vs.* CHB	104 *vs.* 100	77.9	75.3	0.80	[143]
miR-96	Up	Serum	Single	Diagnosis	HCC *vs.* HL	104 *vs.* 120	N/A	N/A	N/A	[143]
miR-96	Up	Serum	Single	Diagnosis	HCC *vs.* LC	104 *vs.* 90	N/A	N/A	N/A	[143]

AUC: area under the receiver-operating characteristic curve; AFP: alpha fetoprotein; BLD: benign liver disease; CH: chronic hepatitis; CHB: chronic hepatitis B; CHC: chronic hepatitis C; CLD: chronic liver disease; DN: dysplastic nodules; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HL: healthy liver; LC: liver cirrhosis; (T): training set; (V): validation set; N/A: not available data

two reports[45,47], but others reported miR-21 high in plasma of HCC patients[48,49]. Analysis of miR-125-b and miR-101 levels also displayed variances between serum and plasma: an upregulation of plasma miR-125-b levels was reported in HCC patients in comparison with healthy controls[43], while downregulation of the same miRNA was found in serum[50]; miR-101 levels were found high in plasma of HCC patients in comparison with healthy controls[51], whereas this miRNA was found downregulated in serum of HCC patients in comparison with healthy controls at least by two reports[52,53]. These several examples support the concept that differences between plasma and serum are common and should be taken in consideration when comparing results from different studies.

The use of plasma or serum is not the only source of variability of achieved results. An analysis of published studies strongly suggests that both pre-analytical and analytical procedures can affect results. Any change in tissue collection steps (like type of blood tubes, centrifugation strength and sample conservation) can generate differences in miRNA levels[54,55]. Considering the different hard-to-control sources of variability, it is not difficult to understand how uneven and sometimes even opposite results can easily derive.

In addition to technical reasons, aspects linked to experimental design can also be added to factors responsible for heterogeneity of results. The existence of various types of control populations represents indeed a source of variability and, in some cases, a limitation to the practical value of such results. In fact, for identifying useful biomarkers for the early detection of HCC it is very important to compare HCC not only with healthy controls but *vs.* cirrhotic patients, considering that 80%-90% of HCCs arise in this group of high-risk patients. A paradigmatic example is miR-122, a liver-specific miRNA whose level was found increased in serum/plasma of HCC patients in comparison with healthy patients[45,51], but studies found no significant differences when HCC patients were compared to cirrhotic or chronic hepatitis patients[51,56]. These findings indicate that increased circulating miR-122 levels likely reflect liver damage rather than the presence of an underlying HCC, indicating the importance of controls to draw conclusions. Among studies that produced results on differential circulating miRNAs between HCC *vs.* cirrhotic patients, Fornari *et al.*[57] found that serum miR-939, miR-595 and miR-494 could separate cirrhotic patients with and without HCC, performing better than AFP. Moshiri *et al.*[51] showed that the combination of three plasma miRNAs, miR-101-3p, miR-106b-3p and miR-1246, exhibited a high diagnostic accuracy in discriminating HCC from cirrhotic patients. Combination of two of the same miRNAs, miR-101-3p and miR-106b-3p, exhibited also an excellent diagnostic accuracy in serum of HCC *vs.* cirrhotic patients. Chen *et al.*[58] proved that plasma miR-

