Development and validation of serum exosomal microRNAs as diagnostic and prognostic biomarkers for hepatocellular carcinoma

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Abstract
Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide. China accounts for over half of the new cases and deaths. Diagnostic imprecision and a lack of complimentary molecular biomarkers are partially responsible for this lack of progress. Herein, serum-derived exosomal microRNA (miRNA) profiling was performed on 80 patients which histologically confirmed HCC and 30 normal controls. A classification of 8 exosomal miRNAs had biologically and statistically significant differences between HCC and normal serum samples, including miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17-5p, and miR-106a. Online algorithm showed strong independent classification accuracy (area under the curve) reached 0.535 to 0.850, separately. The significant correlation between serum exosomal miRNAs and tumor size was observed. In addition, the survival difference of HCC patients with high or low exosomal miR-106a was statistically significant using Kaplan-Meier analysis. Besides, we also measured the proliferation and invasion ability of HCC cells following exosomal miR-106a mimics or inhibitor treatment. After prediction with algorithms, mitogen-activated protein kinase and c-Jun N-terminal kinase pathways were identified associated with miR-106a’s function. In summary, differentially expressed serum exosomal miRNAs can be helpful for diagnostic and prognostic of HCC.

KEYWORDS
diagnosis, exosome, hepatocellular carcinoma, microRNAs, prognosis

1 INTRODUCTION

Hepatocellular carcinoma (HCC) is among the top 6 prevalent cancers and the second leading cause of cancer-related death worldwide, with more than 460,000 new cases diagnosed and 422,100 deaths in China in 2015.1,2 While the survival rate of HCC is the poorest, the age-standardized 5-year relative survival rate is only 10.1%.3

Accumulated studies have demonstrated that infection of hepatitis B virus (HBV) or hepatitis C virus (HCV) contributes to an estimated 75% of all HCC cases.4 In addition, fatty liver disease and alcohol consumption also lead high incidence of HCC in China.3 HCC is an aggressive tumor often with metastasis, and it is also a highly heterogeneous disease. Due to difficulties in early diagnosis, most HCC patients are diagnosed at an advanced stage, losing the opportunity for curative treatments such as resection, sorafenib, epidermal growth factor receptor
(EGFR) inhibitors, and immunotherapy. Therefore, early diagnostic and prognostic are urgently needed.

In the last decades, the development of innovative technology such as next-generation sequencing and serum proteomics have enabled a rapid and dramatic increase in our understanding of the genetic, molecular, and morphological changes occurring in HCC patients. Novel serum biomarkers for HCC including α-fetoprotein (AFP) is widely used diagnostic biomarker for early identification of HCC. In addition, several noncoding RNAs have been identified as new biomarker for HCC diagnosis and prognosis using liquid biopsy, including microRNAs (miRNAs) and long noncoding RNAs.

MiRNAs are approximately 22-nucleotide long noncoding RNAs that modulate essential cellular processes in tumorigenesis at the posttranscriptional level. Circulating miRNA profiles have been investigated and associated with HCC diagnosis, and circulating miRNAs biomarker discovery process, we for the first time have identified 8 exosomal miRNAs are novel biomarkers for HCC, including miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17-5p, and miR-106a. Furthermore, we discovered that exosomal miR-194, miR-17-5p, and miR-106a were significantly higher in patients with a large tumor size. High levels of exosomal miR-106a also predict lower survival rate of HCC by promoting tumorigenesis. Collectively, exosomal miRNAs can be used as new diagnostic and prognostic biomarkers for HCC.

2 | MATERIALS AND METHODS

2.1 | Materials

Cell Counting Kit-8 was purchase from Dojindo (Tokyo, Japan). Polyethylene terephthalate track-etched membranes (12-well) and matrigel were purchased from BD Biosciences (Franklin Lakes, NJ). Antibodies against CD9 and CD63 were purchased from Proteintech (Wuhan, China). MiR-106a mimics were obtained from GenePharma (Suzhou, China). RPMI-1640, fetal bovine serum (FBS) was from Sigma-Aldrich (St Louis, MO).

2.2 | Cell culture and transfection

HepG2 and SMMC-7721 cells were cultured in RPMI-1640 (Sigma-Aldrich) with 10% FBS (GIBCO, Waltham, MA) at 37°C in 5% CO2. Cells were transfected with miR-106a mimics or inhibitors preincubated with exosomes (mimics: 5′-AAAAGUGCUUACAGUGCAGGUAG-3′; inhibitor: 5′-CUACCUGACUGUAAGCACUUUU-3′).

