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Jin-Lei Wang, Director
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Baishideng Publishing Group Inc
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Pleasanton, CA 94588, USA
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REVIEW

Liquid biopsy in patients with hepatocellular carcinoma: Circulating tumor cells and cell-free nucleic acids

Wataru Okajima, Shuhei Komatsu, Daisuke Ichikawa, Mahito Miyamae, Takuma Ohashi, Taisuke Imamura, Jun Kiuchi, Keiji Nishibeppu, Tomohiro Arita, Hirotaka Konishi, Atsushi Shiozaki, Ryo Morimura, Hisashi Ikoma, Kazuma Okamoto, Eigo Otsuji

Wataru Okajima, Shuhei Komatsu, Daisuke Ichikawa, Mahito Miyamae, Takuma Ohashi, Taisuke Imamura, Jun Kiuchi, Keiji Nishibeppu, Tomohiro Arita, Hirotaka Konishi, Atsushi Shiozaki, Ryo Morimura, Hisashi Ikoma, Kazuma Okamoto, Eigo Otsuji, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan

Author contributions: Okajima W and Komatsu S contributed equally to this work; Okajima W and Komatsu S wrote the manuscript; Ichikawa D and Otsuji E helped to draft the manuscript; Miyamae M, Ohashi T, Imamura T, Kiuchi J, Nishibeppu K, Arita T, Konishi H, Morimura R, Shiozaki A, Ikoma H and Okamoto K collected the literature.

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Correspondence to: Shuhei Komatsu, MD, PhD, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachihirokoji, Kamigyo-ku, Kyoto, 602-8566, Japan. skomatsu@koto.kpu-m.ac.jp
Telephone: +81-75-2515527
Fax: +81-75-2515522

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Abstract

Hepatocellular carcinoma (HCC), with its high incidence and mortality rate, is one of the most common malignant tumors. Despite recent development of a diagnostic and treatment method, the prognosis of HCC remains poor. Therefore, to provide optimal treatment for each patient with HCC, more precise and effective biomarkers are urgently needed which could facilitate a more detailed individualized decision-making during HCC treatment, including the following; risk assessment, early cancer detection, prediction of treatment or prognostic outcome. In the blood of cancer patients, accumulating evidence about circulating tumor cells and cell-free nucleic acids has suggested their potent clinical utilities as novel biomarker. This concept, so-called "liquid biopsy" is widely known as an alternative approach to cancer tissue biopsy. This method might facilitate a more sensitive diagnosis and better decision-making by obtaining genetic and epigenetic aberrations that are closely associated with cancer initiation and progression. In this article, we review recent developments based on the available literature on both circulating tumor cells and cell-free nucleic acids in cancer patients, especially focusing on Hepatocellular carcinoma.

Key words: Hepatocellular carcinoma; Biomarker; Liquid biopsy; Circulating tumor cells; Cell-free nucleic acids

Core tip: Accumulating evidence about circulating tumor cells and cell-free nucleic acids in the blood of cancer patients has suggested their potent clinical utilities as novel biomarker. This concept, so-called "liquid biopsy" is widely known as an alternative approach to cancer tissue biopsy. This method might facilitate a more sensitive diagnosis and better decision-making by obtaining genetic and epigenetic aberrations that are closely associated with cancer initiation and progression. In this article, we review recent developments based on the available literature on both circulating tumor cells and cell-free nucleic acids in cancer patients, especially focusing on Hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, but it ranks as the second most common cause of cancer-related death worldwide[1]. Despite recent development of a diagnostic and treatment method, the prognosis of HCC remains poor. Even in major advanced economies, the mortality rates have been increasing. Although HCC is a typical viral infection-related malignancy derived from chronic hepatitis B and C[2,3], HCC has also been strongly associated with lifestyle. Excessive alcohol consumption, obesity, and type 2 diabetes are strongly associated with the carcinogenesis and development of HCC[4-7]. Both the proportion and number of HCC patients with non-viral etiologies have been increasing on a global scale. Therefore, defining the target population should be added to screening as the most important clinical issues.

Early screening of patients for HCC has been reported to confer a survival benefit[8,9]. Patients who are identified early have multiple treatment options leading to improved outcomes. However, in clinical settings, only approximately 30% to 40% of patients with HCC can get effective treatment at the right time[10], and few molecules have been used as clinical biomarkers for HCC. Alpha-fetoprotein (AFP), AFP lectin fraction (AFP-L3), and des-γ-carboxy prothrombin (DCP, also known as proteins induced through vitamin K deficiency or antagonist-Ⅱ, PIVKA-Ⅱ) have been used as conventional serum tumor markers. However, these markers often show false-positive results, and lack sufficient sensitivity and specificity[11-13]. Therefore, to provide optimal treatment for each patient with HCC, more precise and effective biomarkers are urgently needed. Accumulating evidence of liquid biopsy might facilitate a more sensitive diagnosis and individualized decision-making in the duration of treatment of HCC.

In various cancers, many studies have demonstrated a large number of genetic and epigenetic aberrations contribute to carcinogenesis and their clinical utility[14-18]. Traditionally, these tumor-linked alterations has been provided from tissue samples of HCC patients. However, conventional procedures for tissue sampling from HCC patients is not always be conducted due to their clinical difficulties such as anatomical reasons, invasive nature, and/or the patient's poor hepatic status[19,20]. Because of such backgrounds, conventional procedures have some problems: (1) Results from a single biopsy could provide considerably restricted information; and (2) they could not reflect current cancer status, such as treatment sensitivity and therapeutic efficiency. Detecting circulating tumor cells (CTCs) and/or circulating cell-free nucleic acids (cfNAs) in the blood of cancer patients could provide us a so-called "liquid biopsy", which would realize repeated samplings and reflecting the characteristics and dynamics of tumor[21-24].

To date, many study groups have revealed the possibility of CTCs and cfNAs in the blood, as blood-based biomarkers, for several types of cancers[21-24]. These novel biomarkers are thought to have great potential and could provide more detailed individualized decision-making during HCC treatment, including the following; risk assessment, early cancer detection, prediction of treatment or prognostic outcome. In this article, based on the available literature, we review the histological backgrounds, recent developments and prospects for the future of liquid biopsy, particularly focusing on Hepatocellular carcinoma.

BIOLOGY AND DETECTION OF CIRCULATING TUMOR CELLS

CTCs are generally recognized as the "seeds" of tumors, which are shed into peripheral blood from a tumor *in situ* and eventually establish metastatic tumors in other organs[20]. Therefore, theoretically, circulating tumor cells (CTCs) are useful markers for early diagnosis. In 1869, Ashworth initially demonstrated the presence of CTCs[25] in the blood of breast cancer patient. This patients has widespread breast cancer, and the cells similar to those in the primary breast cancer had been detected in her blood. Afterwards, to validate Ashworth's remarks, many researches have challenged to investigate peripheral blood of various cancer patients to identify CTCs.

However, the effort has been hampered by some

difficulties. The problem is that the earlier the stage is, the less the cells are. Namely, the cell tends to be proportional to tumor volume. Moreover, CTCs have estimated infrequencies of approximately 1-10 CTCs in a background of millions of blood cells in patients with metastatic diseases[26]. In addition, less than 0.01% of CTCs introduced into the circulation survive to produce metastases. Furthermore, in phenotype, as well as genotype, CTCs are considered to be quite heterogeneous[27-29]. As CTCs are thought to be derived from the primary cancer or metastases, they are rarely present in patients with non-neoplastic disorders healthy person[30]. Therefore, the performance such as sensitivity and specificity of detection technique should be achieved to a proper level, precise detection of CTCs has been a major problem in this field for researchers.

