Serum miR-375 levels are closely related to disease progression from HBV infection to HBV-related hepatocellular carcinoma

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Research article

Keywords: Hepatitis B virus, hepatocellular carcinoma, microRNA-375, clinical significance, disease progression

Posted Date: September 11th, 2019

DOI: https://doi.org/10.21203/rs.2.14273/v1

Version of Record: A version of this preprint was published at BioMed Research International on April 21st, 2020. See the published version at https://doi.org/10.1155/2020/5819385.

Abstract

Background: There is an urgent need to identify ideal serological biomarkers that not only are closely related to disease progression from hepatitis B virus (HBV) infection to Hepatocellular carcinoma (HCC) but also have high specificity and sensitivity. We conducted this study to determine whether miR-375 has a potential value in the early prediction of the progression of HBV-related HCC (HBV-HCC). Methods: A total of 177 participants were enrolled. Receiver operating characteristic curve (ROC) was used to evaluate the predictive capability of selected miR-375 for HBV-HCC. We upregulated miR-375 expression in HepG2, HepG2.2.15 and HepAD38 cells to determine its effect on cellular proliferation, migration and invasion in vitro using CCK-8 and transwell assays. Results: Serum miR-375 levels decreased in order from healthy controls to CHB without cirrhosis, followed by cirrhosis, and finally, HBV-HCC patients. MiR-375 levels were significantly lower in HBeAg- and HBV DNA-positive patients than negative ($P<0.05$), and significantly lower in patients with elevated alpha-fetoprotein (AFP) and carcino-embryonic antigen (CEA) than normal levels ($P<0.05$). MiR-375 might be a biomarker for HBV-HCC, with a high area under the curve (AUC) of 0.838 (95% confidence interval (CI): 0.780~0.897; sensitivity: 73.9%; specificity: 93.0%). The AUC (0.768 vs 0.584) and sensitivity (93.8% vs 75.0%) for miR-375 were higher than those for AFP. The overexpression of miR-375 noticeably inhibited proliferation, migration and invasion in HepG2, HepG2.2.15 and HepAD38, especially in HepG2.2.15 and HepAD38, which are stably infected with HBV. Conclusions: Serum miR-375 levels are closely related to disease progression from HBV infection to HBV-HCC. Keywords: Hepatitis B virus, hepatocellular carcinoma, microRNA-375, clinical significance, disease progression

Background

It is estimated that almost 240,000,000 people are chronically infected with hepatitis B virus (HBV). Annually, HBV infection accounts for approximately 1 million deaths[1].Hepatocellular carcinoma (HCC) is the major form of primary liver tumor, representing the third leading cause of cancer-related death worldwide. Nearly half of HCC cases and deaths are estimated to occur in China due to the high prevalence of HBV infection. The cancer statistics in China show that the mortality due to HCC is 42.21/100,000[2].Patients with HCC have poor prognosis, with a 5-year survival of approximately 5% because of diagnosis at late stages and limited treatment options[3].Therefore, it is important to predict the progression of HBV-related HCC(HBV-HCC)at an early stage.

Recently, many biomarkers have been widely used for HCC diagnosis, such as alpha-fetoprotein(AFP) [4],SMG-1[5,6],GOLM1[7],Barx2[8],Des-γ-carboxyprothrombin[9],Glypican-3[10],Cytokeratin-19[11],Golgi protein-73[12], and Osteopontin[13]. Nevertheless, most of these proteins are usually detected after HCC occurrence and lack sensitivity and specificity [14]. Moreover, these markers are not specific for HBV-HCC. Hence, it is still difficult diagnose HBV-HCC at an early stage, and particularly, to predict disease progression from healthy liver, chronic hepatitis B infection (CHB) without cirrhosis, CHB with cirrhosis to HBV-HCC. Therefore, there is an urgent need to identify ideal serological biomarkers that not only are

closely related to disease progression from HBV infection to HCC but also have high specificity and sensitivity.

MicroRNAs (miRNAs) are known to play fundamental roles in the regulation of many oncogenes or tumor suppressors and are considered potential diagnostic biomarkers for cancer detection [15, 16].miR-375 is encoded by the chromosomal region 2q35 in humans. Some studies have reported that miR-375 is downregulated in many malignant tumors [17, 20-29]. Research has shown that miR-375 could be used as a serological marker for HCC. When employed as biomarkers, miR-25, miR-375, and let-7f could separate HCC patients from controls. The area under the receiver operating characteristic (ROC) curve (AUC) for miR-375 for HCC prediction was 0.96 (specificity: 96%; sensitivity: 100%)[30]. Mechanistic studies have shown that miR-375 inhibited proliferation, migration and invasion in Hep3B and Huh7 cells. However, there are very few studies on the relationship between miR-375 and HBV-HCC in patients or in HBV-infected cell lines. Moreover, there is a lack of epidemiological studies on the association of serum miR-375 expression with disease progression from healthy liver, CHB without cirrhosis, cirrhosis to HBV-HCC.

In this study, we determined serum miR-375 levels in healthy controls and in patients with CHB without cirrhosis, cirrhosis or HBV-HCC and analyzed the correlation between miR-375 and disease progression, HBV-related serological markers, and clinical features. Then, we upregulated miR-375 expression in HepG2, HepG2.2.15 and HepAD38 cells to determine its effect on cellular proliferation, migration and invasion *in vitro*. Additionally, we evaluated the diagnostic value of serum miR-375 as an ideal serological biomarker for the prediction of HBV-HCC progression.