2.3 | Clinical tumor specimens

All 80 HCC patients’ serum samples were collected from 2015 to 2017 at the First Affiliated Hospital of Soochow University. Pathological analysis confirmed that the cancer tissues were indeed human HCC specimens. Thirty healthy controls receiving examinations at the same period were randomly included into this study. All of the samples were obtained with informed consent from patients and approval from the Ethics Committee of the authors’ institution. Peripheral blood samples of all patients were collected before surgery. The clinical characteristics of patients and healthy controls are shown in Table 1.

2.4 | Exosomes isolation and identification

Serum was extracted by centrifuging the fresh peripheral blood samples at 1200g for 1 minute. Serum exosomes in HCC patients and normal were extracted by the Total Exosome Isolation Kit (GenePharma). Experiments were performed according to the manufacturer’s protocol. Serum-derived exosomes were collected and detected by Western blot analysis. Briefly, equal amounts of protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA). The membranes were then blotted with primary antibodies overnight at 4°C (dilution ratio is 1:1000), which is followed by incubation with the secondary antibody (1:5000). The proteins were developed as described.
2.5 | Quantitative real-time polymerase chain reaction

Total serum exosome RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA). Then, RNA was reverse-transcribed using SuperScript II Reverse Transcriptase kit (Invitrogen); all reverse transcription primers were indicated in Table S1; quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the Power SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA). The relative expression of exosomal miRNAs in serum was normalized to the reference gene, miR-16. All miRNAs primers were described in Table S1.

2.6 | Cell proliferation and invasion assay

Cell proliferation was measured using the Cell Counting Kit-8 from Dojindo. The spectrophotometric absorbance of each sample was measured at 450 nm. All experiments were performed in triplicate, and average values were calculated. Cell invasion assay was performed using cell culture insert membranes coated with diluted matrigel (BD Biosciences). Briefly, $2 \times 10^5$ cells were seeded in serum-free medium. The lower chamber was filled with medium containing 10% FBS. After 48 hours, the cells were fixed and stained using 0.5% crystal violet.

2.7 | Meta-analysis

The relationship between serum exosomal miRNAs and survival rate of HCC patients was estimated by Kaplan-Meier (KM) survival curve and log-rank tests. The 2-year overall survivals of HCC patients were investigated. For online survival meta-analysis, KM curve was generated using the KM Plotter. RNA-seq–derived platform data were analyzed (n = 421).

2.8 | Statistical analysis

Significance tests were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Receiver operating characteristics (ROC) curve was constructed and the area under the curve (AUC) was calculated to evaluate the specificity and sensitivity of predicting cases and controls using SPSS 22.0 software (IBM, NY). Statistical significance of differences between groups was determined using one-way analysis of variance or 2-tailed Student t test. A $P < .05$ was considered significant.

3 | RESULTS

3.1 | Identification and validation of serum exosomal miRNAs upregulated in HCC

We first assessed the exosomes isolated from the serum. Transmission electron microscopy revealed spherical vesicles approximately 40 to 100 nm in diameter of all samples (Figure S1A), which is consistent with previous description of exosomes. We also performed Western blot analysis to detect 2 commonly used exosomal protein markers, namely, CD9 and CD63. With an equal amount of proteins loaded on each lane, the 2 exosome protein markers were highly enriched in the isolated exosomes relative to the serum (Figure S1B). These results confirmed that we have successfully purified entire exosomes from all serum samples.

To explore the potential use of serum exosomal miRNAs as biomarkers for HCC, a panel of 21 cancer-associated miRNAs was chosen on the basis of their reported relevance to HCC. Each exosomal miRNAs were detected by qRT-PCR among 80 HCC patients and 30 healthy controls (Table 1). Using miR-16 as normalization control, levels of 8 serum exosomal miRNAs in HCC patients were significantly overexpressed, including miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17-5p, and miR-106a (Figure 1; Table S2). Thus, these 8 exosomal miRNAs were chosen in further analytic studies.