TECHNIQUES FOR ISOLATION, ENRICHMENT, AND IDENTIFICATION OF CTCS

In recent years, various CTC isolation and enrichment technologies have emerged, their approaches are generally categorized into two methods.

Physical methods

Physical methods mainly depend on the physical properties of CTCs, such as density, size, migratory capacity, deformability and electric charge[31]. Most CTCs originate from epithelial tumors are thought to larger than other blood cells, several filtration-based techniques has been developed[32,33]. However, substantial difference has been demonstrated in cell size not only in an individual cancer patient but also in different cancer patients[34-36]. Thus, novel techniques adopting multiple filters have been studied to solve these issues and improve accuracy of enrichment of CTCs[37,38]. These micro device could isolate cancer cells using their physical properties such as size continuously and deformability. For example, Mohamed *et al*[37] designed a micro-machined device, which had arrays of four successively narrower channels, were able to fractionate cancer cells without interference from the blood cells. Those novel techniques could have substantial possibilities, their utility should be validated in the future.

Biological methods

Another approach is biological methods, which mainly rely on antigen-antibody binding and antibodies against tumor-specific biomarkers including epithelial cell adhesion molecule (EpCAM), human epidermal growth factor receptor2 (Her2), and prostate-specific antigen (PSA) that are typically used in CTCs purification[39]. Currently, Cell-Search™ (Veridex LLC, NJ, United States) is the most commonly used CTC platform. In this platform, immunomagnetic beads coated with EpCAM antibodies capture CTCs, followed by immunostaining with two positive markers, which are cytokeratins 8/18/19 for cytoplasmic epithelium and 4′,6′-diamidino-2-phenylindole hydrochloride for nucleic acids, and a negative marker, leukocyte-specific CD45. Its utility as a clinical indicator has been shown in the patients with metastatic breast, prostate, and colon cancers[40-45]. Therefore, this system has been the only CTC platform to be approved by the United States Food and Drug Administration. However, it could not capture CTCs that have increased the malignant potential, caused by the acquisition of an epithelial-mesenchymal transition (EMT). Concerning the detection and isolation capability and the clinical utility of CTCs, many challenges remain. To overcome its insufficient capability and accuracy, advanced technologies have emerged. "CTC-chip" is the representative technology without being influenced by the heterogeneity of them. It is a unique microfluidic platform, capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated micro-posts[46]. Most recently, CTC-Chip was reported to detect CTCs with high accuracy by using tumor-specific markers, such as human epidermal growth factor (HER2) in breast cancer or prostate-specific antigen (PSA) in prostate cancer, in addition to epithelial markers[46,47]. Another unique approach was reported by Saucedo-Zeni *et al*[48] They captured and enriched CTCs from medical Seldinger guidewire, which were inserted into cubital veins. Despite these advances, the methodology of isolation and enrichment of CTCs has been in the process of development.

The identification process is generally conducted after the isolation and enrichment process. To identify genetic aberrations and other biological characteristics of CTCs, several methodologies, such as immunocytochemistry and molecular techniques, have been used. Conventionally, immunostaining using 4′,6′-diamidino-2-phenylindole hydrochloride as a nuclear stain, CK as an epithelial marker, and CD45 as a hematopoietic marker has been commonly adopted[49]. In various molecular approaches, quantitative reverse transcription-polymerase chain reaction (RT-PCR) has been widely used to identify the molecular characteristics of CKs, CEA, and other markers[50].

CTC DETECTION AND ITS CLINICAL RELEVANCE IN HCC PATIENTS

In the past decades, CTCs in HCC patients have been intensively studied. Table 1 is the summary of previously demonstrated candidates. As described in the last paragraph, these approaches are generally categorized into two methods: physical and biological methods.

Table 1 Circulating tumor cells in hepatocellular carcinoma

Ref.	HCC patients	Ethnicity	Background liver status	Patient background	Controls	Methodology	Positive rate
Matsumura, *et al*[51], 1999	88	Japan	HCV: 85%, HBV: 6%	Pre and post (TAE or PEI)	NA	RT-PCR (AFP)	63.0%
Mou *et al*[56], 2002	30	China	HBV: 100% LC: 100%	Pre resection	25 (HV: 25)	RT-PCR (MAGE1/3)	43.3%
Witzigmann *et al*[55], 2002	85	Germany	NA	Pre, during, post (Resection: 24, LT: 10, TACE: 13, No treatment: 38)	116 (OLT: 50, HD: 39, HV: 27)	RT-PCR (AFP)	28.0%
Vona *et al*[31], 2004	44	France	LC: 89%	Pre/post resection: 22 Unresectable: 22	107 (HV: 38, HD: 69)	ISET	52.2%
Jeng *et al*[53], 2004	81	China	HBV: 77%, HCV: 38% LC: 69%	Pre and post resection	50 (HV: 30, HD: 20)	RT-PCR (AFP)	23.4%
Cillo *et al*[52], 2004	50	Italy	HCV: 50%,HBV: 12% HCV and HBV: 6% Alcohol: 10% LC: 84%	Pre (Resection: 17, LT: 9, PT: 17) No treatment: 7	50 (HD: 6, OT: 44)	RT-PCR (AFP)	40.0%
Kong *et al*[59], 2009	343	South Korea	HBV: 78%, HCV: 10% Alcohol: 6% LC: 52%	Pre (Resection: 12, TACE: 224, RFA: 44, Chemotherapy: 12, Radiotherapy: 12, No treatment: 39)	NA	RT-PCR (AFP) (hTERT)	59.5% 14.0%
Fan *et al*[69], 2011	82	China	HBV: 80%	Pre and post resection	NA	CellSearch™	68.3%
Xu *et al*[70], 2011	85	China	HBV: 84%, HCV: 7% HBV and HCV: 5% nonB, nonC: 4%	Pre Resection: 63 Clinical Diagnosis: 22	71 (HD: 37, HV: 20, OT: 14)	CellSearch™	81.0%
Liu *et al*[67], 2013	60	China	HBV: 93% LC: 93%	Pre resection	NA	Flow cytometry	50.0%
Yao *et al*[57], 2013	123	China	HBV: 72% LC: 93%	NA	276 (HV: 30, HD: 196, OT: 50)	RT-PCR (GPC-3)	70.77%
Sun *et al*[73], 2013	123	China	HBV: 75% LC: 76%	Pre/post resection	NA	CellSearch™	66.6%
Schulze *et al*[72], 2013	59	Germany	Alcohol: 38% HBV: 17%, HCV: 13% LC: 89%	Pre (resection or systemic therapy)	19 (HD: 19)	CellSearch™	30.5%
Li *et al*[71], 2013	60	China	HBV: 92%, HCV: 3% nonB, nonC: 7% LC: 88.7%	NA	30 (HD: 10, HV: 10, OT: 10)	CellSearch™	76.6%
Bahnassy *et al*[68], 2014	70	Egypt	HCV: 100%	NA	63 (HD: 30, HV: 33)	Flow cytometry (CK19, CD90, 133) RT-PCR (Telomerase, MAGE1/3)	73.0%, 49.8%, 69.5% 55.7%, 60.0%, 62.9%
Li *et al*[76], 2014	27	China	NA	NA	61 (HD: 34, HV: 15, OT: 12)	CellSearch™	88.9%
Mu *et al*[78], 2014	62	China	HBV: 95.2%	NA	22 (HD: 7, HV: 15)	CellSearch™	48.3%
Fang *et al*[74], 2014	42	China	LC: 55%	Pre and post TACE	20 (HV: 10, HD: 10)	CellSearch™	52.3%
Morris *et al*[77], 2014	52	United Kingdom	Alcohol: 38%, HBV: 8% Diabetes: 12%	No treatment	NA	CellSearch™ ISET	28% 100%
Guo *et al*[75], 2014	299	China	HBV: 90% LC: 90%	Pre/post (Resection: 157, TACE: 76, RFA: 66)	120 (HV: 71, HD: 25, BT: 24)	CellSearch™	42.6%
Choi *et al*[58], 2015	81	South Korea	HBV: 80%, HCV: 11% Alcohol: 4% LC: 59%	Pre and post (Resection: 64, LT: 17)	16 (LHD: 16)	RT-PCR (K19, CD44)	22.2%
Kelley *et al*[19], 2015	20	Caucasian: 55%, Asian: 35%, American: 10% (African-5%) (Native-5%)	HBV: 25%, HCV: 45% HBV and HCV: 10% Alcohol: 5% NAFLD: 10%	NA	10 (HD: 10)	CellSearch™	40.0%
Wang *et al*[79], 2016	42	China	HBV: 81%, HCV: 2% nonB, nonC: 17%	NA	NA	CTC-Chip	59.5%
Zhang *et al*[207], 2016	36	China	NA	NA	NA	CTC-Chip	100%