Table 3. Circulating miRNAs as prognostic biomarkers of hepatocellular carcinoma

miRNA	Expression[1]	Body fluid	Experimental setting	Clinical setting	Sample size	Kaplan-Meier analysis (*P* value)	Ref.
miR-101	Down	Plasma	Single	Prognosis (DFS)	163	*P* < 0.001	[144]
miR-122	Down	Serum	Single	Prognosis (OS)	122	*P* < 0.01	[145]
miR-1247-3p	Up	Serum	Single	Prognosis (OS)	85	*P* < 0.05	[131]
miR-1247-3p	Up	Serum	Single	Prognosis (DFS)	85	*P* < 0.01	[131]
miR-125a-5p	Down	Serum	Single	Prognosis (OS)	120	*P* < 0.01	[76]
miR-125b	Down	Exosomes	Single	Prognosis (OS)	128	*P* < 0.01	[77]
miR-143	Down	Serum	Single	Prognosis (OS)	131	*P* < 0.05	[132]
miR-148a	Down	Serum	Single	Prognosis (OS)	76	*P* < 0.001	[71]
miR-152	Down	Serum	Single	Prognosis (OS)	76	*P* < 0.05	[71]
miR-192-5p	Up	Serum	Single	Prognosis (OS)	74	*P* < 0.01	[125]
miR-192-5p	Up	Serum	Single	Prognosis (PFS)	74	*P* < 0.01	[125]
miR-29a-3p	Up	Serum	Single	Prognosis (OS)	74	*P* < 0.01	[125]
miR-29a-3p	Up	Serum	Single	Prognosis (PFS)	74	*P* < 0.05	[125]
miR-21, lncRNA-ATB	Up	Exosomes	Single + lncRNA	Prognosis (OS)	79	*P* < 0.05	[80]
miR-21, lncRNA-ATB	Up	Exosomes	Single + lncRNA	Prognosis (PFS)	79	*P* < 0.05	[80]
miR-218	Down	Serum	Single	Prognosis (OS)	156	*P* < 0.05	[73]
miR-221	Up	Serum	Single	Prognosis (OS)	46	*P* < 0.05	[72]
miR-224	Down	Serum	Single	Prognosis (OS)	182	*P* < 0.05	[146]
miR-24-3p	Up	Serum	Single	Prognosis (OS)	84	*P* < 0.01	[67]
miR-24-3p	Up	Serum	Single	Prognosis (DFS)	84	*P* < 0.01	[67]
miR-638	Down	Exosomes	Single	Prognosis (OS)	126	*P* < 0.01	[75]
miR-96	Up	Serum	Single	Prognosis (OS)	49	*P* < 0.05	[143]

[1]Expression in the group with the poorest prognosis. lncRNA: long noncoding RNA; OS: overall survival; PFS: progression free survival; DFS: disease-free survival

125b could differentiate HCC from cirrhotic patients, and Guo *et al.*[59] found that serum miR-21 analysis had also a good diagnostic efficacy in discriminating HCC patients both from non-HCC populations or from cirrhotic patients. Analyses of miRNA panels have been used for discriminating cirrhotic patients with or without HCC. For example, two different miRNA panels have been employed to efficiently distinguish HCC patients from cirrhotic patients, one in serum (miR-122, miR-885-5p, miR-221, miR-181b[60]) and one in plasma (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-803[49]). More recently, Tat Trung *et al.*[61] showed that a miRNA panel including miR-21, miR-122 and miR-192 had a good diagnostic performance in discriminating HCC patients from other groups, in particular from cirrhotic and chronic hepatitis patients.

Another potential limitation of several published studies is the fact that they were based on the assumption that cell-free miRNA levels were altered as a consequence of their release by neoplastic cells, and tried to validate as circulating cancer biomarkers the same miRNAs deregulated in tumor tissues. This assumption may not be correct[62,63]. In fact, if we consider that ctDNA represents a small or very small fraction of cfDNA (approximately 0.1%-1% or less for non-metastatic tumors and 1%-10% for large metastatic tumors), it is difficult to conceive that cancer cells could instead release such a high amount of RNA to significantly change content and levels of specific circulating RNAs. While this consideration raises doubts about the source of circulating RNAs, a number of studies (see review by McAllister and Weinberg[64]) offer a possible explanation by indicating that cancer should be considered a systemic disease. In this view, tumor-associated systemic processes may unbalance the release of miRNAs from non-tumor cells and therefore change circulating RNA profiles. Thus, finding altered circulating miRNA profiles would represent evidence of a systemic pathophysiological process closely linked to the presence of a tumor, and such RNAs would then represent actual circulating tumor biomarkers, although largely not directly released by tumor cells. In this viewpoint, the numerous evidences that the altered levels of miRNAs in circulation do not necessarily reflect the miRNA deregulation found in cancer tissues would become plausible and results should be re-