3.2 | Exosomal miRNAs as potential diagnostic biomarkers of HCC

To evaluate whether these 8 exosomal miRNAs can be used as potential diagnostic marker for HCC, ROC curve analyses were performed. It was revealed that these serum exosomal miRNAs were potential marker for
discriminating HCC patients from healthy controls with the areas under the ROC curve (AUC) of 0.535 to 0.850 (Figure 2). In addition, increased levels of exosomal miR-194, miR-17-5p, and miR-106a in serum were significant in HCC patients with larger tumor size (>3 cm) (Figure 3). But no difference of tumor stage, cirrhosis, HBV, and AFP were observed. Taken together, exosomal miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17-5p, and miR-106a in serum might set as diagnostic biomarkers of HCC.

3.3 | Serum exosomal miR-106a influenced the survival of HCC patients

To evaluate the prognostic ability of these 8 exosomal miRNAs, the KM survival curves were analyzed. As shown in Figure 4A, HCC patients with high levels of serum exosomal miR-106a had poor 2-year overall survival compared with those with low levels of serum exosomal miR-106a. Interestingly, patients with high expression of other 7 exosomal miRNAs did not have significant difference of
survival rate. In addition, KM curves were generated using the online meta-analysis tool KM Plotter. From a clinical data for 421 individuals, we find that high expression of miR-106a also predicts a worse 10-year outcome for those patients (Figure 4B). These data suggest that exosomal miR-106a is a prognostic factor for HCC.

3.4 Serum exosomal miR-106a promotes tumorigenesis by regulating several pathways

Toward this end, the function of exosomal miR-106a in HCC tumorigenesis was observed. By preincubation miR-106a mimics or inhibitors with serum-derived exosome, HepG2 and SMMC-7721 cell growth and invasion were measured. Serum exosome-encapsulated miR-106a mimics increased HCC cell proliferation and invasion separately (Figure 5A,B). While, after incubation with miRNA inhibitors, decreased exosomal miRNAs abolished the stimulation in cell proliferation and invasion (Figure 5A,B). These results indicated that exosomal miR-106a could influence HCC tumorigenesis by promoting cancer cell proliferation and invasion, leading a low survival rate of HCC patients.

To further investigate the potential signaling pathway which contributes to miR-106a-mediated tumorigenesis, 4 prediction algorithms were utilized to predict the targets, including miRDB, Targetscan, DIANA, and PicTar (Table S3). Cross-reference analysis indicates that there is a substantial overlap in 4 datasets, with 187 targets commonly predicted (Figure 6A). We performed pathway analysis using DAVID 6.7 and found that regulation of signaling transduction such as mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways was significantly enriched (Figure 6B; Table S4). Taken together, these results suggest that exosomal miR-106a promotes HCC tumorigenesis by regulating MAPK and JNK signal transduction.

4 DISCUSSION

Liver cancer, consisting mainly of HCC, is one of the most common cancers worldwide, with especially high...
prevalence in China and a growing incidence of 782,000 cases reported worldwide in 2012. Late diagnosis, metastasis, and recurrence account for the high mortalities. The early diagnosis of HCC is desirable and urgent in clinical. Up to now, AFP has mainly been used for diagnosis of primary HCC. However, the sensitivity and specificity are not satisfying. Toward this end, several studies raised that circulating serum exosomal miRNAs were proposed as a potential resource of biomarkers for a lot of disease. Although the clinical significance of these findings has not been elucidated in detail, those studies demonstrated that serum exosomal miRNAs could be novel diagnostic or prognostic markers for malignant disease such as HCC.

Here, we first isolated serum exosomes with high and stable purity, ensuring a reliable exosomal miRNA source. Using qRT-PCR analysis, 21 miRNAs in serum exosome were quantified. MiR-16 levels were used for normalization based on previously
published results.\textsuperscript{15,19} We found that the levels of exosomal miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17-5p, and miR-106a were significantly upregulated in HCC patient serum. In addition, these exosomal miRNAs except miR-145 yielded AUC of 0.650 to 0.850 for discriminating HCC from healthy subjects (Figure 2). More importantly, it was found that the levels of exosomal miR-194, miR-17-5p, and miR-106a were correlated with tumor size (Figure 3). But there was no significant difference between these exosomal miRNAs and HBV, cirrhosis, AFP levels.

