miR-155, miR-96 and miR-99a as potential diagnostic and prognostic tools for the clinical management of hepatocellular carcinoma

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Abstract. Increasing evidence has demonstrated that circulating microRNAs (miRNAs) can be utilized as potential biomarkers for the diagnosis of cancer, as well as a prognostic tool for the management of the disease. Therefore, the present study aimed to evaluate the predictive ability of miRNA (miR)-155, miR-96 and miR-99a for the diagnosis and prognosis of hepatocellular carcinoma (HCC). Tissues were collected from 30 patients with HCC and their matched adjacent normal liver tissues, as well as from serum samples from 30 patients with HCC and 30 healthy controls. Reverse transcription-quantitative PCR was used to measure the expression levels of miR-155, miR-96 and miR-99a. The expression levels of miR-155 and miR-96 were upregulated in the tissues and serum of patients with HCC, whereas miR-99a expression levels were decreased. Receiver operating characteristics (ROC) curve analysis revealed that circulating miR-155, miR-96, miR-99a and a combination of these three miRNAs could serve as diagnostic biomarkers for HCC with areas under the curve (AUC) of 0.84, 0.824, 0.799 and 0.931, respectively. Serum α-fetoprotein (AFP) was detected using electrochemiluminescence immunoassay analyzer. The addition of AFP with the combination of these three miRNAs offered a higher accuracy of HCC diagnosis (AUC, 0.979; sensitivity, 90.0%; specificity, 100.0%). In addition, elevated expression levels of miR-155 and miR-96 were associated with poor survival time of patients with HCC. The panel of miR-155, miR-96, miR-99a and AFP had a higher sensitivity and specificity for the diagnosis of HCC when compared with a single marker. Furthermore, the present data suggested that miR-155 and miR-96 may be potential prognostic markers for the clinical management of patients with HCC.

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Introduction

Liver cancer is one of the most frequently diagnosed types of cancer and it was identified as a leading cause of cancer-associated mortality, with an estimated 782,500 new cases and 745,500 mortalities occurring worldwide during 2012 (1). The incidence and mortality rates of liver cancer in China account for ~50% of cases worldwide (1). Of the primary liver cancers occurring worldwide, 70-90% are hepatocellular carcinoma (HCC) (1). At present, surgery remains the first-line treatment option for HCC (2). However, the early clinical symptoms of HCC are not obvious and are often overlooked. The majority of patients with HCC are diagnosed at an advanced stage of the disease when there are limited therapeutic options available, which results in a high mortality rate (3). Therefore, the identification of specific and sensitive biomarkers for the early diagnosis of HCC is required.

MicroRNAs (miRNAs) are a family of single-stranded non-coding RNAs, which are typically 20-25 nucleotides in length and do not encode for any proteins (4). miRNAs can regulate protein expression at the post-transcriptional level via the negative regulation of mRNA translation by binding to specific sequences in the 3' untranslated region of their target mRNAs (4). Notably, certain miRNAs are dysregulated in cancers and can serve as a critical determinant of cancer initiation and malignant progression (5). Aberrant expression levels of miRNAs have been detected in a variety of different types of cancer, including HCC. Cancer cells may also release miRNAs into the blood circulation. Previous studies revealed that there are different levels of miRNA expression between tumors and corresponding non-cancerous tissues, and that serum miRNAs can discriminate cancer patients from healthy subjects (6,7). Circulating miRNAs have been demonstrated to possess high levels of stability and can easily be obtained from patients with cancer (8). Furthermore, blood samples have the advantage of being minimally invasive and can be subjected to continuous *in vitro* testing, as well as being highly reproducible. The discovery of dysregulated miRNAs as potential circulating biomarkers in the plasma and/or serum may represent a useful approach for future diagnosis, prognosis and personalized therapy of patients with cancer (9,10).

In a previous study, aberrant expression of miR-155, miR-96 and miR-99a between HCC tissues and their matched adjacent normal liver tissues were validated through miRNA microarray assay and reverse transcription-quantitative PCR (RT-qPCR) (11). Given that blood samples can be easily obtained, and it has been demonstrated that miRNAs are stably maintained in human serum/plasma, it was suggested that they can be utilized as potential biomarkers for cancer diagnosis and prognosis. In the present study, 30 HCC tissues and their matched adjacent normal liver tissues were collected, as well as 30 HCC and 30 healthy serum samples, and the expression levels of miR-155, miR-96 and miR-99a were measured using RT-qPCR. The diagnostic and prognostic potential of these miRNAs for HCC were also investigated.