NA: Not applicable; LC: Liver cirrhosis; NAFLD: Non-alcoholic fatty liver disease; TAE: Trans-arterial embolization; PEI: Percutaneous ethanol injection; LT: Liver transplantation; TACE: Trans-catheter arterial chemoembolization; RFA: Radiofrequency ablation; PT: Percutaneous treatment; HV: Healthy volunteers; HD: Hepatic disease without evidence of HCC; OLT: Other malignant liver tumors; BT: Benign tumor; OT: Other cancerous disease; LHD: Liver healthy donors; RT-PCR: Reverse transcriptase polymerase chain reaction; ISET: Isolation by size of epithelial tumor cells.

Physical methods

Vona *et al*[31] first reported the isolation by size of epithelial tumor cell (ISET) method to detect CTCs in HCC patients. By cytomorphologic analysis, they demonstrated that the spontaneous circulation of CTCs in peripheral blood reflects tumor progression and tumor spread in patients with HCC. Compared with expensive and cumbersome molecular techniques, ISET is a unique, inexpensive methodology. In this method, we can apply the cytopathological diagnosis of tumor cells, which were widely used in clinical oncology, as peripheral blood samples without any special equipment[32]. However, ISET device is still hard to release CTCs from the membrane. This may limit the application of downstream genetic analysis.

Biological methods

The presence and clinical utility of CTCs in HCC was first reported by Matsumura *et al*[51] using RT-PCR. They demonstrated the following. (1) the presence of alpha-fetoprotein (AFP) messenger RNA (mRNA) in peripheral blood could be a marker of circulating HCC cells; (2) the status of AFP mRNA in blood were investigated at entry, extrahepatic metastasis developed more frequently among the AFP mRNA-positive patients than among the AFP mRNA-negative patients; and (3) after treatment, AFP mRNA was investigated, and cumulative metastasis-free survival and overall survival were significantly better in patients whose AFP mRNA became negative after treatment than in patients with persistently positive AFP mRNA. In summary, they demonstrated that the presence or absence of AFP mRNA in blood (CTCs' positivity) could be a predictor of outcome in patients with HCC.

Following this study, the clinical utility of peripheral AFP mRNA was validated by other groups[52,53]; however, the significance as prognostic marker has not been adequately confirmed[54,55]. Thus, other tumor-specific molecules in the bloodstream, such as MAGE-1, MAGE3[56], glypican-3 (GPC-3)[57], keratin 19 (K19), cluster of differentiation 44 (CD44)[58], and hTERT[59] mRNA, have been investigated for markers of circulating HCC cells. For example, MAGE gene transcripts have been considered as HCC-specific markers[60]. Mou *et al*[56] demonstrated that detection of MAGE transcripts in blood with a follow-up survey could predict the prognosis and monitor the response to therapy. GPC-3 is a membrane-anchored heparin sulfate proteoglycan, known to be a reliable biomarker for HCC[61]. Yao *et al*[57] demonstrated that GPC-3 mRNA abnormality is useful as clinical biomarkers from early cancer detection to evaluating metastasis. Furthermore, K19 and CD44 have been shown to be cancer stem cell markers in HCC[62-65], their significance of prognostic factor in peripheral blood were also demonstrated by Choi *et al*[58]. However, HCC associated genes were not always candidates for the markers of CTCs. Although serum human telomerase reverse transcriptase protein (hTERT) mRNA expression has been suggested as a potential candidate diagnostic marker for HCC[66], the significance as prognostic marker has not been adequately confirmed[59].

Liu et *al*[67] and Bahnassy *et al*[68] used flow cytometry to analyze intercellular adhesion molecule 1 (ICAM-1) expression, cytokeratin 19, CD133, and CD90 in HCC blood samples and demonstrated their prognostic value. Among various techniques, EpCAM-based Cell-Search™ is currently the most widely used CTC platform[19,69-78]. Using this method, Sun *et al*[73] collected blood samples from 123 HCC patients who underwent curative resection and suggested that $EpCAM^+$ CTCs could be useful for real-time parameter for monitoring treatment response and be also used for therapeutic target in HCC recurrence. Guo *et al*[75] collected blood samples from 299 HCC patients with various kinds of treatment and 120 control subjects, and demonstrated that this method could be useful in early decision-making to tailor the most effective antitumor strategies. Most recently, Wang *et al*[79] suggested that novel CTC-Chip platform might be a new method for a simple and efficient detection of CTCs in HCC patients. They created biocompatible and transparent Hydroxyapatite/chitosan nanofilm coated by aptamer for carbohydrate sialyl Lewis Xto and demonstrated that it could be useful as prognostic marker.

Overall, the usefulness of CTCs as biomarkers in HCC might be practically guaranteed. However, several challenges that must be overcome remain. Firstly, it is possible that etiological differences of patients and controls, such as background liver disease, haptic status, and race, could be responsible for the heterogeneity of the results. Secondly, a novel methodology for the detection should be provided for solving the problem of the rarity and heterogeneity of CTCs. Thirdly, the techniques and results of past research have greatly differed. Consequently, a large-scale validation using patients with homogeneous backgrounds and development of a unified methodology are required for future applications.