Methods

2.1. Subjects

From August 2015 to August 2017, healthy individuals and patients with HBV-related diseases who were older than 18 years were recruited from the Department of Hepatobiliary Surgery, Xijing Hospital and the Center of Infectious Diseases, Tangdu Hospital, Xi'an City, Shaanxi Province, China. All patients were positive for HBsAg, and none of the patients had any other type of liver disease, such as chronic hepatitis C infection, alcoholic liver disease, autoimmune liver disease, or metabolic liver disease. The diagnosis of HCC and cirrhosis was histopathologically confirmed. CHB in the liver was caused by persistent infection with HBV. The diagnostic criteria included the following four points: 1. $HBsAg^{+}$>6 months; 2. serum HBV DNA>20,000 IU/mL(10^{5}copies/mL), lower values 2,000-20,000 IU/mL(10^{4}-10^{5} copies/mL) are often seen in HBeAg-negative patients with CHB; 3. Persistent or intermittent elevation in alanine transaminase (ALT)/aspartate aminotransferase (AST) levels; and 4. Liver biopsy showing chronic hepatitis with moderate or severe necroinflammation.

A standard questionnaire was used by trained investigators to collect information from face-to-face interviews, pathology reports and medical records. The questionnaire recorded the following information:

basic demographic characteristics of the subjects, family history of HBV infection, cause of diseases, tumor pathology and clinical indicators of cancer. Blood samples for miRNA detection were collected in tubes and processed within 1 h of collection. The blood samples were centrifuged at 12000 g for 10 min at 4 °C to spin down blood cells, and the supernatants were transferred into microcentrifuge tubes, followed by a second centrifugation at 12000 g for 10 min at 4 °C. The supernatants were transferred to RNase-free tubes and stored at −80 °C.

2.2 Ethics, consent and permissions

The Ethics Committee of The Air Force Medical University approved the study protocol. All participants were fully informed the details of research study, and the participants or legal guardians of the participants signed written informed consent before inclusion in the study.

2.3 Serological examination of HBV

Blood samples of participants were obtained by forearm venipuncture. The serum samples were laboratory tested after collection. The same lot of enzyme-linked immunosorbent assay(ELISA) reagents from Wantai Production Company (Beijing, China)was used for the initial testing of HBsAg and HBeAg and the anti-HBs, anti-HBe and anti-HBc antibodies. All specimens were evaluated for the presence of HBV DNA by using a diagnostic kit (for PCR-based fluorescence probing) according to the instructions of the manufacturer(Roche Molecular Systems). Viral loads higher than a linear range were determined by dilution as recommended by the manufacturer.

2.4. Detection of miR-375 via real-time quantitative RT-PCR

2.4.1 RNA isolation

Total RNA was isolated from serum samples using TRIzol reagent (Invitrogen, USA). Briefly, 250μl serum samples were used to extract total RNA. Each sample was eluted in 50μl of RNase-free water. The concentration and purification of RNA were spectrophotometrically determined by measuring its optical density (OD, A260/280>2.0 and A260/230>1.8) using a NanoDrop ND-2000 Spectrophotometer (Thermo Scientific Wilmington, DE, USA).

2.4.2 RNA reverse transcription and qRT-PCR

The primers used for PCR were as follows: miR-375forward primer,(5’-TTTGTTCGTTCGGCTCGC-3’), reverse primer and U6 (5’-CGCTTCGGCAGCACATATAC-3’) purchased from TaKaRa (Dalian, China). Reverse transcription of miRNAs was performed with the SYBR Prime ScriptmiRNA RT-PCR Kit (TaKaRa, Dalian, China). The expression of mature miRNAs was determined using miRNA-specific qRT-PCR (TaKaRa, Dalian, China). Fluorescence was automatically detected during amplification, and the melting curve for the product was obtained. The expression levels were normalized to the level of U6 endogenous control and calculated using the comparative cycle threshold (Ct) method.

2.5 Cell culture

The following HCC cell lines were used in this study: HepG2, HepG2.2.15 and HepAD38. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 380 mg/L G418 sulfate (Promega, USA) at 37 °C in an atmosphere with 5% CO_2.HepAD38 cells, which are a variant of HepG2 cells, express the HBV genome under the control of a tetracycline (Tet)-off promoter.

2.6 Cell transfection

miR-375 mimics and negative control (NC)oligonucleotides were designed and synthesized by Gene Pharma(Shanghai, China). The sequences were as follows: miR-375 mimic, 5'-UUUGUUCGUUCGGCUCGCGUGA-3' and random miRNAmimics(NC), 5'-UUCUCCGAACGUGUCACGUTT-3'. miR-375 mimics were transfected into cells at a final oligonucleotide concentration of 20nmol/L. Transfection was performed with Lipofectamine 2000 reagent (Invitrogen, CA, USA)following the manufacturer's protocol. Briefly, cells were trypsinized, counted and seeded in plates on the day before transfection to ensure suitable cell confluency on the day of transfection.

2.7 Proliferation assay

The Cell Counting Kit-8 (CCK-8) assay was carried out to assess cell viability. HepG2 cells, HepG2.2.15 cells and HepAD38 were seeded in 96-well culture plates at 2×10^5 cells per well. The next day, the cells were transfected with miR-375 mimics or NC as above. Then, 10 mL of CCK-8 reagent was added to each well before transfection and at 24 h, 48 h or 72 h after transfection. Cell proliferation rates were determined by the measurement of optical density (OD) at 490 nm via a microplate reader (Bio-Rad).

2.8 Transwell assay

For invasion assays, transfected HepG2 cells, HepG2.2.15 cells and HepAD38 were plated in the top chamber of a transwell plate with a BD Matrigel-coated membrane. The cells were plated in serum-free medium, and medium supplemented with 10% serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h at 37 °C with 5% CO_2 in a cell culture incubator. After 24 h, the uninvaded cells were removed from the upper surface of the transwell membrane. The invaded cells on the lower surface of the inserts were stained with crystal violet and counted.