Materials and methods

Tissue and serum samples. In total, 30 HCC tissues and their matched adjacent normal liver tissues (≥5 cm from the tumor site) were collected from patients from the Affiliated Tumor Hospital of Guangxi Medical University (Nanning, China) between January 2012 and December 2013. All 30 patients with HCC (age range, 26-67; female to male ratio, 7:23) were given a follow-up examination 58-70 months following hepatectomy. Overall survival was defined based on the elapsed time between the time of surgery and mortality. According to the median cut-off values, 30 patients with HCC were categorized into high-expression and low-expression of miRNA. In addition, the 30 HCC (age range, 31-70; mean age, 50.7±10.56) and 30 healthy (age range, 34-69; mean age, 49.8±10.76) blood samples (2 ml) were also obtained from the Affiliated Tumor Hospital of Guangxi Medical University between January 2014 and December 2014. There was no significant difference in the age of the patients observed between the HCC group and healthy group (P>0.05). All the participants provided written informed consent. Patients included in this study were patients with HCC diagnosed by histopathological examination and according to the National Comprehensive Cancer Network clinical practice guidelines for oncology. The exclusion criteria were as follows: i) Patients with HCC who received anticancer therapy prior to surgery; and ii) Patients with HCC who also suffered from other severe diseases or malignant tumors simultaneously. Furthermore, healthy volunteers with autoimmune diseases, severe liver and kidney diseases, hematologic diseases, and infectious diseases were excluded from this study. All tissues were diagnosed independently following liver resection and were snap frozen in liquid nitrogen and immediately stored at -80˚C. Serum samples were centrifuged immediately at 13,900 x g for 10 min at 4˚C after collection and then stored at -20˚C for the future experiments. The present study was approved by the Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University. The Tumor-Node-Metastasis (TNM) stage was determined according to the 6th edition of the TNM Classification of Malignant Tumor. The clinical characteristics of the included participants are summarized in Table I.

Total RNA extraction from tissues. Total RNA was isolated from all test tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The isolated total RNA was then purified using a NucleoSpin® RNA clean-up kit (Macherey-Nagel GmbH and Co.), and the quantity and quality of total RNA were determined by using a NanoDrop 2000 spectrophotometer (version 1.6.198; Thermo Fisher Scientific, Inc.) and agarose gel electrophoresis. RNA was stored at -80˚C for subsequent RT-qPCR analysis.

Total RNA extraction from serum. Total RNA of serum was extracted using miRNeasy Serum/Plasma kit (Qiagen GmbH) according to the manufacturer's protocol. The NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) was used to measure the quality of total RNA, and agarose gel electrophoresis was used to ensure the integrity of the total RNA. RNA was stored at -80˚C for subsequent RT-qPCR analysis.

RT-qPCR. For the miRNA RT-qPCR, 1 μg RNA was reverse transcribed to cDNA using a miScript II RT kit (Qiagen GmbH) according to the manufacturer's protocol. RT-PCR assays were performed as previously described (11). RT-qPCR was performed using an Agilent MX 3000 real-time PCR system (Agilent Technologies, Inc.) with the miScript SYBR-Green PCR kit (Qiagen GmbH) and reactions were performed in triplicate. U6 was used as an endogenous control to normalize the expression level of miRNA extracted from the tissue samples, and miR-16 was used as an endogenous control for the serum samples (12). The thermocycling conditions were the following: Initial denaturation for 95˚C for 15 min, followed by 40 cycles of 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 70˚C for 30 sec. The primers used in the present study were purchased from Qiagen GmbH. The sequences for the primers used were as follows: miR-155 forward, 5'-CCTTTGCTGGAA TGGACAAGAAC-3'; miR-96 forward, 5'-TTGGGTGAA ATATATTGTGCGTCTC-3'; miR-99a forward, 5'-GAGTCC TGGACACCCAACTACAAG-3'; miR-16 forward, 5'-UAG CAGCACGUAAAUAUUGGCG-3'; U6 forward, 5'-GCACCG TCAAGGCTGAGAAC-3'; miScript universal reverse primer, 5'-AGCCGAAGTGAGCCACTGAA-3'. Relative levels of the three miRNAs were calculated using the $2^{-\Delta\Delta Cq}$ method (13).