BIOLOGY AND DETECTION OF CELL-FREE NUCLEIC ACIDS

cfNAs in peripheral blood of cancer patients, comprised of DNA, mRNA, and miRNA, are known to come from apoptotic and necrotic cells or are released from living eukaryotic cells[21]. The first discovery of cfNAs in human peripheral blood was in 1948 by Mandel and Metais[80]. However, their work did not gain attention for a long time due to insufficient understanding of that new concept, completely different from conventional ones. Cell free DNAs were first discovered by Leon *et al*[81] in the serum of cancer patients in 1977. They also suggested it could be a clinical indicator of treatment

outcome, showing decreased cfDNA levels in response to radiotherapy. Since then, numerous alterations in cfNAs have been demonstrated in various cancer patients. Cell free DNA with cancer characteristics was first discovered by Vasioukhin *et al*[82] in 1989. Tumours can shed DNA into the circulation. Their discovery indicated the possibility that cancers could release DNA into the blood of the cancer patients. This hypothesis was validated in the plasma of cancer patients: *KRAS* mutation in the pancreatic cancer patients[83] and *NRAS* mutation in the leukemia patients[84].

The state of RNA in the blood is easy-to degrade by the presence of endogenous ribonuclease, however, cell-free RNA has been demonstrated in blood. The presence of cfRNA was first discovered in 1999 from the serum of patients with nasopharyngeal carcinoma[85] and malignant melanoma[86]. Afterwards, many study group have demonstrated the presence and utility of mRNA in the blood of various cancer patients[87-89].

In 2008, Mitchell *et al*[90] first demonstrated that circulating microRNAs (miRNAs) in patients with solid cancers could be a promising biomarker. Since then, circulating noncoding RNAs have been intensively studied. Among them, miRNAs has especially gained attention. Other noncoding RNAs, such as small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), piwi-interacting RNA (piRNA), and long noncoding RNA (lncRNA), have been also expected to be biomarkers, however, there are few studies of these. In the future, further research will probably be necessary.

CIRCULATING CELL-FREE DNA DETECTION AND ITS CLINICAL RELEVANCE IN HCC PATIENTS

Circulating cfDNA, a naturally occurring biological material, is generally considered to be a potential novel biomarker for a long time[91]. These abnormalities can be divided into two changes, such as quantitative changes and qualitative changes. Quantitative changes appear as higher concentrations of total circulating cfDNA, qualitative changes are gene mutations, DNA copy number variations, tumor-specific methylation, microsatellite instability (MSI) and loss of heterozygosity. Thus, analysis of circulating cell-free DNA in the plasma/serum can be mainly categorized into two strategies. One of these strategies is to measure the quantity of cell-free DNA in circulation. The other strategy is to detect tumor-specific genetic aberrations. Most researchers have adopted studies the later one as liquid biopsy[92-95].

Table 2 is the summary of previously demonstrated candidates. Some researchers have adopted a quantitative analysis[96-102]. Huang *et al*[100] and Chen *et al*[99] demonstrated that plasma DNA or serum DNA levels were significantly higher in HCC patients and they were associated with a poorer prognosis; however, it has not become a mainstream of cfDNA studies because the elevated levels of cfDNA were not specific for HCC. Although single nucleotide mutation[103] and copy number variation[95,104] were representative changes for the qualitative strategy, the "methylation pattern" has been the most intensively investigated. Initially, the presence and the clinical utility of circulating cell-free DNA in HCC was reported by Wong *et al*[105]. Using methylation-specific PCR, they analyzed p15 methylation patterns in three kinds of samples such as plasma, serum, and tissues surgically resected from HCC patients and showed the following: (1) in the blood samples, methylated p15 sequences were detected in 25% of patients with p15 methylation in the tissue; (2) nearly all patients showing p15 and p16 methylation in the tissue had detectable methylation abnormalities in their blood samples; and (3) clinical metastasis or recurrence were developed in the patients with p15/p16 methylation. In summary, these epigenetic markers could serve as diagnostic and prognostic markers. As previous studies revealed that changes of DNA methylation exited in various malignancies and played an important role in carcinogenesis[106,107], following this study, many researchers investigated the cfDNA methylation profile in HCC patients[108-124]. For example, Iyer *et al*[115] compared the tumor methylation profile for tumor suppressor genes, such as APC, FHIT, p15, p16, and E-cadherin, and in tumor tissues and plasma from the same HCC patients, and demonstrated that concordance between the two types of specimens was statistically significant for all five genes. It suggested that plasma DNA reliably predicts methylation events in tissue DNA; therefore, plasma DNA could be used for methylation studies. Huang *et al*[116] analyzed the plasma methylation status of four genes (APC, GSTP1, RASSF1A, and SFRP1) and showed the sufficient diagnostic value of cfDNAs. Although the area under the receiver-operation characteristic curve (AUC-ROC) for an individual gene was not adequate, the combination analysis of these four genes indicated higher AUC (0.933) in discriminating HCC from the normal control. Furthermore, they demonstrated methylated RASSF1A in plasma could be an independent prognostic factor for overall survival.

MS-PCR has been widely used for methylation research, because it provides a rapid and simple method with high sensitivity and accuracy. More recently, droplet digital PCR[125-128] and genome-wide high-throughput sequencing[128,129] has been reported as a further accurately detection tools for rare and multiple types of mutations in circulating DNA. These novel approaches has revealed that genetic aberrations in cell-free DNA gained from the bloodstream of cancer patients and drug resistance were correlating[130-132]. It is required that the potent clinical utility of cell-free DNA, such as risk assessment, early cancer detection, prediction of drug resistance and prognostic outcome,