2.9 Statistical analysis

RT-PCR was performed in triplicate, and average Ct values were calculated. The average expression level of miR-375 in all samples was normalized using U6 as the reference, and the $2^{-\Delta Ct}$ method (ΔCt=miR-375 Ct-U6 Ct) was to express the level of miR-375 in serum samples. The normalized data were analyzed using t-test or ANOVA, with asymptotic p value computations at $P<0.05$. Groups of patients with different miR-375 levels in their sera were evaluated by the two–tailed non-parametric Mann–Whitney U test. Furthermore, Spearman correlation was used to analyze the correlation between the expression level of

miR-375 and patient characteristics. ROC curve analysis was employed to determine the diagnostic utility of miR-375. Forcytological experiments, differences among experimental groups were statistically evaluated using t-tests. Statistical analyses were performed using SPSS version 19.0 (IBM, USA). All graphs were constructed using GraphPadPrism 5 (GraphPad Software, USA).

Results

3.1. Description and clinical features of patients

A total of 177 participants were enrolled into the study at the Department of Hepatobiliary Surgery, Xijing Hospital and the Center of Infectious Diseases, Tangdu Hospital from August 2015 to August 2017. A total of 63 HBV-HCC patients, 74 patients with CHB and 40 healthy controls were enrolled in this study. Among the 74 patients with CHB, 54 had cirrhosis, including 20 patients with compensated cirrhosis and 34 patients with decompensated cirrhosis. The average age of all the subjects was 47.24±10.72 years. The average age of the healthy controls and HBV-infected subjects was 45.63±9.43 and 48.99±10.93 years, respectively. There was no significant difference in the distribution of age and sex between the above two groups (P>0.05). All patients were positive for HBsAg, and 62 patients were positive for HBV DNA. In addition, 1.5% of the patients were positive for anti-HBs antibodies, 39.4% were positive for HBeAg, 40.9% were positive for anti-HBe antibodies, and 92.7% were positive for anti-HBc antibodies. There were 74 patients with elevated ALT levels. The tumor markers AFP, CEA and CA19-9 were elevated in 72, 32 and 51 patients, respectively. None of the patients had any other type of liver disease, such as chronic hepatitis C infection, alcoholic liver disease, autoimmune liver disease, or metabolic liver disease. The characteristics of the HBV-infected subjects are presented in Table 1.

3.2. Expression of miR-375 in the serum

To investigate whether serum miR-375 levels are abnormally altered in healthy individuals, inpatients with CHB with or without cirrhosis, and in patients with HCC, serum miR-375 levels were measured in 137 patients and 40 healthy controls. The expression of serum miR-375 by qRT-PCR analysis decreased in order from healthy controls to patients with CHB without cirrhosis, followed by patients with cirrhosis, and finally, patients with HBV-HCC. Compared with those in healthy controls, serum miR-375 levels were significantly decreased in cirrhosis and HBV-HCC patients (P<0.05). Among the HBV-infected patients, serum miR-375 levels were significantly higher in the CHB group than in the HBV-HCC group (P<0.05) (Fig. 1).

3.3. Relationship between serum miR-375 levels and HBV serological markers in HBV-infected patients

To assess whether serum miR-375 levels were associated with viral replication-related and HBV serological markers, the correlation between serum miR-375 levels and concentrations of HBV DNA, HBeAg, and anti-HBe, anti-HBc, and anti-HBs antibodies were analyzed in HBV-infected patients. The expression of miR-375 was significantly lower in HBeAg-positive patients than in HBeAg-negative patients (P<0.05). The expression of miR-375 was significantly lower in anti-HBe-negative patients than in anti-

HBc-positive patients (P<0.05). Additionally, the expression of miR-375 was significantly lower in HBV DNA-positive patients than in HBV DNA-negative patients (P<0.05) (Table2).

3.4 Relationship between serum miR-375 levels and the clinical features of HBV-infected patients

We next analyzed the correlation between the expression of serum miR-375 and clinical features of patients, such as ALT, AFP, carbohydrate antigen 19-9(CA19-9), carcino-embryonic antigen (CEA) levels. The expression of miR-375 was significantly lower in patients with elevated AFP and CEA levels than in patients with normal AFP and CEA levels (P<0.05, Table3).

3.5. Diagnostic value of serum miR-375 for HBV-HCC

ROC curve analysis was performed to verify the accuracy of serum miR-375 in diagnosing HBV-HCC. Indeed, serum miR-375 levels could serve as valuable biomarkers for differentiating HCC patients from the whole cohort with an AUC of 0.838(95% confidence interval (CI): 0.780~0.897, Fig. 2) and sensitivity and specificity of 73.9% and 93.0%, respectively. We compared the diagnostic accuracy of miR-375 and AFP using the same serum samples (from 137 HBV-infected patients). miR-375 could differentiate HCC patients from other HBV-infected patients with an AUC of 0.768 (95% CI: 0.644~0.891, Fig. 2), sensitivity of 93.8%, and specificity of 63.9%. When we used AFP to separate HBV-HCC patients from HBV-infected patients, the AUC was 0.584 (95% CI: 0.456~0.713), the sensitivity was 75.0%, and the specificity was 65.5%.

3.6. miR-375 was downregulated in HBV-HCC cell lines

HepG2.2.15, HepAD38 and parental HepG2 cells were selected for *in vitro* experiments. HepG2.2.15 and HepAD38cells were positive for HBsAg. However, HepG2 cells were negative for HBsAg, and HBV DNA copy numbers in these cells were below the level of detection. Based on qRT-PCR analysis, miR-375 expression were clearly lower in HepG2.2.15 cells and HepAD38 than in HepG2 cells (P<0.05, Fig. 3), which is consistent with the results of serological analyses.