Serum detection of α-fetoprotein (AFP). Serum AFP level was quantitatively measured using electrochemiluminescence immunoassay analyzer kit (Cobas e 601 module; Roche Diagnostics) according to the manufacturer's protocol. Normal reference values for AFP were 0-20 ng/ml.

Statistical analysis. One-way ANOVA followed by Student-Newman-Keuls post hoc was used to assess differences in the miRNA expression levels between the group of normal and pathological samples. Student's t-test was used to compare the ages of the two groups. The receiver operating characteristic (ROC) curve was applied to assess the diagnostic values of the three miRNAs investigated. Sensitivity and specificity levels were obtained according to the Youden's index (sensitivity + specificity-1) (9). The ROC curves of the combinations were constructed using binary logistic regression analysis. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Statistical analysis was performed with SPSS software (version 17.0; SPSS, Inc.), and P<0.05 was considered to indicate a statistically significant difference.

Table I. Demographic and clinical characteristics of the patients with hepatocellular carcinoma and 30 healthy subjects included in the present study.

Characteristic	Tissue sample, n (%)	Serum sample from patients, n (%)	Serum sample from healthy controls, n (%)
Sex			
Male	23 (76.7)	18 (60.0)	16 (53.3)
Female	7 (23.3)	12 (40.0)	14 (46.7)
Age			
≤50 years	17 (56.7)	16 (53.3)	15 (50.0)
>50 years	13 (43.3)	14 (46.7)	15 (50.0)
AFP			
>20 ng/ml	18 (60.0)	18 (60.0)	0 (0)
≤20 ng/ml	12 (40.0)	12 (40.0)	30 (100)
Tumor size			
≤5 cm	12 (40.0)	11 (36.7)	-
>5 cm	18 (60.0)	19 (63.3)	-
Liver cirrhosis			
Present	12 (40.0)	22 (73.3)	-
Absent	18 (60.0)	8 (26.7)	-
Differentiation			
Well + moderate	10 (33.3)	20 (66.7)	-
Poor	20 (66.7)	10 (33.3)	-
TNM stage			
I+II	17 (56.7)	12 (40.0)	-
III+IV	13 (43.3)	18 (60.0)	-

AFP, α-fetoprotein; TNM, tumor node metastasis.

Results

Expression of miRNAs in tissue and serum of patients with HCC. In the present study, the expression levels of miR-155, miR-96 and miR-99a were analyzed in 30 HCC tissues and their matched adjacent normal liver tissues. The present results indicated that compared with the adjacent normal tissues, the expression levels of miR-155 and miR-96 in HCC tissues were significantly upregulated, whereas miR-99a was significantly downregulated (P<0.05; Fig. 1A-C). Consistent with the results from tissue samples, the expression levels of miR-155 and miR-96 were also identified to be upregulated in the sera of patients with HCC compared with healthy controls. In addition, the serum levels of miR-99a were significantly downregulated in patients with HCC compared with healthy controls (P<0.05; Fig. 1D-F).

Diagnostic value of the combination of the serum levels of miRNAs and AFP in patients with HCC and healthy controls. To determine whether the serum levels of miR-155, miR-96, miR-99a and AFP exhibited diagnostic value in HCC, the ROC curves were plotted to analyze their diagnostic sensitivity, specificity and area under the curve (AUC) (Fig. 2). The AUC of the ROC analyses for four tested biomarkers in HCC were: i) miR-155, 0.840; ii) miR-96, 0.824; iii) miR-99a, 0.799; and iv) AFP, 0.839 (Table II). When miR-155, miR-96 and miR-99a were combined to form a panel, the combination had a higher accuracy than each miRNA separately in discriminating patients with HCC from healthy controls [AUC, 0.931; 95% CI, 0.870-0.992; sensitivity, 76.7%; specificity, 96.7%]. The addition of AFP to the combination of the aforementioned miRNAs resulted in the highest diagnostic accuracy (AUC, 0.979; 95% CI, 0.903-0.999; sensitivity, 90.0%; specificity, 100.0%).

Association between miRNA expression levels and prognosis. In order to evaluate the possible prognostic value of miR-155, miR-96 and miR-99a, an overall survival analysis for patients with HCC was performed. Kaplan-Meier curves were plotted between the high or low miRNA expression levels in HCC tissues and overall survival. The median survival time of patients with HCC was 58.5 months (Fig. 3). Kaplan-Meier analysis revealed that high levels of miR-155 and miR-96 were significantly associated with the decreased overall survival time of patients with HCC (P=0.004 and P=0.019, respectively), indicating that miR-155 and miR-96 may possess prognostic value for patients with HCC. In contrast, the patients with low miR-99a expression had slightly reduced survival times compared with those with high miR-99a. However, these results were not statistically significant (P=0.179).