MiR-122, miR-125b, miR-192, miR-194, miR-29a, miR-17-5p, and miR-106a are evolutionary conserved across species and were identified as the most abundant liver-specific miRNA.\textsuperscript{11,21,23,25,26} Nonetheless, little is known about the role of exosomes derived miRNAs in the prognosis of HCC patients. In this study, we investigated the expression of these 8 exosomal miRNAs in serum and their effects on the prognosis of HCC patients. KM survival curve showed that aberrantly expressed exosomal miR-106a was related to the survival of patients with HCC. The AUC of miR-122, miR-192, miR-194, and miR-17-5p is higher than miR-106, but these 4 exosomal miRNAs were not associated with patients’ survival since our observation period is just 2 years. Furthermore, the role of exosomal miR-106a on tumorigenesis was observed. In the current study, when cocultured with exosomes with miR-106a mimics, the proliferation of 2 HCC cells were obviously promoted, as well as the invasion ability, suggesting an oncogenic role of serum exosomal miR-106a in hepatocellular carcinogenesis. In addition, pretreatment with miR-106a inhibitors reduced the HCC cell proliferation and invasion, indicating a therapeutic role of exosomal miR-106a (Figure 6). Sushrut et al. has demonstrated that exosomes facilitate therapeutic targeting of KRAS in pancreatic cancer.\textsuperscript{27} Exosome-encapsulated miR-638 from serum samples inhibits HuH7 and SMCC7721 cell growth. Consistent with these results, our results offers insight into the therapeutic potential of exosomal miR-106 in HCC.

Apart from the modifying effect on HCC progression, miR-106a in HCC tissue have been demonstrated to promote invasion in our previous study.\textsuperscript{28} The potential targeted genes and underlying molecular mechanisms including TP53INP1, CDKN1A, and TIMP2 mediated cell cycle and apoptosis. Furthermore, more potential genes and underlying molecular mechanisms were also analyzed in this study by comparing 4 common miRNA prediction algorithms including miRDB, PicTar, DIANA, and Targetscan. Hundred and eighty-seven overlapped targets were selected and analyzed by DAVID. MAPK and JNK pathways that are well known to regulate cancer cell proliferation and invasion were enriched significantly.\textsuperscript{29,30}

Collectively, our study indicates the evidence that 8 serum exosomal miRNAs distinguish HCC patients from normal controls. Furthermore, exosomal miR-106a promotes tumorigenesis by regulating MAPK and JNK pathways, which may serve as a novel serum prognostic biomarker, offering a new approach for HCC treatment.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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REFERENCES

1. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. \textit{CA Cancer J Clin}. 2016;66:115-132.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. \textit{CA Cancer J Clin}. 2015;65:87-108.
3. Zeng H, Zheng R, Guo Y, et al. Cancer survival in China, 2003-2005: a population-based study. \textit{Int J Cancer}. 2015;136:1921-1930.
4. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. \textit{Gastroenterology}. 2007;132:2557-2576.
5. Hou G, Liu G, Yang Y, et al. Neuraminidase 1 (NEU1) promotes proliferation and migration as a diagnostic and prognostic biomarker of hepatocellular carcinoma. \textit{Oncotarget}. 2016;7:64957-64966.
6. Chen C, Li K, Jiang H, et al. Development of T cells carrying two complementary chimeric antigen receptors against glypican-3 and asialoglycoprotein receptor 1 for the treatment of hepatocellular carcinoma. \textit{Cancer Immunol Immunother}. 2017;66:475-489.
7. Wang MD, Wu H, Fu GB, et al. Acetyl-coenzyme A carboxylase alpha promotion of glucose-mediated fatty acid synthesis enhances survival of hepatocellular carcinoma in mice and patients. \textit{Hepatology}. 2016;63:1272-1286.
8. Ma W, Wang H, Teng L. Correlation analysis of preoperative serum alpha-fetoprotein (AFP) level and prognosis of hepatocellular carcinoma (HCC) after hepatectomy. \textit{World J Surg Oncol}. 2013;11:212.
9. Xue X, Fei X, Hou W, Zhang Y, Liu L, Hu R. MiR-342-3p suppresses cell proliferation and migration by targeting AGR2 in non-small cell lung cancer. \textit{Cancer Lett}. 2018;412:170-178.
10. Huang YH, Liang KH, Chien RN, et al. A circulating microRNA signature capable of assessing the risk of hepatocellular carcinoma in cirrhotic patients. Sci Rep. 2017;7:523.