Table 4. Circulating miRNAs as predictive biomarkers of therapy response in hepatocellular carcinoma

miRNA	Expression in circulation[1]	Body fluid	Experimental Setting	Clinical setting	Treatment	Sampling (pre/post therapy)	Ref.
miR-1246	Up	Plasma	Single	Monitoring	LT	Pre/post	[82]
miR-182	Up	Serum	Single	Monitoring	TACE	Pre/post	[135]
miR-331-3p	Up	Serum	Single	Monitoring	TACE	Pre/post	[135]
miR-122	Up	Plasma	Single	Response prediction	TACE	Pre	[84]
miR-122	Up	Plasma	Single	Response prediction	RFA	Pre	[147]
miR-181a-5p	Down	Serum	Single	Response prediction	Sorafenib	Pre	[89]
miR-200	Up	Serum	Single	Response prediction	TACE	Pre	[87]
miR-21	Up	Serum	Single	Response prediction	Resection	Pre	[79]
miR-21, miR-26a, miR-29a-3p		Plasma	Multiple	Response prediction	TACE	Pre	[148]
miR-221	Up	Serum	Single	Response prediction	Sorafenib	Pre	[88]
miR-26a	Down	Plasma	Single	Response prediction	Resection or RFA	Pre	[149]
miR-29a	Down	Plasma	Single	Response prediction	Resection or RFA	Pre	[149]
miR-339-5p	Down	Serum	Single	Response prediction	Sorafenib	Pre	[89]
miR-34a	Down	Serum	Single	Response prediction	Resection	Pre	[150]
miR-665	Up	Serum exosomes	Single	Response prediction	Resection	Pre	[74]
miR-718	Down	Serum exosomes	Single	Response prediction	LT	Pre	[151]
miR-1246	Up	Plasma	Single	Responsive *vs*. non responsive	LT	Post	[82]
miR-148, miR-1246	Up	Plasma	Multiple	Responsive *vs*. non responsive	LT	Post	[82]
miR-148a	Up	Plasma	Single	Responsive *vs*. non responsive	LT	Post	[82]
miR-148a, miR-148b, miR-152	Down	Serum	Multiple	Responsive *vs*. non responsive	Resection	Post	[71]
miR-182	Up	Serum	Single	Responsive *vs*. non responsive	TACE	Post	[135]
miR-221	Down	Serum	Single	Responsive *vs*. non responsive	Sorafenib	Post	[88]
miR-331-3p	Up	Serum	Single	Responsive *vs*. non responsive	TACE	Post	[135]
miR-335	Down	Serum	Single	Responsive *vs*. non responsive	TACE	Post	[86]
miR-423-5p	Down	Serum	Single	Responsive *vs*. non responsive	Sorafenib	Post	[152]
miR-122	Down	Exosomal	Single	Responsive *vs*. non responsive	TACE	Pre/Post	[85]

[1]Circulating miRNA levels in non-responsive patients. LT: liver transplantion; RFA: radiofrequency ablation; TACE: transcatheter arterial chemoembolisation

interpreted in accordance. In this context, an example is miR-101-3p, which was found downregulated in HCC tissues[65] but upregulated in plasma[51]. Other examples include miR-21, upregulated in HCC tissues[66] but in some cases they are reported to be downregulated in patients serum[45,47] or the previously mentioned miR-122, whose altered circulating level is predominantly a sign of hepatic injury[51,56].

The combination of biomarkers can potentially overcome the individual limitations. For this reason, miRNAs have been tested in association with AFP for improving test performance. Some studies indicated that the combination of miR-21 and AFP improved the discrimination between HCC patients *vs.* chronic hepatitis patients[48]. Meng *et al.*[67] showed that the combination of miR-24-3p and AFP allowed to better separate HCC from chronic liver disease affected patients and also a combination of miRNA panels with AFP provided a very good discriminating power between HCC *vs.* cirrhotic or chronic hepatitis patients[52,60,61].

Circulating miRNAs for HCC prognosis

Differences in median level of plasma/serum miRNAs as a cut-off value provided information about tumor stage and prognosis in HCC patients [Table 3]. Some studies have shown correlations of miRNA levels with pathological characteristics associated with prognosis. Members of the miR-148/152 family (miR-148a, miR-148b and miR-152) are important modulators of cell growth and progression of HCC[68-70]. Wang *et al.*[71] showed that low levels of miR-148a and miR-148b were significantly associated with tumor size and TNM stage, whereas low levels of miR-152 correlated with TNM stage. Additionally, the combination of circulating