Discussion

miRNAs have been reported to be involved in a series of biological processes, in particular in the occurrence and progression

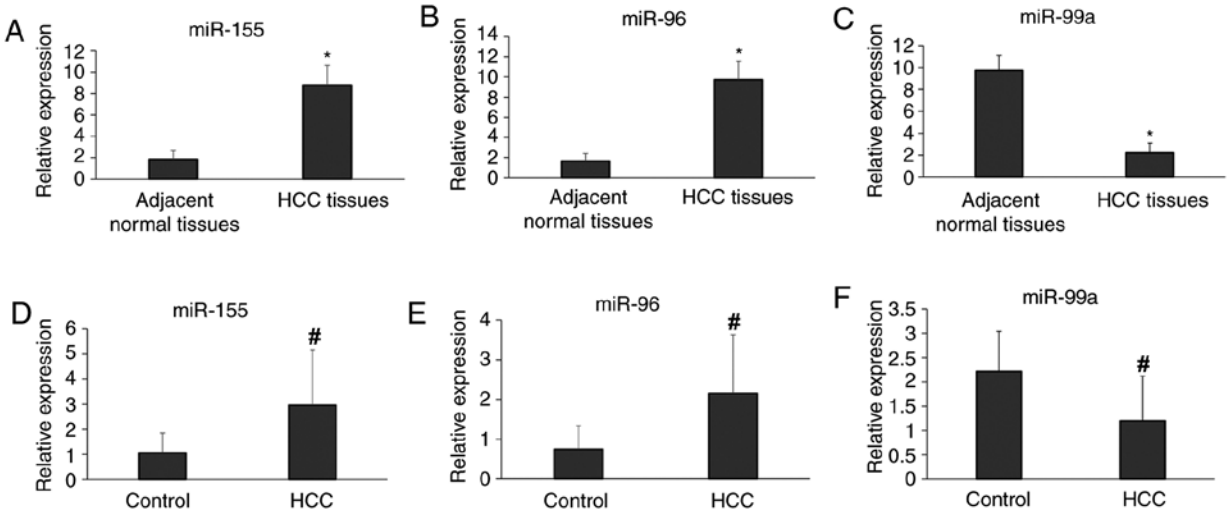

Figure 1. Expression levels of miR-155, miR-96 and miR-99a in HCC tissues and serum. Relative expression levels of (A) miR-155, (B) miR-96 and (C) miR-99a in HCC tissues and their matched adjacent normal liver tissues. Relative expression levels of (D) miR-155, (E) miR-96 and (F) miR-99a in the serum of patients with HCC and healthy controls. Relative expression levels are represented as the mean ± SD. *P<0.05 vs. adjacent normal tissue; #P<0.05 vs. healthy control. miR, microRNA; HCC, hepatocellular carcinoma.