11. Zhu HT, Hasan AME, Liu RB, et al. Serum microRNA profiles as prognostic biomarkers for HBV-positive hepatocellular carcinoma. Oncotarget. 2016;7:45637-45648.

12. Hoshino A, Costa-Silva B, Shen TL, et al. Tumour exosome integrins determine organotropic metastasis. Nature. 2015;527:329-335.

13. Tavakolizadeh J, Roshanaei K, Salmaninejad A, et al. MicroRNAs and exosomes in depression: potential diagnostic biomarkers. J Cell Biochem. 2018;119(5):3783-3797.

14. Ebrahimkhani S, Vafaee F, Young PE, et al. Exosomal microRNA signatures in multiple sclerosis reflect disease status. Sci Rep. 2017;7:14293.

15. Shi M, Jiang Y, Yang L, Yan S, Wang YG, Lu XJ. Decreased levels of serum exosomal miR-638 predict poor prognosis in hepatocellular carcinoma. J Cell Biochem. 2018;119(6):4711-4716.

16. Won Sohn JK, Kang So Hee, Yang Se Ra, et al. Serum exosomal microRNAs as novel biomarkers for hepatocellular carcinoma. Exp Mol Med. 2015;47:e184.

17. Shushan Yan YJ, Liang Caihong, Jin Chengwen, et al. Exosomal miR-6803-5p as potential diagnostic and prognostic marker in colorectal cancer. J Cell Biochem. 2018;119(5):4113-4119.

18. Hu R, Huffman KE, Chu M, Zhang Y, Minna JD, Yu Y. Quantitative secretomic analysis identifies extracellular protein factors that modulate the metastatic phenotype of non-small cell lung cancer. J Proteome Res. 2016;15:477-486.

19. Tomimaru Y, Eguchi H, Nagano H, et al. Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J Hepatol. 2012;56:167-175.

20. Győrfy B, Lanczky A, Eklund AC, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat. 2010;123:725-731.

21. Shi KQ, Lin Z, Chen XJ, et al. Hepatocellular carcinoma associated microRNA expression signature: integrated bioinformatics analysis, experimental validation and clinical significance. Oncotarget. 2015;6:25093-25108.

22. Van Caster P, Brandenburger T, Strahl T, et al. Circulating microRNA-122, -21 and -223 as potential markers of liver injury following warm ischaemia and reperfusion in rats. Mol Med Rep. 2015;12:3146-3150.

23. Gao F, Sun X, Wang L, Tang S, Yan C. Downregulation of microRNA-145 caused by hepatitis B virus X protein promotes expression of CUL5 and contributes to pathogenesis of hepatitis B virus-associated hepatocellular carcinoma. Cell Physiol Biochem. 2015;37:1547-1559.

24. Yu D, Wu Y, Shen H, et al. Exosomes in development, metastasis and drug resistance of breast cancer. Cancer Sci. 2015;106:959-964.

25. Varnholt H, Drebber U, Schulze F, et al. MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. Hepatology. 2008;47:1223-1232.

26. Wu Q, Liu HO, Liu YD, et al. Decreased expression of hepatocyte nuclear factor 4alpha (Hnf4alpha)/microRNA-122 (miR-122) axis in hepatitis B virus-associated hepatocellular carcinoma enhances potential oncogenic GALNT10 protein activity. J Biol Chem. 2015;290:1170-1185.

27. Kamerkar S, LeBlu VS, Sugimoto H, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. Nature. 2017;546:498-503.

28. Yuan R, Zhi Q, Zhao H, et al. Upregulated expression of hepatocyte nuclear factor 4alpha (Hnf4alpha)/microRNA-122 (miR-122) axis in hepatitis B virus-associated hepatocellular carcinoma enhances potential oncogenic GALNT10 protein activity. J Biol Chem. 2015;290:1170-1185.

29. Igarashi K, Pagliarini RA, Xu T. Loss of cell polarity drives tumor growth and invasion through JNK activation in Drosophila. Curr Biol. 2006;16:1139-1146.

30. Rao W, Li H, Song F, et al. OVA66 increases cell growth, invasion and survival via regulation of IGF-1R-MAPK signaling in human cancer cells. Carcinogenesis. 2014;35:1573-1581.

**SUPPORTING INFORMATION**

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