3.7. miR-375 inhibited the proliferation of HBV-HCC cells

We tested the expression level of miR-375 by qRT-PCR in HepG2 cells, HepG2.2.15 cells and HepAD38 cells at 48 h after transfection to determine the transfection efficiency. The expression of miR-375 in the transfected cells was significantly higher than that in the non-transfected control cells by nearly 15~20 folds, indicating that the expression of miR-375 in the cells was enhanced after transfection.

We performed the CCK-8 assay inHepG2 cells,HepG2.2.15 cells and HepAD38 cells at 24 h, 48 h and 72 h after transfecting the cells with miR-375 mimics. The proliferation rates of miR-375-transfected cells were lower than those of non-transfected cells (transfected vs non-transfected HepG2.2.15cells, transfected vs non-transfected HepAD38 cells and transfected vs non-transfected HepG2 cells). The proliferation rates of HepG2.2.15 cells and HepAD38 cells were lower than those of HepG2 cells (transfected HepG2.2.15 cells and HepAD38 vs transfected HepG2cells, non-transfected HepG2.2.15 and HepAD38cells vsnon-

transfected HepG2 cells). The proliferation rate of transfected HepG2.2.15 cells and HepAD38 were the lowest among all groups. Therefore, the overexpression of miR-375 significantly inhibited cellular proliferation (Fig. 4).

3.8. miR-375 inhibited invasion of HBV-HCC cell lines

Matrigel invasion assays demonstrated that after being transfected with miR-375 mimics for 48 h, the number of invasive cells was 87.333±6.658 in transfected HepG2 cells, which was lower than that in non-transfected HepG2 cells (206.000±16.523) (t=11.538, P=0.0003). Similarly, the number of invasive cells was 69.667±7.095 in transfected HepG2.2.15 cells, which was lower than that in non-transfected HepG2.2.15 cells (248.000±9.849) (t=25.447, P<0.0001). The number of invasive cells was 30.000±3.000 in transfected HepAD38 cells, which was lower than that in non-transtected HepAD38 cells (170.000±17.692) (t=17.200, P<0.0001). The number of invasive transfected HepG2.2.15 (t=3.145, P<0.05) and HepAD38 (t=13.600, P=0.0002) cells was clearly lower than that of invasive transfected HepG2 cells, respectively (Fig. 5).

Discussion

The purpose of the present study was to explore novel serum biomarkers for the prediction of HBV-HCC progression. Because of the lack of reliable serum biomarkers with high sensitivity and specificity, we conducted the present study to identify whether miR-375 has a potential diagnostic value in predicting HBV-HCC progression.

First, we analyzed serum miR-375 levels in healthy controls and in patients with CHB, cirrhosis, or HBV-HCC. Compared with those in healthy controls, serum miR-375 levels were significantly decreased in cirrhosis and HBV-HCC patients (P<0.05). Among the HBV-infected patients, serum miR-375 levels were significantly higher in the CHB group than in the HBV-HCC group (P<0.05). These results were consistent with our previous study, which compared miR-375 expression in tumor and adjacent normal tissues from patients with HBV-HCC [31]. In that study, miR-375 levels were significantly lower in tumor tissues than in adjacent tissues from HBV-HCC patients. In other words, miR-375 expression is downregulated in both tissues and sera of HBV-HCC patients. Previous studies have also demonstrated that miR-375 is significantly downregulated in multiple types of cancer and its plays the role of a tumor suppressor (3, 17).Moreover, our data showed that the expression of serum miR-375 decreased in order from healthy controls to patients CHB without cirrhosis, followed by patients with cirrhosis, and finally, patients with HBV-HCC. Serum miR-375 expression was the lowest in the HBV-HCC group. Therefore, the expression of serum miR-375 decreases with disease progression.

Next, we analyzed the relationship between serum miR-375 levels and HBV serological markers in HBV-infected patients. Serum miR-375 expression was significantly lower in HBeAg- and HBV DNA-positive patients than in HBeAg- and HBV DNA-negative patients. HBV DNA is a quantitative virologic marker reflecting the degree of replication of HBV. A number of studies have found that the amount of HBV DNA is related to the extent of liver injury and severity of liver fibrosis and that it can be used as an

independent factor to predict response to antiviral treatment [31-33]. HBeAg is a serologic marker associated with a high degree of viral replication and infectivity. In general, HBeAg-positivity is correlated with a high HBV DNA load [34].In this study, the expression of serum miR-375 was relatively lower in HBV DNA-positive and HBeAg-positive patients. The results of our previous study showed that the abnormal expression of miR-375 in HBV-HCC patient tissues was closely related to the replication status of HBV. In addition, the expression level of miR-375 in tissues was negatively correlated with the HBV DNA load. The higher was the titer of HBV DNA, the lower was the expression level of miR-375 in tissues[31].These results were consistent with the results of the current study.

Next, we analyzed the relationship between serum miR-375 levels and commonly used serum tumor markers. miR-375 expression was significantly lower in patients with elevated AFP and CEA levels than in patients with normal AFP and CEA levels(P<0.05). The negative correlation between miR-375 and tumor markers also suggests that miR-375 may serve as a serum marker for the diagnosis of HCC. Subsequently, we performed ROC curve analyses to verify the diagnostic accuracy of serum miR-375 for HCC. Serum miR-375 could differentiate HCC patients from the whole cohort with an AUC of 0.838 (95% CI: 0.780~0.897) and sensitivity and specificity of 73.9% and 93.0%, respectively. Additionally, serum miR-375 could differentiate HCC patients from other HBV-infected patients with an AUC of 0.768 (95% CI: 0.644~0.891) and sensitivity and specificity of 93.8% and 63.9%, respectively. Therefore, serum miR-375 may serve as a valuable biomarker for distinguishing HCC patients from other groups of HBV-infected patients.