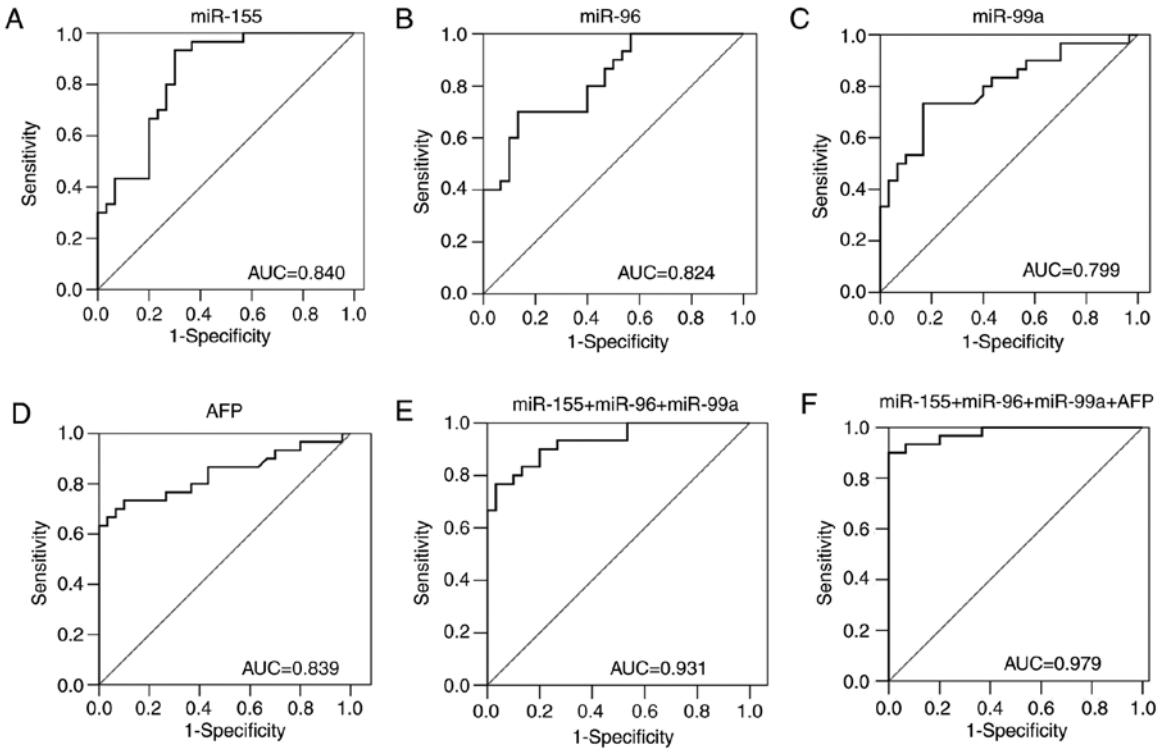

Figure 2. ROC curves analyses for distinguishing patients with HCC from healthy controls. ROC curves assessing the diagnostic value of (A) miR-155, (B) miR-96, (C) miR-99a and (D) AFP levels in the serum of patients with HCC compared with 30 healthy controls. (E) Combination ROC curve of miR-155 + miR-96 + miR-99a in distinguishing patients with HCC from healthy controls. (F) Combination ROC curve of miR-155 + miR-96 + miR-99a + AFP in distinguishing patients with HCC from healthy controls. ROC, receiver operating characteristic; miR, microRNA; AFP, α-fetoprotein; HCC, hepatocellular carcinoma.

of tumors. Accumulating evidence suggest that numerous miRNAs are aberrantly expressed in tumor tissues, blood and cells, and dysregulation of these miRNAs is connected with the occurrence, progression and prognosis of cancer (14). Certain miRNAs serve oncogenic roles, with some of these being upregulated in patients with cancer; whereas other miRNAs are downregulated, acting as tumor suppressors (6). However, all these miRNAs are highly associated with the carcinogenesis, progression and prognosis of patients with cancer. Thus, it is possible that many miRNAs could serve as biomarkers for the diagnosis and prognosis of various types of cancer, including HCC (7). Based on previous microarray assay and RT-PCR data (11), miR-155, miR-96 and miR-99a were selected as candidate miRNAs for the diagnosis and prognosis of HCC.

miR-155 acts as an oncogenic miRNA, which has been found to be upregulated in various types of tumor, such

Table II. AUC and the corresponding 95% CI of serum microRNA and AFP in patients with hepatocellular carcinoma compared with healthy controls.

Tumor markers	Sensitivity, %	Specificity, %	AUC	SE	95% CI	P-value
miR-155	93.3	70.0	0.840	0.051	0.739-0.941	<0.001
miR-96	70.0	86.7	0.824	0.052	0.722-0.927	<0.001
miR-99a	73.3	83.3	0.799	0.058	0.687-0.912	<0.001
AFP	66.7	96.7	0.839	0.054	0.733-0.946	<0.001
miR-155 + miR-96 + miR-99a	76.7	96.7	0.931	0.031	0.870-0.992	<0.001
miR-155 + miR-96 + miR-99a + AFP	90.0	100.0	0.979	0.015	0.903-0.999	<0.001

AUC, area under the curve; miR, microRNA; AFP, α-fetoprotein; SE, standard error.