miR-148/152 family could discriminate HCC from non-malignant chronic liver diseases[71]. Other studies associated circulating miRNA levels with HCC patients' prognoses. For example, Li *et al.*[72] showed that high serum levels of miR-221 correlated with tumor size, cirrhosis, tumor stage and with a lower OS in comparison with patient with low miR-221 expression levels, suggesting serum miR-221 as an independent risk factor for poor prognosis. A study by Yang *et al.*[73] reported that low serum levels of miR-218 were associated with clinic-pathological features such as tumor size, vascular invasion and TNM stage, as well as OS of patients. All these reports supported the potential role of circulating miRNAs in the assessment of prognosis in HCC. Studies described also the correlation between exosomal serum miRNA levels with pathological features and survival in HCC patients. For example, HCC patients with low levels of miR-665 showed a strong association with large tumour size (> 5 cm), local tumour invasion and metastases[74]; Shi *et al.*[75] showed that decreased levels of exosomal miR-638 had poor OS. Zheng *et al.*[76] reported that low levels of serum miR-125a-5p were associated with a lower OS compared with those exhibiting higher expression levels, and more recently Liu *et al.*[77] confirmed by a Kaplan-Meier analysis that HCC patients with lower serum miR-125b levels showed reduced time to recurrence and OS. Many studies involved the analysis of circulating miR-21. In one study, high levels of serum miR-21 were found correlated with cirrhosis and tumor stage[78], another revealed an association with metastasis[79]; Lee *et al.*[80] reported that exosomal miR-21 was an independent predictor of disease progression in HCC patients and high circulating levels of exosomal miRNA-21 were associated with lower OS and progression-free survival. These data support the role of miRNAs as potential prognostic biomarker in HCC.

Circulating miRNAs for prediction of HCC recurrence and treatment response
Many studies focused their attention on miRNAs ability to predict treatment response and monitor disease relapse after surgery or drug therapy [Table 4].

Surgery is the treatment of choice for early HCC, however relapse is common[81]. Levels of circulating miRNAs were studied in patients who underwent surgical resection, revealing a correlation with the post-operative survival. For example, subjects with low serum miR-21 levels had a 29% 5-year survival rate, whereas those with high expression had a 14.3% 5-year survival rate[79]. Wang *et al.*[71] showed that levels of serum miR-148/152 family decreased in case of relapse after surgery. Ng *et al.*[82] showed that miR-1246 was an independent predictor of OS and disease-free survival of HCC patients after liver transplant.

In patients at intermediate HCC stage, recommended first-line therapy is trans-arterial chemoembolization (TACE)[83], while sorafenib is the standard first line-systemic therapy for patients with advanced tumors (BCLC C)[81]. The association between circulating levels of miR-122 and treatment outcome after TACE was evaluated in two recent studies. Kim *et al.*[84] found that high plasma miR-122 expression levels could be predictive for early and overall TACE insufficient responses and refractoriness in HCC patients. Suehiro *et al.*[85] found that exosomal miR-122 expression levels were significantly decreased after TACE, especially in patients with cirrhosis, and suggest that the reduction in exosomal miR-122 levels may reflect a decrease in the liver function, rather than the anti-tumor effects of the procedure. Other miRNAs were studied in HCC patients treated with TACE. For example, lower serum miR-335 levels were associated with a shorter OS[86], while lower expression of miR-200 in HCC patients predicted a better prognosis in HCC patients treated with TACE[87]. Considering the data reported, circulating miRNAs could be associated with clinical outcome of HCC patients treated with TACE.

There are no biomarkers to predict response to sorafenib. A small number of studies evaluated whether circulating miRNAs could predict or anticipate therapy responsiveness. From the analysis of miR-221 levels in sera from HCC patients who received sorafenib, Fornari *et al*[88] found that the treatment determined an increase of miR-221 only in responders. Moreover, analyzing miR-221 levels in sera from HCC patients before sorafenib treatment, lower miR-221-circulating levels were associated with better response to the drug[88]. Analysing serum miRNA profiles during sorafenib therapy, Nishida *et al.*[89] found that miR-181a-