Table 2 Circulating cell-free DNA in hepatocellular carcinoma

Ref	HCC patients	Sample	Ethnicity	Background liver	Controls	cfDNA abnormalities Methodolgy	Target
Wong *et al*[105], 2000	25 15	Plasma/serum Buffy coat	Hong-Kong	HBV: 88% HCV: 2%	55 (HD: 35, HV: 20) 35 (HD: 15, HV: 20)	Methylation MS-PCR	P16
Wong *et al*[108], 2003	29/22 29	Plasma/serum Buffy coat	Hong Kong	NA	50 (HD and HV) 35 (HD: 15, HV: 20)	Methylation MS-PCR	P16INK4A
Chu *et al*[109], 2004	46	Serum	Korea	HBV: 65% HCV: 22%	23 (HD: 23)	Methylation MS-PCR	P16INK4A
Yeo *et al*[110], 2005	40	Plasma	Hong-Kong	HBV: 83%	10 (HV: 10)	Methylation MS-PCR	RASSF1A
Iizuka *et al*[96], 2006	52	Serum	Japan	HCV: 100%	46 (HD: 30, HV: 16)	Quantitative analysis Real-time PCR	GSTP1
Ren *et al*[97], 2006	79	Plasma	China	HBV: 85% LC: 86%	40 (HD: 20, HV: 20)	Quantitative analysis Real-time PCR Allelic imbalance analysis	NA D8S258 and D8S264
Zhang *et al*[112], 2007	50	Serum	Taiwan	HBV: 22% HCV: 16%	50 HV: 50	Methylation MS-PCR	P15, P16
Tan *et al*[111], 2007	8	Serum	Singapore	NA	72 (OT: 62, HV: 10)	Methylation MS-PCR	RUNX3
Chan *et al*[113], 2008	85	Serum	Hong-Kong	HBV: 92%	135 (HD: 63, HV: 72)	Methylation RT-PCR	RASSFIA
Chang *et al*[114], 2008	19	Plasma	China	HBV: 89%	17 (LC: 17)	Methylation MS-PCR	APC, GSTP1, RASSF1A, P16, E-cadherin
Iyer *et al*[115], 2010	28	Plasma	Egypt	HCV: 79% HNV: 18%	NA	Methylation MS-PCR	APC, FHIT, P15, P16 and E-cadherin
Yang *et al*[98], 2011	60	Plasma	China	NA	50 (HD: 21, HV: 29)	Quantitative analysis FQ-PCR	hTERT
Szymańska *et al*[103], 2011	14	Plasma	China	Mostly HBV	NA	Single nucleotide mutation SOMA	R249S (TP53 mutation)
Iizuka *et al*[117], 2011	220	Serum	Japan	HCV: 100%	202 (HD: 202)	Methylation MS-PCR	SPINT2, SRD5A2
Huang *et al*[116], 2011	72	Plasma	China	HBV: 85%	37 (HD: 37)	Methylation MSRE-qPCR	APC, GSTP1, RASSF1A, and SFRP1
Huang *et al*[100], 2012	72	Plasma	China	HBV: 85%	115 (HD: 74, HV: 41)	Quantitative analysis Real-time PCR	NA
Chen *et al*[99], 2012	80	Serum	China	HBV: 100%	130 (HD: 80, HV: 50)	Quantitative analysis Real-time PCR	NA
Mohamed *et al*[118], 2012	40	Serum	Egypt	HCV: 100%	60 (HD: 40, HV: 20)	Methylation Real-time PCR	RASSF1A
Chen *et al*[101], 2013	39	Serum	China	HBV: 79%	45 (HV: 45)	Quantitative analysis Real-time PCR	NA
Piciocchi *et al*[102], 2013	66	Plasma	Italy	HCV: 51% Alcohol: 27%	76 (HD: 76)	Quantitative analysis Real-time PCR	hTERT
Chan *et al*[95], 2013	4	Plasma	China	NA	20 (HD: 4, HV: 16)	Copy number variation MPS	NA
Sun *et al*[119], 2013	43	Serum	China	HBV: 86%	50 (HD: 24, HV: 26)	Methylation MS-PCR	TFPI2
Zhang *et al*[120], 2013	37	Serum	China	HBV: 100%	33 (HD: 33)	Methylation Bead Chip, Hot-start PCR, Pyrosequencing	DBX2, THY1
Huang *et al*[122], 2014	66	Serum	United States	HCV: 100% HCV and HBV: 6%	43 (HD: 43)	Methylation Pyrosequencing, MS-PCR	INK4A
Han *et al*[121], 2014	160	Serum	China	HBV: 22%	133 (HD: 88, HV: 45)	Methylation MS-PCR	TRG5
Ji *et al*[123], 2014	121	Serum	China	HBV: 83%	68 (HD: 37, HV: 31)	Methylation MS-PCR	MT1M
Kuo *et al*[124], 2014	40	Plasma	Taiwan	NA	34	Methylation MS-PCR	HOXA9
Jiang *et al*[104], 2015	90	Plasma	Hong-Kong	NA	135 (HD: 103, HV: 32)	Copy number variation CAZA	NA

NA: Not applicable; HD: Hepatic disease without evidence of HCC; HV: Healthy volunteers; RT-PCR: Reverse transcriptase polymerase chain reaction; SOMA: Short oligonucleotide mass analysis; CAZA: Chromosome arm-level z-score analysis; MPS: Massively parallel sequencing; MS-PCR: Methylation-specific PCR; RT-PCR: Real time polymerase chain reaction; MSRE-qPCR: Methylation-sensitive restriction enzymes-based quantitative PCR; FQ-PCR: Real-time quantitative fluorescent polymerase chain reaction.

could be demonstrated in the patients with HCC.

CIRCULATING CELL-FREE MRNA IN PLASMA/SERUM AND HCC

Although RNA is fragile, easily degraded by ribonuclease (RNase) and the concentration of RNase in plasma/serum is known to be elevated in cancer patients[133], many researchers have successfully demonstrated the stable presence of cell-free mRNAs in the bloodstream of cancer patients. Recently, novel mechanistic insights have been gained that these RNAs can be incorporated into other surrounding such as exosomes, microvesicles and multivesicles, which considered to be sufficiently protected from the degradation by RNases and released from the cellular surface to the blood[134]. There are many studies of cell-free mRNA in the blood of patients with various solid cancers, and most of them targeted the mRNAs in plasma/serum whose up-regulation were previously validated in cancer tissues[87-89,135-137]. Regarding HCC, several study groups investigate mRNA in peripheral blood mononuclear cells as a marker for the detection of CTCs[51-53,55-57,59]; however, the quantity of cell-free mRNAs in plasma/serum is exceedingly small. Further studies of cell-free mRNA in patients with HCC may provide new knowledge to the research field of liquid biopsy.

CIRCULATING NONCODING RNA IN PLASMA/SERUM

Although as much as 80% of genomic DNA had already demonstrated to be transcribed into RNAs[138], the Human Genome Project revealed that the open reading frames of protein genes is only 2% of the 3.2 billion bases[139,140]. It can be paraphrased as there are only a very few human genomic DNAs that actually code proteins. It is gradually reveled that various noncoding RNAs (ncRNAs) play crucial roles in several cellular processes in the transition from DNA to protein. Therefore, the expression patterns of ncRNAs could be promising molecular biomarkers in novel diagnostic techniques[141].

For circulating ncRNAs, particular attention has been paid to miRNAs. MiRNAs are small non-coding RNAs that play crucial roles in various cellular processes. A single miRNA could regulate the expression of genes as follows: A guide strand of mature miRNA is taken into the RNA-induced silencing complex and then hybridizes to the 3′-untranslated region of their target mRNAs to translate or degrade these mRNAs. Thus, miRNAs have occupied important place in all cellular processes, some alterations in miRNA expression has come to draw a lot of attention in the association with various disease. Particularly, some researchers have demonstrated that specific miRNAs could act like oncogenes or tumor suppressors. Several studies in recent years on this subject have also shown that some extracellular miRNAs were generated from both cell lysis and active secretion[21,142,143]. Furthermore, several researchers have detected miRNAs in the plasma/serum in a remarkably stable form. In this regard, Kosaka *et al*[143] proved that secretory mechanisms and intercellular transfer of microRNAs in living cells. A group of miRNAs is packaged into small membrane vesicles called exosomes and released through a ceramide-dependent secretory machinery. Furthermore, miRNAs are remarkably stable form in plasma as they bind to certain proteins, such as argonaute 2 and high-density lipoproteins[144]. Therefore, all circulating miRNAs, regardless of whether they are taken into certain protein complexes and/or cell-derived microvesicles, has been thought to be sufficiently protected against the degradation by RNases in the bloodstream. These findings of recent years have pioneered a novel research field in cancer science.