AFP is one of the most widely used tumor markers for the diagnosis of HCC, and it has a sensitivity of 60% at a cutoff value of 20 ng/Ml[4, 35].To compare the diagnostic accuracy of miR-375 and AFP, we analyzed the AUC of the two markers using the same serum samples. The AUC (0.768 vs 0.584) and sensitivity (93.8% vs 75.0%) of serum miR-375 were higher than those for AFP. Therefore, serum miR-375 is better than AFP for diagnosing HBV-HCC. Nevertheless, these results need to be validated in larger cohorts in the future.

In recent years, some studies have shown that abnormal expression of miR-375 can affect the proliferation, migration and invasion of HCC cell lines including Hep3B and Huh7 [36]. However, these studies determined the expression of miR-375 and its downstream regulatory genes in pure hepatoma cell lines but not HBV-HCC cell lines. Therefore, the effect of the abnormal expression of miR-375 on the biological function of HBV-HCC cell lines remained unknown. The cell line HepG2.2.15 and HepAD38 that were used in this study has been derived by the stable expression of HBV in HepG2 cells. We routinely cultured and confirmed the identity of HepG2, HepG2.2.15and HepAD38 cells. HepG2.2.15 and HepAD38 cells were positive for HBsAg, and HBV DNA titers in these cells indicated moderate viral replication, whereas HepG2 cells were negative for HBsAg and HBV DNA. Therefore, HepG2.2.15 and HepAD38 displayed characteristics of HBV infection, and there was no cross contamination between the three cell lines.

In vitro, we determined the expression level of miR-375 in HepG2.2.15, HepAD38 cells and parental HepG2 cells using qRT-PCR. Compared with that in HepG2 cells, which are not infected with HBV, the expression level of miR-375 was clearly reduced in HepG2.2.15 cells and HepAD38, which are infected with HBV. In other words, the expression level of miR-375 in liver cancer cells is affected by HBV infection. Some studies have also shown that other miRNAs are differentially expressed between cell lines with stable HBV infection and their parental counterparts [31]. MiRNAs play a critical role in virus-host interactions. Cellular miRNAs in the host modulate the expression of various viral genes, thus playing a pivotal role in the host-pathogen interaction network. In addition, viruses also encode miRNAs for protection against cellular antiviral responsesor may even exploit host miRNA pathways to their own advantage. The persistence of virus in host cells in turn affects the expression of miRNAs in the host.

We upregulated miR-375 expression in HepG2, HepG2.2.15 and HepAD38 cells and analyzed the effects of miR-375 overexpression on proliferation and invasion *in vitro* using the CCK-8 and transwell assays. The overexpression of miR-375 significantly inhibited cellular proliferation. InHepG2, HepG2.2.15 and HepAD38 cells, the proliferation rate of miR-375-transfected cells was lower than that of non-transfected cells. Maximum inhibition was observed at 48 h post-transfection. Additionally, the proliferation rates of HepG2.2.15 cells and HepAD38 with or without miR-375 overexpression were significantly lower than the respective proliferation rates HepG2 cells with or without miR-375 overexpression. Between 48 and 72hpost-transfection with miR-375, the size of the HepG2.2.15 cell population remained almost constant. Therefore, miR-375 could inhibit the proliferation of liver cancer cells, and the inhibition was more pronounced in the presence of HBV infection.

Transwell assays were used to simulate tumor cell invasion *in vitro*. The results of transwell assays showed that compared with respective non-transfected cells, miR-375-transfected HepG2, HepG2.2.15and HepAD38 cells exhibited markedly reduced invasion. Furthermore, miR-375-transfected HepG2.2.15 andHepAD38 cells were less invasive thanmiR-375-transfected HepG2 cells, indicating that miR-375 could inhibit the invasion of liver cancer cells and that this inhibition was more pronounced in the presence of HBV infection.

The present study has two limitations. First, during the transition from healthy liver, HBC, liver cirrhosis to HBV-HCC, there is an asymptomatic phase of HBV infection. However, our cohort did not included such HBV carriers. In the future, we will further expand the sample size and evaluate patients at various stages of HBV infection, including chronic asymptomatic HBV carriers. Thus, we will perform in-depth analysis to further confirm the diagnostic value of serum miR-375 for HBV-HCC. Second, this study only examined miR-375, and other potentially relevant markers including some classic HCC risk factors could not be evaluated. In the future, we will assess these markers together with miR-375 and construct a predictive model for HBV-HCC.

Conclusions

Serum miR-375 expression decreased in order from healthy controls to patients with CHB without cirrhosis, followed by patients with CHB with cirrhosis, and finally, patients with HBV-HCC. Serum miR-375 expression was correlated with HBeAg, HBV DNA, and AFP and CEA levels. ROC curve analyses revealed that serum miR-375may be a promising biomarker for HBV-HCC detection, with a relatively high AUC, sensitivity, and specificity. The AUC and sensitivity of serum miR-375 were higher than those for AFP. In addition, compared with that in HepG2 cells, miR-375 expression was markedly reduced in HepG2.2.15 and HepAD38 cells. The overexpression of miR-375 clearly inhibited proliferation, migration and invasion *in vitro*, especially in HepG2.2.15 cells and HepAD38, which are stably infected with HBV. Therefore, serum miR-375 might serve as a potential biomarker to predict the progression of HBV-HCC.

Declarations

Disclosure Statement

Authors declare no conflicts of interest for this article.