Table 5. Circulating long noncoding RNAs as biomarkers of hepatocellular carcinoma

lncRNA	Expression	Body fluid	Experimental setting	Clinical setting	Cohorts comparison	Ref.
AF085935	Up	Serum	Single	Diagnosis (early)	HCC *vs.* HL	[95]
AF085935	Up	Serum	Single	Diagnosis (early)	HCC *vs.* HBV carriers	[95]
CTBP + LAMP2 + miR-16-2 + miR-21-5p		Serum	Multiple + miRNA	Diagnosis	HCC *vs.* HL + CHC	[153]
CTBP + LAMP2 + miR-16-2 + miR-21-5p		Serum	Multiple + miRNA	Prognosis (PFS)	HCC	[153]
DANCR	Up	Plasma	Single	Diagnosis	HCC *vs.* HL + CHB + LC	[105]
DANCR	Up	Plasma	Single	Prognosis	HCC	[105]
ENSG00000258332.1 + LINC00635	Up	Serum	Multiple + AFP	Diagnosis	HCC *vs.* HL + LC + CHB	[96]
ENSG00000258332.1 + LINC00635	Up	Serum	Single	Prognosis (OS)	HCC	[96]
HULC	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	[104]
HULC	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	[154]
HULC + LINC00152	Up	Plasma	Multiple + AFP	Diagnosis	HCC *vs.* HL	[104]
JPX	Down	Plasma	Single + AFP	Diagnosis	HCC *vs.* HL	[101]
JPX	Down	Plasma	Single	Diagnosis	HCC *vs.* HL	[101]
JPX	Down	Plasma	Single	Prognosis	HCC	[101]
JUN + UCA1	Up	Serum	Multiple	Diagnosis (early)	HCC *vs.* HL + CHC	[155]
JUN + UCA1	Up	Serum	Multiple + AFP	Diagnosis (early)	HCC *vs.* HL + CHC	[155]
LRB1	Up	Serum	Single + AFP/DCP	Diagnosis	HCC *vs.* HL	[102]
LRB1	Up	Serum	Single	Prognosis (OS)	HCC	[102]
LINC00152	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	[104]
LINC00974	Up	Plasma	Single	Diagnosis (early)	HCC *vs.* HL	[156]
LINC01225	Up	Serum	Single	Diagnosis	HCC *vs.* HL	[157]
lnc-PCDH9-13:1	Up	Saliva	Single	Diagnosis (early)	HCC *vs.* HL + HBV carrier + CHB + LC	[107]
LOC149086 + RP11-160H22.5 + XLOC_014172	Up	Plasma	Multiple	Diagnosis	HCC *vs.* cancer free (T)	[158]
LOC149086 + RP11-160H22.5 + XLOC_014172	Up	Plasma	Multiple	Diagnosis	HCC *vs.* cancer free (V)	[158]
PIVKAII + MALAT1	Up	Plasma	Multiple + AFP	Diagnosis	HCC *vs.* HL	[159]
PVT1 + uc002mbe.2	Up	Serum	Multiple	Diagnosis	HCC *vs.* HL	[100]
SNHG1	Up	Plasma	Single	Diagnosis	HCC *vs.* HL + CHB + LC	[97]
SNHG1	Up	Plasma	Single + AFP	Diagnosis	HCC *vs.* HL + CHB + LC	[97]
SPRY4-IT1	Up	Plasma	Single + AFP	Diagnosis	HCC *vs.* HL	[160]
SPRY4-IT1	Up	Plasma	Single	Diagnosis	HCC *vs.* HL	[160]
uc001ncr + AX800134		Serum	Multiple	Diagnosis (early)	HCC *vs.* HL + HBV carriers	[161]
uc003wbd	Up	Serum	Single	Diagnosis (early)	HCC *vs.* HL	[95]
uc003wbd	Up	Serum	Single	Diagnosis (early)	HCC *vs.* HBV carriers	[95]
UCA1	Up	Serum	Single	Diagnosis	HCC *vs.* HL + CHC	[99]
UCA1	Up	Serum	Single	Prognosis (PSF)	HCC	[103]
UCA1 + WRAP53	Up	Serum	Multiple + AFP	Diagnosis	HCC *vs.* HL + CHC	[99]
UCA1 + WRAP53	Up	Serum	Multiple	Diagnosis	HCC *vs.* HL + CHC	[99]
WRAP53	Up	Serum	Single	Diagnosis	HCC *vs.* HL + CHC	[99]
WRAP53	Up	Serum	Single	Prognosis (PSF)	HCC	[99]
XIST	Down	Plasma	Single	Prognosis	HCC	[101]
ZFAS1	Up	Plasma	Single + AFP	Diagnosis	HCC *vs.* HL (T)	[98]
ZFAS1	Up	Plasma	Single + AFP	Diagnosis	HCC *vs.* HL + CHB + LC (V)	[98]
ZFAS1	Up	Plasma	Single	Diagnosis	HCC *vs.* HL (T)	[98]
ZFAS1	Up	Plasma	Single	Diagnosis	HCC *vs.* HL + CHB + LC (V)	[98]

lncRNA: long noncoding RNA; AFP: alpha fetoprotein; DCP: des-carboxyprothrombin; CHC: chronic hepatitis C; CHB: chronic hepatitis B; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HL: healthy liver; LC: liver cirrhosis; (T): training set; (V): validation set; OS: overall survival; PFS: progression free survival

5p was decreased in non-responder patients compared to responders, suggesting this miRNA as a candidate serum biomarker for predicting response to sorafenib.