In 2008, Mitchell *et al*[90] first reported that circulating miRNAs could be useful for stable blood-based markers for cancer detection. Since then, circulating miRNAs in the blood of cancer patients have been intensively studied to validate their potential as biomarkers. Table 3 is the summary of previously demonstrated candidates. In 2010, Li *et al*[145] first demonstrated that serum miRNAs expression profile could be useful as novel noninvasive biomarkers for the distinction between HBV infection and HBV-positive HCC. Since then, several research groups have reported the potential utility of miRNAs circulating in plasma/serum in clinical applications. Concerning circulating miRNAs in HCC, more than 70 miRNAs have been thought to be useful for biomarkers[145-195]. Some miRNAs had been used in combination with AFP, conventional serum tumor marker, to improve diagnostic accuracy[158,171,177,188,191,195]. Moreover, one miRNA could influence various mRNAs, more and more miRNAs and related mRNAs continues to be reported by numerous research groups. However, they are not always superimposable due to the large variances in the results. Thus, to realize more accurate diagnosing, some researchers have tried to use miRNAs in combination. Zhou *et al*[150] using the unique panel consisting of 7 mRNAs (miR-122, -192, -21, -223, -26a, -27a, and -801), based on the expression in plasma, could differentiate HCC from healthy (AUC = 0.941), chronic hepatitis B (AUC = 0.842), and cirrhosis (AUC = 0.884). More recently, Tan *et al*[166] reported that a combination of eight miRNAs could provide high diagnostic accuracy for HCC.

In terms of diagnosis, it should fully consider that HCC is an extremely prominent cancer among high-risk group patients. Patients who are already infected with HBV and HCV, and/or liver cirrhosis are at risk of developing liver cancer; however, some candidate miRNAs could not discriminate HCC patients from patients with liver chronic hepatitis, or cirrhosis[146,147,149,160,162,179,187]. They were useful for detecting HCC from general population, but not suitable for further screening, narrowing down patients

Table 3 Circulating cell-free microRNA in hepatocellular carcinoma

miR	Expression	Sample	HCC patients	Ethnicity	Background liver	Controls	Value	Ref.
miR-1	Up	Serum	195	Germany	HCV: 45%, Alcohol: 33%, HBV: 17%	54 (HD: 54)	P	Köberle *et al*[155] 2013
miR-10b	Up	Blood	27	China	Alcohol: 23%	81 (HD: 81)	D	Jiang *et al*[175] 2015
miR-15b	Up	Serum	153	China	HBV: 88%	59 (HD: 29, HV: 39)	D	Liu *et al*[152] 2012
miR-15b-5p	Down	Plasma	37	China	NA	60 (HD: 29, HV: 31)	D	Chen *et al*[170] 2015
miR-16	Down	Serum	105	United States	HCV: 64%, HBV: 20%	178 (HD: 107, HV: 7)	D	Qu *et al*[148] 2011
			90	China	NA	60 (HV: 60)	D	Ge *et al*[161] 2014
			40	Egypt	HCV: 100%	60 (HD: 40, HV: 20)	D	El-Abd *et al*[174] 2015
miR-17-5p	Up	Serum	136	China	NA	NA	P	Zheng *et al*[159] 2013
			8	Turkey	HCV: 100%	84 (HD: 56, HV: 28)	D	Oksuz *et al*[178] 2015
miR-18a	Up	Serum	101	China	HBV: 100%	90 (HD: 30, HV: 60)	D	Li *et al*[151] 2012
	Up	Serum	20	South Korea	HBV: 70%	40 (HD: 40)	D	Sohn *et al*[179] 2015
miR-19a	Down	Serum	112	Egypt	HCV: 100%	167 (HD: 125, HV: 42)	D	Motawi *et al*[177] 2015
miR-21	Up	Plasma	457	China	HBV:100%	477 (HD: 310, HV: 167)	D	Zhou *et al*[150] 2011
			136	Japan	HCV:68%, HBV: 23%	80 (HD: 30, HV: 50)	D, P	Tomimaru *et al*[153] 2012
		Serum	101	China	HBV: 75%	137 (HD: 48, HV: 89)	D	Xu *et al*[149] 2011
			136	China	HBV: 95%	NA	P	Liu *et al*[164] 2014
			97	China	HBV: 62%	30 (HV: 30)	D, P	Wang *et al*[180] 2015
			23	Egypt	HCV: 87%, HBV: 13%	17 (HD: 17)	D	Amr *et al*[186] 2016
	Down	Serum	70	China	HBV: 100%	72 (HD 48, HV: 24)	D	Qi *et al*[147] 2011
			90	China	NA	60 (HV: 60)	D, P	Ge *et al*[161] 2014
			52	China	HBV: 63%, HCV: 4%	85 (HD: 42, HV: 43)	D	Zhuang *et al*[195] 2016
miR-22	Down	Serum	192	Egypt	HCV: 100%	192 (HD: 192)	D	Zekri *et al*[194] 2016
miR-24-3p	Up	Serum	84	China	HBV: 100%	77 (HD: 31, HV: 46)	D, P	Meng *et al*[165] 2014
miR-26a	Down	Plasma	457	China	HBV: 100%	477 (HD: 310, HV: 167)	D	Zhou *et al*[150] 2011
		Serum	52	China	HBV: 63%, HCV: 4%	85 (HD42, HV: 43)	D	Zhuang *et al*[195] 2016
miR-26a-5p	Down	Serum	261	China	HBV: 100%	406 (HD 233, HV: 173)	D	Tan *et al*[166] 2014
miR-27a	Down	Plasma	457	China	HBV: 100%	477 (HD: 310, HV: 167)	D	Zhou *et al*[150] 2011
miR-29b	Down	Serum	192	Egypt	HCV: 100%	192 (HD: 192)	D	Zekri *et al*[194] 2016
miR-30c	Down	Serum	242	China	HCV: 63%	NA	P	Liu *et al*[176] 2015
miR-30c-5p	Down	Serum	8	Turkey	HCV: 100%	84 (HD: 56, HV: 28)	D	Oksuz *et al*[178] 2015
miR-34a	Up	Serum	112	Egypt	HCV: 100%	167 (HD: 125, HV: 42)	D	Motawi *et al*[177] 2015
miR-92a-3p	Up	Plasma	20	Turkey	HBV: 100%	74 (HD: 46, HV: 28)	D	Giray *et al*[162] 2014
miR-96	Up	Serum	104	China	HBV: 100%	400 (HD: 280, HV: 120)	D	Chen *et al*[171] 2015
miR-101	Up	Serum	25	China	HBV: 100%	20 (HV: 20)	D	Fu *et al*[154] 2013
	Down	Serum	67	China	HBV: 100%	170 (HD: 140, HV: 3,)	D	Xie *et al*[167] 2014
			20	South Korea	HBV: 70%	40 (HD: 40)	D	Sohn *et al*[179] 2015
			52	China	HBV: 63%, HCV: 4%	85 (HD: 42, HV: 43)	D	Zhuang *et al*[195] 2016
miR-106b	Up	Blood	27	China	Alcohol: 23%	81 (HD: 31, HV: 50,)	D	Jiang *et al*[175] 2015
miR-122	Up	Serum	70	China	HBV: 100%	72 (HD: 48, HV: 24,)	D	Qi *et al*[147] 2011
			101	China	HBV: 75%	137 (HD: 48, HV: 89,)	D	Xu *et al*[149] 2011
			195	Germany	HCV: 45%, Alcohol: 33%, HBV: 17%	54 (HD: 54)	P	Köberle *et al*[155] 2013
			30	Egypt	HCV: 100%	70 (HD: 60, HV: 10)	D	El-Garem *et al*[160] 2014
			136	China	HBV: 95%	NA	P	Liu *et al*[164] 2014
			192	Egypt	HCV: 100%	192 (HD: 192)	D	Zekri *et al*[194] 2016
	Down	Plasma	457	China	HBV: 100%	477 (HD: 310, HV: 167)	D	Zhou *et al*[150] 2011
		Serum	20	South Korea	HBV: 70%	40 (HD: 40)	D	Sohn *et al*[179] 2015
			122	China	NA	NA	P	Xu *et al*[181] 2015
miR-122a	Down	Serum	85	China	HBV: 88%	HV (HV: 85)	D	Luo *et al*[156] 2013
miR-122-5p	Up	Plasma	20	Turkey	HBV: 100%	74 (HD: 46, HV: 28)	D	Giray *et al*[162] 2014
		Plasma	120	South Korea	HBV: 100%	NA	P	Cho *et al*[172] 2015
		Serum	120	China	HBV: 100%	DN: 30	D	Hung *et al*[189] 2016
	Down	Serum	261	China	HBV: 100%	406 (HD: 233, HV: 173)	D	Tan *et al*[166] 2014
miR-125b	Down	Plasma	64	China	HBV: 100%	178 (HD: 122, HV: 56)	D	Chen *et al*[187] 2016
miR-125b-5p	Up	Plasma	20	Turkey	HBV: 100%	74 (HD: 46, HV: 28)	D	Giray *et al*[162] 2014
miR-126	Up	Plasma	59	India	HBV: 100%	38 (HD: 20, HV: 18)	D	Ghosh *et al*[188] 2016
	Down	Serum	23	Egypt	HCV: 100%	55 (HD: 55)	D	Khairy *et al*[190] 2016
miR-128-2	Up	Serum	222	China	HBV: 87%	NA	P	Zhuang *et al*[184] 2015
miR-129	Down	Serum	23	Egypt	HCV: 100%	55 (HD: 55)	D	Khairy *et al*[190] 2016
miR-130a	Up	Serum	112	Egypt	HCV: 100%	167 (HD: 125, HV: 42)	D	Motawi *et al*[177] 2015
miR-130b	Up	Serum	153	China	HBV: 88%	59 (HD: 29, HV: 30)	D	Liu *et al*[152] 2012
miR-139	Down	Plasma	31	China	NA	31 (HD: 31)	D, P	Li *et al*[163] 2014
miR-141-3p	Up	Serum	261	China	HBV: 100%	406 (HD: 233, HV: 173)	D	Tan *et al*[166] 2014
miR-143	Up	Serum	95	China	NA	245 (HD: 118, HV: 127)	D	Zhang *et al*[168] 2014
miR-143-3p	Up	Plasma	59	India	HBV: 100%	38 (HD: 20, HV: 18)	D	Ghosh *et al*[188] 2016
miR-146a	Up	Serum	112	Egypt	HCV: 100%	167 (HD: 125, HV: 42)	D	Motawi *et al*[177] 2015