Funding

This study was funded by National Natural Science Foundation of China (81773488, 81472988), and national key project for prevention of infectious disease (2017ZX10105011).

Conflict of Interest: No conflict of interest to declare.

Authors' contributions

The first three authors contributed equally to this paper. WLZ, TF and ZJG: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript and obtaining funding; YZ, LZ, HXS, YL, JG and ZHJ: acquisition of data, technical support and analysis of data; YPY and ZJS: study concept and design, critical revision of the manuscript for important intellectual content and obtaining funding.

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Tables

Table 1. Characteristics of the HBV-infected subjects in the study [n(%)]

Clinical features	Number (%)
Gender	
Male	111 (81.0%)
Female	26 (19.0%)
Anti-HBs	
Positive	2 (1.5%)
Negative	135 (98.5%)
HBeAg	
Positive	54 (39.4%)
Negative	83 (60.6%)
Anti-HBe	
Positive	56 (40.9%)
Negative	81 (59.1%)
Anti-HBc	
Positive	127 (92.7%)
Negative	10 (7.3%)
HBV DNA	
Positive	62 (45.3%)
Negative	75 (54.7%)
ALT	
Elevated	74 (54.0%)
Normal	63 (46.0%)
CEA	
Elevated	32 (23.4%)
Normal	105 (76.6%)
CA19-9	
Elevated	51 (37.2%)
Normal	86 (62.8%)
AFP	
Elevated	72 (52.6%)
Normal	65 (47.4%)

[Categorized by serum levels of ALT (<40 U/L and ≥40 U/L), AFP (<20 ng/L and ≥20 ng/L), CA19-9 (<37 U/mL and ≥37 U/mL) and CEA (<5 μg/mL and ≥5 μg/mL) at baseline.]

Figures

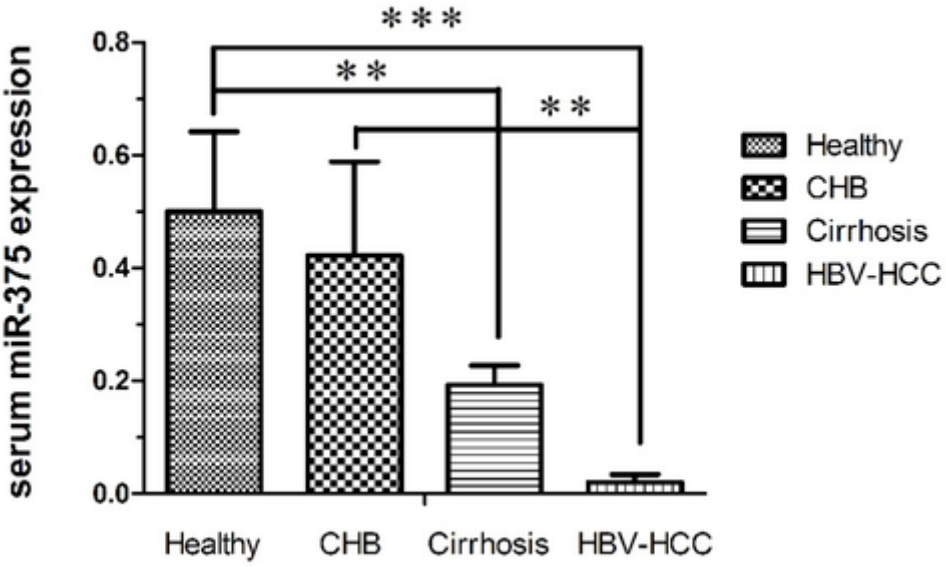

Figure 1

The differential serum levels of miR-375 in the HCC, cirrhosis, CHB and healthy control groups [The expression of serum miR-375 decreased in order from healthy controls to patients with chronic hepatitis B infection (CHB) without cirrhosis, followed by patients with CHB with cirrhosis (cirrhosis), and finally, patients with HBV-HCC. Serum miR-375 levels were significantly higher in healthy controls than in cirrhosis patients (**P<0.01). Serum miR-375 levels were significantly higher in healthy controls than in HBV-HCC patients (***P<0.001). Serum miR-375 levels were significantly higher in patients with CHB than in patients with HBV-HCC (**P<0.01).]