LNCRNAS

lncRNAs are a class of > 200 nt RNA transcripts linked to the modulation of several pathways through various different molecular mechanisms[90,91]. Their expression differs between cancer and non-cancer tissues and their role in cancer biology is well recognized[92,93]. Expression deregulation of several lncRNAs has been reported in HCC[94] and abnormal levels of an increasing number of lncRNAs are being found in serum/plasma of HCC patients, suggesting their potential use as circulating tumor biomarkers. Table 5 summarizes the studies that have linked circulating lncRNAs to HCC. Technologies for detection and quantification of lncRNAs in biological samples are the same previously described for miRNAs.

A number of studies demonstrated that circulating lncRNAs could discriminate HCC from healthy controls or patients with non-malignant chronic liver diseases. In several cases, the studies involved detection of early HCCs, a crucial factor for the application of curative strategies. lncRNAs were evaluated either as single biomarkers or in combination. As single biomarkers, results indicated a sensitivity ranging from 51% to 92%. In this experimental setting, lncRNA - uc003wbd[95], ENSG00000258332.1[96], small nucleolar RNA host gene 1 (SNHG1)[97], zinc finger antisense 1 (ZFAS1)[98] were able to differentiate HCC patients from chronic hepatitis B virus (HBV) patients or healthy controls, urothelial carcinoma associated-1 (UCA1) or WRAP53[99] were significantly higher in HCC patients' sera in comparison with chronic hepatitis C virus (HCV) patients or healthy volunteers. Diagnostic accuracy improved when lncRNAs were combined among them or with AFP and DCP. Combination of the lncRNAs plasmacytoma variant translocation 1 (PVT1) and uc002mbe.2 could discriminate early HCC patients from either HBV or HCV positive patients[100]. ZFAS1[98] or the expressed neighbor of XIST (Enox or JPX)[101] levels in combination with AFP could discriminate HCC from healthy individuals or chronic liver diseases patients with a better accuracy than each biomarker considered individually. The same was found for the combination of serum lncRNA uc007biz.1 (LRB1) with AFP and DCP biomarkers[102] or the combination of PVT1 and uc002mbe.2 with AFP[100].

Many circulating lncRNAs were also found to correlate with unfavourable pathologic features[96,97,100-105] and their association with prognosis was also evaluated. The increased levels of UCA1[106] or differentiation antagonizing non-protein coding RNA (DANCR)[105] or LRB1[102] were all associated to a poorer OS. Some of the studies investigated the combination of lncRNAs. The combined up-regulation of ENSG00000258332.1 and LINC00635[96], or of SNHG and UCA1[106] or low levels of JPX and X inactive-specific transcript (XIST)[101] significantly correlated with a poorer prognosis in HCC patients.

lncRNAs were also evaluated as biomarkers for monitoring tumor recurrence after surgery. In this clinical setting, it was found that patients with higher DANCR levels after surgery were prone to develop HCC recurrence[105]. It was also reported that the circulating levels of PCDH9-13:1 were significantly reduced after curative hepatectomy. However, it increased again in case of a relapse, suggesting that this lncRNA could be used to monitor patients after surgery[107]. Similarly, based on the decreased levels of SNHG1[97] or PVT1[100] after surgery, it was speculated that a subsequent increase of their expression might serve as biomarkers to monitor patients for HCC relapse.

CONCLUSION

Analysis of cell free DNA and RNA in body fluids, the so-called liquid biopsy, represents a very promising strategy for the early detection of cancer at an early stage or during monitoring of patients for the early detection of cancer relapse. The approach has the potential to significantly improve the clinical management of HCC cancer patients. Studies on circulating DNA/RNA in HCC originate from the need to identify more effective biomarkers than those currently in use, AFP and DCP. This review presents the results obtained so far in HCC. Although the specific studies still require further validation, overall they demonstrate a good sensitivity and specificity, higher than the current biomarkers and, once present limitations are over, can successfully find a valuable clinical use.