miR-150	Down	Serum	120	China	HBV: 100%	230 (HD: 110, HV: 120)	D, P	Yu *et al*[183] 2015
miR-155	Down	Serum	23	Egypt	HCV: 100%	55 (HD: 55)	D	Khairy *et al*[190] 2016
miR-181a	Down	Blood	27	China	Alcohol: 23%	81 (HD: 31, HV: 50)	D	Jiang *et al*[75] 2015
miR-181b	Up	Serum	192	Egypt	HCV: 100%	192 (HD: 192)	D	Zekr *et al*[194] 2016
miR-182	Up	Serum	103	China	NA	135 (HD: 95, HV: 40)	D, P	Chen *et al*[169] 2015
miR-192	Up	Plasma	457	China	HBV: 100%	477 (HD: 310, HV: 167)	D	Zhou *et al*[150] 2011
		Serum	112	Egypt	HCV: 100%	167 (HD: 125, HV: 42)	D	Motawi *et al*[177] 2015
miR-192-5p	Down	Serum	261	China	HBV: 100%	406 (HD: 233, HV: 173)	D	Tan *et al*[166] 2014
miR-195	Down	Serum	112	Egypt	HCV: 100%	167 (HD: 125, HV: 42)	D	Motawi *et al*[177] 2015
			20	South Korea	HBV: 70%	40 (HD: 40)	D	Sohn *et al*[179] 2015
miR-199a	Down	Serum	105	United States	HCV: 64%, HBV: 20%	178 (HD: 107, HV: 71)	D	Qu *et al*[148] 2011
			40	Egypt	HCV: 100%	60 (HD: 40, HV: 20)	D, P	El-Abd *et al*[174] 2015
			78	China	NA	156 (HV: 156)	D	Yin *et al*[182] 2015
			23	Egypt	HCV: 87%, HBV: 13%	17 (HD: 17)	D	Amr *et al*[186] 2016
miR-199a-3p	Down	Serum	192	Egypt	HCV: 100%	192 (HD: 192)	D	Zekri *et al*[194] 2016
miR-199a-5p	Down	Serum	261	China	HBV: 100%	406 (HD: 233, HV: 173)	D	Tan *et al*[166] 2014
miR-200a	Up	Serum	136	China	HBV: 95%	NA	P	Liu *et al*[176] 2015
miR-203	Down	Serum	23	Egypt	HCV: 100%	55 (HD: 55)	D	Khairy *et al*[190] 2016
miR-203a	Down	Serum	242	China	HCV: 63%	NA	P	Liu *et al*[176] 2015
miR-206	Up	Serum	261	China	HBV: 100%	406 (HD: 233, HV: 173)	D	Tan *et al*[166] 2014
miR-215	Up	Serum	95	China	NA	245 (HD: 118, HV: 127)	D	Zhang *et al*[168] 2014
miR-218	Down	Serum	156	China	HBV: 72%	162 (HD: 98, HV: 64)	D, P	Yang *et al*[193] 2016
miR-221	Up	Serum	20	South Korea	HBV: 70%	40 (HD: 40)	D	Sohn *et al*[179] 2015
			192	Egypt	HCV: 100%	192 (HD: 192)	D	Zekri *et al*[194] 2016
	Down	Serum	30	Egypt	HCV: 100%	70 (HD: 60, HV: 10)	D	El-Garem *et al*[174] 2014
miR-222	Up	Serum	70	China	HBV: 100%	72 (HD: 48, HV: 24)	D	Qi *et al*[147] 2011
			20	South Korea	HBV: 70%	40 (HD: 40)	D	Sohn *et al*[179] 2015
miR-223	Up	Serum	70	China	HBV: 100%	72 (HD: 48, HV: 24)		Qi *et al*[147] 2011
		Serum	101	China	HBV: 75%	137 (HD: 48, HV: 89)	D	Xu *et al*[149] 2011
	Down	Plasma	457	China	HBV: 100%	477 (HD: 310, HV: 167)	D	Zhou *et al*[150] 2011
		Serum	23	Egypt	HCV: 100%	55 (HD: 55)	D	Khairy *et al*[190] 2016
miR-223-3p	Down	Plasma	20	Turkey	HBV: 100%	74 (HD: 46, HV: 28)	D	Giray *et al*[162] 2014
		Serum	8	Turkey	HCV: 100%	84 (HD: 56, HV: 28)	D	Oksuz *et al*[178] 2015
miR-224	Up	Plasma	107	Japan	HCV: 41%, HBV: 18%, Alcohol: 15%	102 (HD: 27, HV: 75)	D, P, T	Okajima *et al*[192] 2016
		Serum	20	South Korea	HBV: 70%	40 (HD: 40)	D	Sohn *et al*[179] 2015
			182	China	HBV: 87%	NA	D, P	Zhuang *et al*[185] 2015
			122	China	HBV: 100%	157 (HD: 135, HV: 22)	D	Lin *et al*[191] 2016
miR-224-5p	Up	Serum	136	China	HBV: 95%	NA	P	Liu *et al*[164] 2014
miR-296	Up	Serum	112	Egypt	HCV: 100%	167 (HD: 125, HV: 42)	D	Motawi *et al*[177] 2015
miR-302c-3p	Down	Serum	8	Turkey	HCV: 100%	84 (HD: 56, HV: 28,)	D	Oksuz *et al*[178] 2015
miR-331-3p	Up	Serum	103	China	NA	135 (HD: 95, HV: 40)	D, P	Chen *et al*[169] 2015
miR-335	Down	Serum	125	China	NA	250 (HD: 125, HV: 125)	D, P	Cui *et al*[173] 2015
miR-338-5p	Up	Plasma	37	China	NA	60 (HD: 29, HV: 31)	D	Chen *et al*[170] 2015
miR-375	Up	Serum	120	China	HBV: 100%	393 (HD: 183, HV: 210)	D	Li *et al*[145] 2010,
	Down		78	China	NA	156 (HV: 156)	D	Yin *et al*[182] 2015.
miR-433-3p	Up	Serum	261	China	HBV: 100%	406 (HD: 233, HV: 173)	D	Tan *et al*[166] 2014
miR-483-5p	Up	Serum	69	United States	HCV: 63%, HBV: 14%	69 (HV: 69)	D	Shen *et al*[157] 2013
			112	China	NA	141 (HD: 56, HV: 85)	D	Zhang *et al*[158] 2013
miR-500a	Up	Serum	112	China	NA	141 (HD: 56, HV: 85)	D	Zhang *et al*[158] 2013
miR-764	Up	Plasma	37	China	NA	60 (HD: 29, HV: 31)	D	Chen *et al*[170] 2015
miR-801	Up	Plasma	457	China	HBV: 100%	477 (HD: 310, HV: 167)	D	Zhou *et al*[150] 2011
miR-885-5p	Up	Serum	46	China	HBV: 72%	105 (HD: 64, HV: 24, GC: 17)	D	Gui *et al*[146] 2011
			192	Egypt	HCV: 100%	192 (HD: 192)	D	Zekri *et al*[194] 2016
miR-1228-5p	Up	Serum	261	China	HBV: 100%	406 (HD: 233, HV: 173)	D	Tan *et al*[166] 2014
let-7b	Up	Serum	120	China	HBV: 100%	30 (DN: 30)	D	Hung *et al*[189] 2016
let-7f	Down	Serum	90	China	NA	60 (HV: 60)	D, P	Ge *et al*[161] 2014