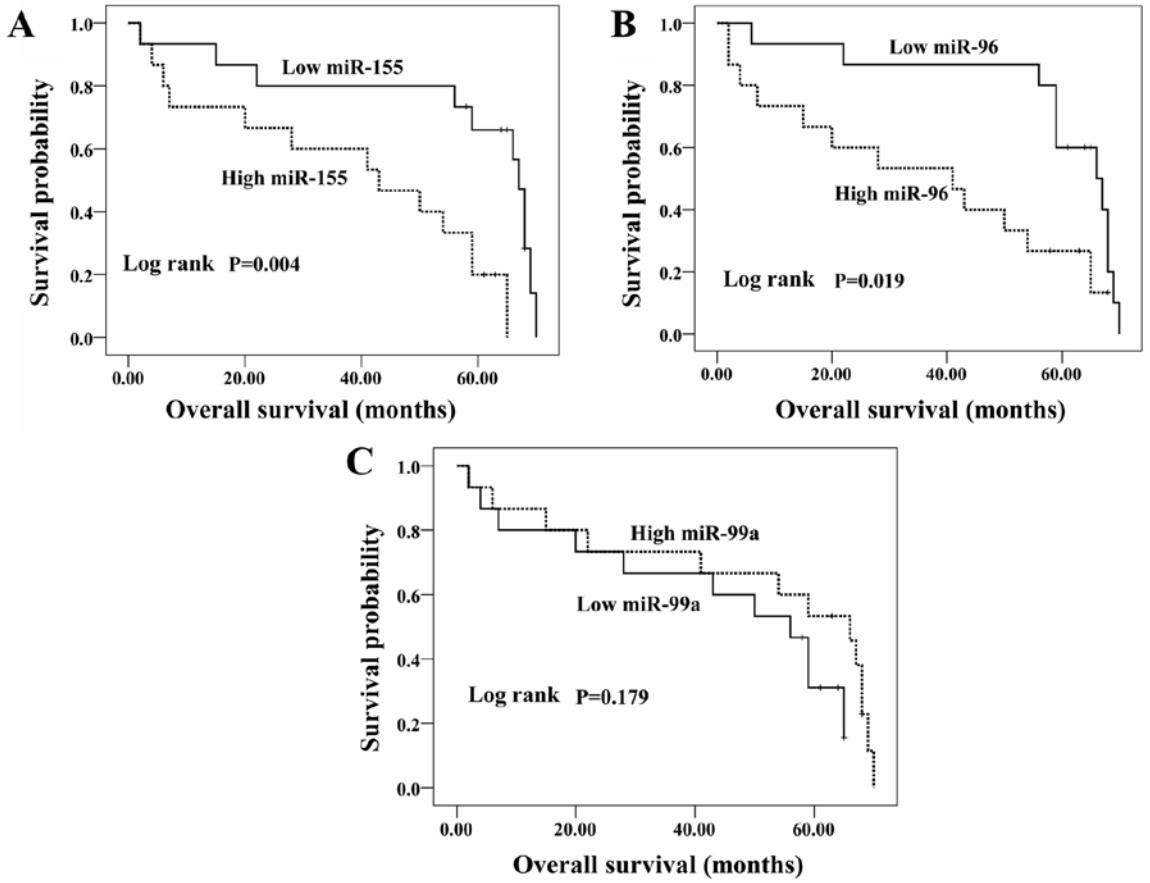

Figure 3. Kaplan-Meier curves for overall survival according to the relative expression levels of (A) miR-155, (B) miR-96 and (C) miR-99a in primary hepatocellular carcinoma tissues. Patients were categorized into two groups based on the median expression levels of miRNA. miR, microRNA.

as breast cancer (15), gastric cancer (16), lung cancer (17), colorectal cancer (18) and other solid malignancies. Bašová *et al* (15) indicated that there was a positive correlation between miR-155 levels and the pathogenesis of early breast cancer relapse, particularly at the time of post-surgery. These previous data suggested that oncogenic miRNAs (miR-155 and miR-24) in serum may enable not only the monitoring of early breast cancer, but may also become a highly valuable tool for the detection of relapse in patients with early breast cancer (15). The expression levels of miR-155 were significantly increased in gastric cancer tissues, and the upregulation of miR-155 promoted proliferation and migration of gastric cancer cells (16). A previous study has demonstrated that miR-155 exerts an oncogenic role in non-small cell lung cancer (17). It has also been demonstrated that an enhanced expression level of miR-155 is correlated with a higher frequency of distant metastases in patients with colorectal cancer (18). Furthermore, targeting miR-155-5p may be a useful therapeutic strategy against colon cancer metastasis (19). A previous study (20) revealed that the expression levels of miR-155 were highly upregulated in HCC tissues, and that miR-155 may be involved in the tumorigenesis of HCC, which is consistent with the results of the present study.