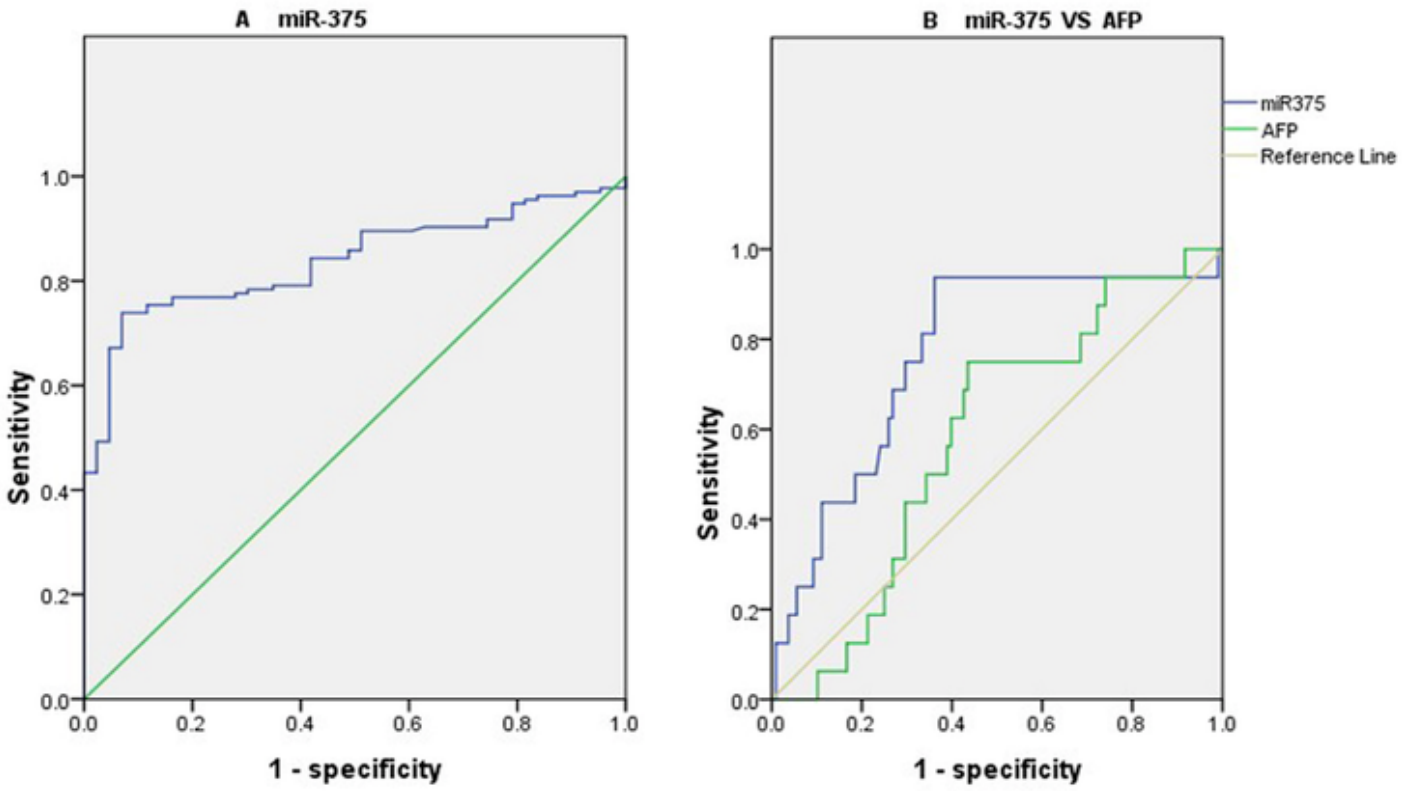

Figure 2

OC curve analysis of the diagnostic value of serum miR-375 for HCC [(A) AUC estimation for the value of serum miR-375 in identifying HCC patients within the whole cohort (AUC=0.838; sensitivity=73.9%; specificity=93.0%). (B) AUC estimation of the value of serum miR-375 in distinguishing HCC patients from HBV patients (AUC=0.768; sensitivity=93.8%; and specificity=63.9%). AUC estimation of the value of AFP in distinguishing HCC patients from HBV patients (AUC=0.584; sensitivity=75.0%; and specificity=65.5%).]

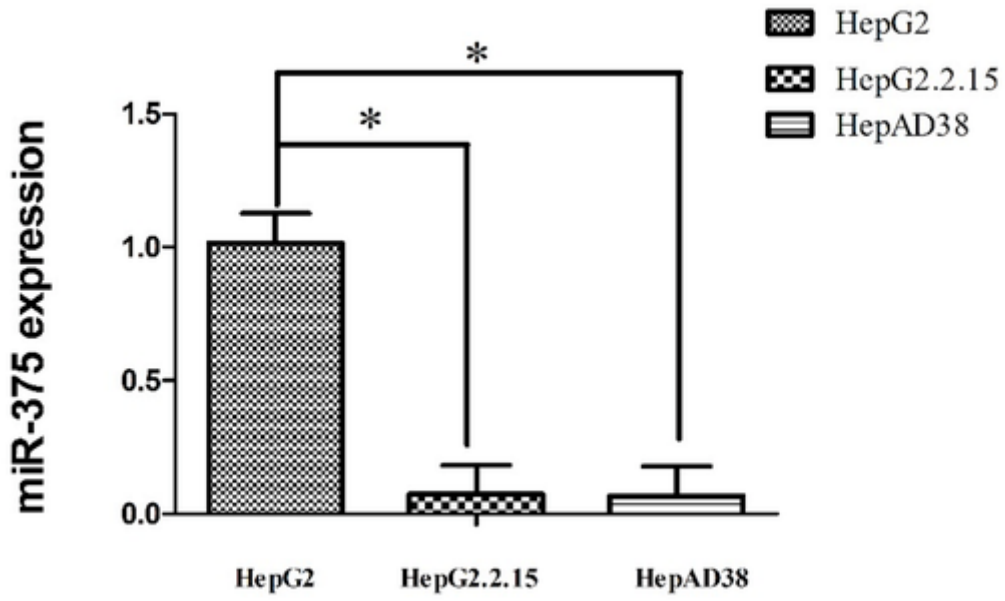

Figure 3

The expression of miR-375 in HepG2, HepG2.2.15 and HepAD38 cell lines [The expression of miR-375 was clearly lower in HepG2.2.15 cells and HepAD38 cells than in HepG2 cells, respectively.*P<0.05.]