NA: Not applicable; HV: Healthy volunteers; HD: Hepatic disease without evidence of HCC; GC: Gastric cancer; DN: Dysplastic nodule; D: Diagnostic marker; P: Prognostic marker; T: Treatment outcome marker.

who already have some risks for HCC. Recently, Zhou *et al*[150], Okusuz *et al*[178], Lin *et al*[191], and Zekri *et al*[194] defined three subgroups of healthy volunteers, with patients with chronic hepatitis and cirrhosis as controls. Most recently, Motawi *et al*[177] and our group[192] set subgroups according to fibrosis stage. To demonstrate the clinical utility for diagnosis, it is necessary to select appropriate controls.

Accumulating evidence of circulating cell-free miRNAs made clear their clinical utility as prognostic biomarker as well as a marker for the detection of HCC[184,196-205]. In terms of the two types of curative treatment, Zheng *et al*[159] demonstrated that the level of serum miR-17-5p could serve as a novel prognostic

marker for HCC patients who underwent surgical resection, and Cho *et al*[172] demonstrated that high plasma miR-122 expression was associated with poor overall survival in patients with HBV-related HCC who underwent radiofrequency ablation (RFA). In terms of the treatment for unresectable HCC, trans-arterial chemoembolization (TACE), Liu *et al*[164] demonstrated that miR-200a was the independent prognostic factor associated with survival.

Most recently, our group found that miR-224 may be an indicator of residual tumor in non-surgical treatment, such as percutaneous ablation therapy and/or TACE, although this was preliminary result because the number of cases was small[192]. Despite accumulating evidence, we should recognize that several challenges remain for clinical application. Regarding inter- and intra-individual variation, the kind of blood samples such as serum, plasma or all blood for better clinical application of miRNAs as a liquid-based biomarker, many issues should be addressed. Furthermore, it is necessary to build a consensus what molecule is suitable for clinical application.

Recent research has demonstrated that several noncoding RNAs regulate oncogenic and/or tumor-suppressive functions. PTEN[150,180], Stathmin1[150], RUNX3[152], Rho-kinase 2[164], Mcl-1[167], SOX9[167], p21/E2F5[175], FNDC3B[168], VEGF[177], TP53INP1[169], LIN28B[187], ADAM17[194], ISRE[194], CDKN1B/p27[194], CDKN1C/p57[194], TIMP3[194], HDAC4[194], and mTOR[194], have been demonstrated to have cancer-related functions, and validated as targets for specific miRNAs in the blood of patients with HCC[206]. The noncoding RNAs, such as lncRNA, snoRNA, snRNA, and piRNA, in the blood of patients with HCC remain unexplored. We hope further studies of circulating noncoding RNAs based on the knowledge of recent years in HCC will shed more light on this research field.

CONCLUSION

Blood-based molecular biomarkers, the most typical one of the so-called liquid biopsy, are promising as diagnostic, therapeutic and/or prognostic markers for HCC because researchers has got over their clinical difficulties such as anatomical reasons, invasive nature, and/or the patient's poor hepatic status. Although the clinical utility of liquid biopsy in HCC has been practically guaranteed by many research groups, there remains large variance in the results. The lack of a standardized technical approach has contributed to the lack of consensus. The techniques adopted, patient's hepatic status, sample type, storage conditions and target molecules have differed according to study groups. Thus, large-scale study, which is performed in a uniform methodology through all processes, is required. Another important reason is the etiological difference in each cohort. HCC is a marked regional clustering cancer, the background liver is different for each study group. For example, as indicated in each of the Tables, while all patients were infected with HBV in some reports from China and South Korea, all patients infected with HCV in other reports from Egypt. Their results were too biased by sample characteristics, it is desirable to be validated in another cohort before further clinical application.

The utility of the current serum biomarkers, such as AFP, AFP-L3, and proteins induced through vitamin K deficiency, and imaging modalities, such as ultrasonography, computed tomography, and gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced liver magnetic resonance imaging (GdEOB-DTPA-enhanced MRI) is far from satisfactory. What is now required is less invasive and repeatable methodology. Accumulating evidence of liquid biopsy might facilitate a more sensitive diagnosis and individualized decision-making in the duration of treatment of HCC. A challenge is how to achieve further development based on recent studies. Many issues should be addressed before these promising results can be translated into a real clinical settings.

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