The miR-99 family consists of three members, miR-99a, miR-99b and miR-100. Recent studies demonstrated that the miR-99 family had potential functions as tumor suppressors, which may affect tumor cell growth, invasion and migration (21). Dysregulation of the miR-99 family members has been reported to serve an important role in multiple types of cancer (22-25). The expression levels of miR-99a, miR-99b and miR-100 were all significantly decreased in glioma tissues compared with non-neoplastic brain tissues. Further *in vitro* experiments indicated that the overexpression of miR-99a, miR-99b and miR-100 markedly suppressed cellular migration and invasion in glioma cells (21). In HCC, oral squamous cell carcinoma (OSCC) and non-small cell lung cancer, miR-99a also functions as a tumor suppressor (22-24). A previous study revealed that the low-level expression of miR-99a was correlated with poor tumor-free survival and overall survival for patients with HCC (25). For miR-99b, a previous study demonstrated that miR-99b-5p is expressed significantly less in liver metastasis lesions compared with paired primary cancer and can function as a tumor-suppressive microRNA in liver metastasis of colorectal cancer (26). He *et al* (27) revealed that the expression levels of miR-99b-3p were decreased in the tissues of patients with OSCC, and validated the suppressive role of miR-99b-3p in OSCC via glycogen synthase kinase 3 β downregulation. In the present study, the expression levels of miR-99a in HCC tissues and serum were significantly downregulated compared with healthy controls, in line with previous studies (22,25).

miR-96 belongs to the miR-183 family, which is located in the human chromosome 7q32.2. miR-96 is highly expressed in various types of cancer and is a well-known oncogenic miRNA (28-30). A previous study demonstrated that miR-96 was significantly upregulated in breast cancer tissues and that it could promote breast tumorigenesis both *in vitro* and *in vivo* (28). Yoshino *et al* (29) demonstrated that miR-96 was overexpressed in bladder cancer tissue. The upregulation of miR-96 in tissues, serum and serum exosomes has also been observed in patients with lung cancer (30). Furthermore, exosomal miR-96 could act as a serum biomarker for identifying malignant lung cancer (30). Iwai *et al* (31) reported that miR-96-5p expression levels were markedly upregulated in primary HCC tumors compared with paired non-tumorous tissues, and suggested that miR-96-5p inhibits HCC cell apoptosis by targeting caspase-9 mRNA. A previous study showed that serum miR-96 levels discriminated patients with HCC from patients with chronic hepatitis B with an AUC of 0.803, and suggested that serum miR-96 is a promising biomarker for patients with HCC (32). Another previous study indicated that upregulation of miR-96 confers an oncogenic function in HCC dissemination, which could promote cell invasion and serve as a potential prognostic factor for patients with HCC (33). The present study suggested that miR-96 expression was upregulated in tissues and sera of patients with HCC, and its upregulation was associated with reduced overall survival time.

In the present study, it was identified that miR-155 and miR-96 were upregulated in tissues and sera of patients with HCC, whereas miR-99a level was decreased. In addition, the results revealed that circulating miR-155, miR-96 and miR-99a, and the combination of the three miRNAs could serve as valuable biomarkers for the diagnosis of HCC with AUCs of 0.840, 0.824, 0.799 and 0.931, respectively. At present, AFP is one of most frequently used biomarkers for HCC (7). The addition of AFP to the combination of miR-155, miR-96 and miR-99b offered a higher accuracy of HCC diagnosis. The main limitation of the present study is the small sample size. Further studies using a larger cohort of patients are therefore required to validate the diagnostic value of these miRNAs.

In summary, the present study highlighted the potential role of serum-circulating miR-155, miR-96 and miR-99b levels for the diagnosis of HCC. The combination of these miRNAs with AFP could potentially improve the sensitivity and specificity for HCC diagnosis compared with using either miRNAs or AFP alone. In addition, elevated expression levels of miR-155 and miR-96 were identified to be associated with poor survival times in patients with HCC. Collectively, the present results suggested that miR-155 and miR-96 can act as prospective prognosis predictors of HCC.

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Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SN wrote the manuscript, designed the study and interpreted the data. HL performed the statistical analysis and interpreted the results. BG performed the experiments. WW, JL and AY were involved in the sample collection and data analysis. LZ was involved in obtaining the funds for the present study, designing the study and critically revising the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of the Affiliated Tumor Hospital of Guangxi Medical University (Nanning, China). Written informed consent was obtained from each participant included within the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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