Although there are small amounts of nucleic acids in the circulation and only a fraction originate from tumor cells, the current technologies allow to pinpoint the changes induced by the presence of a tumor both qualitatively and quantitatively. Deep sequencing-based approaches for high-throughput and quantitative PCR analyses for targeted investigations demonstrated the appropriate ability to highlight such decisive traces of disease. However, it is clear that there are differences deriving from the analysis of DNA or RNA in the circulation.

From this point of view, DNA analysis is simpler and provides a straightforward interpretation: the presence, even in traces, of genetic or epigenetic alterations typically associated with a neoplastic disease provides a tangible sign of the presence of tumor cells. However, ctDNA analysis can also provide additional information. In fact, the technologies either based on NGS or ddPCR are quantitative and therefore allow to monitor the quantitative evolution of genetic or epigenetic alterations over time, thereby providing an assessment of therapy effectiveness. Furthermore, it is possible that distinct tumor clones may differentially respond to therapy. The mutational analysis of ctDNA offers the possibility of highlighting tumor heterogeneity, that tissue biopsy does not allow, thus making possible the detection of tumor clones differentially responsive to therapy. In this regard, identification of mutations in specific "actionable" genes offers the clinician the possibility of using targeted therapies if a molecular target is spotted. At present, the situation in HCC is limited, but auspiciously in evolution. Although sorafenib and regorafenib were the only few available options in advanced HCC until now, two new kinase inhibitors, namely lenvatinib and cabozantinib, have been recently approved by FDA for first-line and second-line treatment[108,109]. Unfortunately, an important limitation is that none of these drugs is associated with specific molecular alterations. As seen in other tumor types, it is however reasonable to expect that molecular subgroups of HCCs might be recognized for their differential responsiveness to specific targeted therapies. From these considerations, it is therefore clear that ctDNA analysis, which widely surpasses AFP and DCP circulating biomarkers in many aspects, can find clinical application for the management of HCC patients. It possibly presents a limitation, namely the possibility of detecting the presence of gene mutations in the early stages of disease, which can be difficult due to the very little amount of DNA released by small tumors. Overcoming this current limit will certainly be a goal of future research. Another limitation in HCC is the availability of a limited number of effective drugs against the most frequently mutated genes in HCC, such as catenin, as currently there are no target drugs capable of acting against the encoded oncoproteins.

The results from circulating RNA studies have probably a different conceptual meaning and so far the produced outcomes are not yet ready for clinical use. From a practical point of view, unlike genetic or epigenetic DNA-based tests that display characteristics intrinsically distinctive of neoplastic cells, in the case of circulating RNAs, they are also present in circulation of unaffected individuals and differences with cancer patients are exclusively quantitative. This indicates that for clinical application it will be necessary to define significance thresholds and appropriate methods to obtain reliable data. Quantitative PCR methods can be easily applied to diagnostic applications and, at present, ddPCR emerged as a robust method to quantify circulating miRNAs[37,39,40,110,111].

In summary, the analysis of circulating miRNA/lncRNA is potentially useful in different phases of the disease including early stages and the ddPCR method is optimally effective for performing the analysis. However, the approach is at present immature for clinical use both for methodological and scientific issues that need to be solved. Both pre-analytical and analytical procedures need to be standardized to guarantee solid results from independent laboratories[112]. On the scientific side, the levels of circulating RNAs show a normal variability among individuals. To diagnose HCC is essential to reaching an understanding of the variability in circulating miRNA/lncRNA levels not only in healthy individuals, but also and more importantly in patients affected by chronic liver diseases, such as chronic HBV/HCV infection, alcoholic and non-alcoholic fatty liver disease, steatohepatitis and cirrhosis. To this end, large study cohorts are needed[113].

Albeit often very exciting, results from the scientific literature are so far inconsistent. To change this status and open the way to translational applications, prospective well-designed large multicenter trials are needed.

DECLARATIONS

Authors' contributions
Conceived and designed the study: Callegari E, Sabbioni S, Negrini M
Wrote the manuscript: Guerriero P, Moshiri F, Lupini L, Callegari E, Negrini M
Read and approved the manuscript: All authors

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