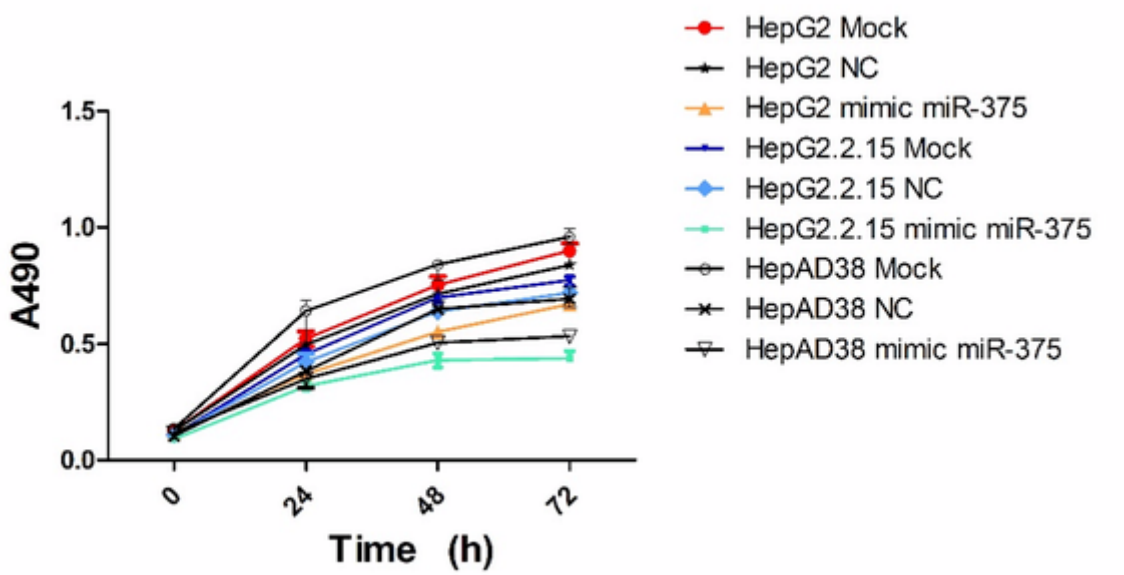

Figure 4

The effect of miR-375 on the proliferation of HCC cells [The proliferation rates of miR-375-transfected cells were lower than those of non-transfected cells (transfected vs non-transfected HepG2.2.15 cells, transfected vs non-transfected HepAD38 cells and transfected vs non-transfected HepG2 cells). The proliferation rates of HepG2.2.15 cells and HepAD38 cells were lower than those of HepG2 cells (transfected HepG2.2.15 cells vs transfected HepG2 cells, transfected HepAD38 cells vs transfected HepG2 cells and non-transfected HepG2.2.15 cells vs non-transfected HepG2 cells).]

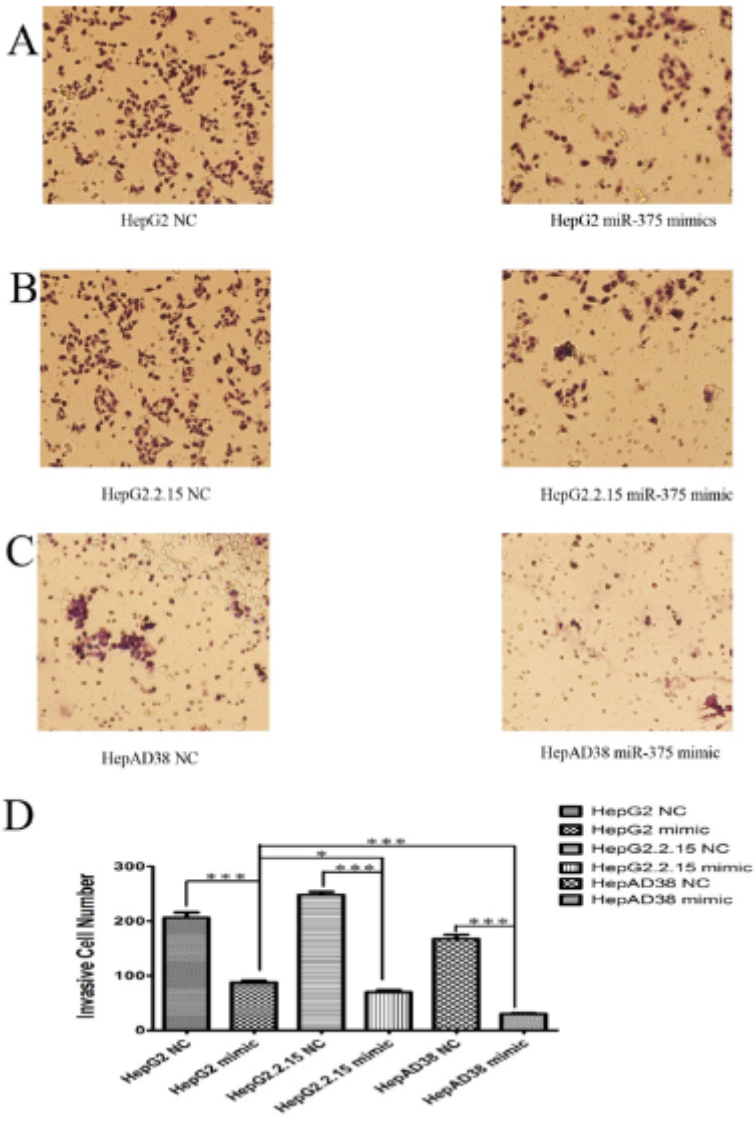

Figure 5

The effect of miR-375 on the invasion of HCC cells [After 48 h of transfection, the number of invasive HepG2 cells (A) was lower in the mimic-transfected group than in the NC-transfected group (***P<0.001). Similarly, the number of invasive HepG2.2.15 cells (B) was lower in the mimic-transfected group than in the NC-transfected group (***P<0.001). The number of invasive HepAD38 cells (C) was lower in the mimic-transfected group than in the NC-transfected group (***P<0.001). The number of invasive miR-375 mimic-transfected HepG2.2.15 cells was lower than that of invasive miR-375 mimic-transfected HepG2 cells (*P<0.05). The number of invasive miR-375 mimic-transfected HepAD38 cells was lower than that of invasive miR-375 mimic-transfected HepG2 cells(***P<0.001).]