# Urinary miR-93-5p as A Promising Biomarker for Early HBV-Related Hepatocellular Carcinoma

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**Research**

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# Abstract

**Background:** The mortality rate of early HBV-related hepatocellular carcinoma (HCC) is increasing annually due to sensitive and readily available diagnostic tools are lacking. This study aims to find such diagnostic biomarkers.

**Methods:** MiR-93-5p was chosen as a candidate biomarker base on the analysis of relevant datasets of Gene Expression Omnibus (GEO). It was subjected to validation using qPCR for the quantification of its expression levels in tissue, plasma and saliva sample sets.

**Results:** miR-93-5p was found to be significantly upregulated in HBV-related HCC tissue. Notably, miR-93-5p in plasma and urine was also significantly increased in patients with early HBV-related HCC. The expression of miR-93-5p was significantly and positively correlated between any two kinds of samples (tissue vs. plasma; tissue vs. urine, plasma vs urine). Moreover, miR-93-5p in plasma and urine reduced significantly over one month after the surgery and returned to normal levels. Finally, ROC analysis showed both plasmatic and urinary miR-39-5p could detect early, advanced and total HBV-relative HCC cases with over 85% of sensitivities and over 93% of specificities.

**Conclusion:** plasma and urine miR-93-5p show great promise as novel and potential biomarkers for early HBV-related HCC.


# Background

Liver cancer ranks the sixth most common cancer and the fourth leading cause of cancer death worldwide, with estimated 841,000 new cases and 782,000 deaths annually. According to the histological types of liver cancer, hepatocellular carcinoma (HCC) comprises 75%-85% of liver cancers [1]. The incidence rate of liver cancer continues to increase rapidly and the mortality rates rose for liver cancer by 2.7% per year in women and by 1.6% per year in men during 2011 through 2015 worldwide [2], with the incidence rate increasing most rapidly, by 2–3% annually during 2007 through 2016 and mortality rates rising over the past decade in the USA [3]. Chronic hepatitis B virus (HBV) infection causes about 55%-60% of HCC globally, and is the most important etiologic agent of HCC in Asia (with China representing half of the world's cases of HCC) and sub-Saharan Africa. Hepatectomy and liver transplantation are the only curative treatments for HCC [4]. HCC is highly malignant and lethal, with an overall 5-year survival rate of around 8.2% from the time of clinical diagnosis [1]. If HCC patients develop distal metastasis, the overall 5-year survival rate is just about 3% [5].However, curative surgery can improve the 5-year survival rate to over 70% [6]. Due to asymptomatic onset and lack of sensitive imaging modalities and biomarkers for HCC, HCC patients are still diagnosed at a stage when curative treatments are lacking. Hence, effective tools for early detection of HCC are urgently needed to help reduce its mortality.

MicroRNAs (miRNA) are non-coding RNAs with 17 to 25 nucleotides that control important cellular processes, such as proliferation, development, differentiation, and apoptosis [7]. Accumulating studies

demonstrate that aberrantly expressed miRNAs in tissue and blood can serves as biomarkers for diagnosing and monitoring various diseases. The kidneys have an extensive blood supply via the renal arteries. Urine is formed in the kidneys through a filtration of blood. The urine is then passed through urinary tract to the outside of the body. Hence, it is reasonably postulated that urinary miRNAs may have the potential to outplace circulating microRNA biomarkers in diagnosis diseases. Dozens of studies reveal that cancers of urinary tract, such as prostate, bladder etc may shed cancer cells into the urine. Some reports have suggested that a few urinary miRNAs are promising biomarkers for cancers of urinary tract. Additionally, a few studies indicate that miRNAs in urinary supernatant that without effects of exfoliated cells and debris from the urinary tract and are also potential biomarkers for non-urinary diseases, including heart diseases [8], breast, endometrial, ovarian cancer [9], gastric cancer [10]. Due to the easy and non-invasive collection of urine samples, it is highly intriguing to identify more urinary biomarkers for early detection of lethal diseases, such as HCC.

As mentioned above, HBV-related HCC is still increasing in incidence and mortality. Thus far, to the best of our knowledge, there is no study with regard to finding miRNA biomarkers in urine to detect early HBV-related HCC. This work was undertaken to identify robust biomarker in urine to help diagnose early HBV-related HCC.

## Methods

# The selection of candidate microRNA biomarkers

In order to seek candidate microRNA biomarkers from relative datasets regard to microarray-based microRNA profiles, and comparing the miRNA expression in HBV-related HCC tissue with normal liver tissue, relevant datasets from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) were collected and further assessed. The following criteria for data inclusion were used: (a) data were miRNA profiling by microarray; (b) sample size in each study had $n > 5$; and (c) the study analyzed liver tissue profiles from patients with HBV-related HCC and controls. Two datasets, GSE10694 (including 78 HBV-related HCC cancer tissue, and 10 normal liver tissues) and GSE69580 (including 5 HBV-related HCC tissues and 5 adjacent non-tumor liver tissues), met the aforementioned criteria and were chosen for subsequent analysis. GEO2R tool (http://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to identify aberrantly expressed miRNAs from the GEO datasets [11]. Candidate miRNA biomarkers were defined as upregulated with P-values < 0.05 in both datasets. Finally, miR-93-5p was selected as a candidate biomarker for HBV-related HCC.

# Participants and samples

The study was conducted mainly according to the updated Standards for Reporting of Diagnostic Accuracy (STARD) reporting guideline for diagnostic biomarker studies [12]. Samples from all participants were collected if they met the inclusion criterion mentioned below between July 2014 and July 2020 at The Fifth People's Hospital of Ganzhou and The First Affiliated Hospital of Gannan Medical University, Ganzhou, China. The 65 healthy controls were defined as the participants with normal results

by health examinations, including chest X-rays, routine blood and urine tests, abdominal ultrasounds, faecal occult-blood testing, blood cancer biomarker assays (AFP, CEA, CA19–9), HBV antigen, HCV, HIV, and syphilis antibodies. Normal human liver tissues were obtained from distal normal liver tissues of liver hemangioma. HBV-related HCC tissues from 64 early HBV-related HCC patients and normal liver tissues were obtained. Some parts of tissue was snap-frozen in liquid nitrogen within five minutes after surgical removal. Other parts of tissue were preserved by fixing them in formalin for pathological examinations. HCC and normal liver tissues were determined by pathology after hepatectomy. 66 advance HCC patients (Stage IV) was diagnosed based on biopsy of the tumor or CT/MRI. All participants were enrolled without diagnosed concurrent urinary, infectious, autoimmune diseases and diabetes mellitus. All HCC patients were positive with HBsAg and without exposure to other well-recognized causes of HCC, i.e. Hepatitis C virus (HCV), alcohol, aflatoxin, fatty liver, chronic biliary disease, genetic and metabolic liver diseases [13]. HBV- related HCC patients with a diagnosis of concurrent other cancers and those receiving chemotherapy and radiotherapy prior to sampling were also excluded. Plasmatic and urinary samples was obtained from all of 64 patients with early HBV-related HCC over 1 week before and over 1 month after curative surgery. Whole blood was collected into commercially available anticoagulant-treated tubes. Blood Cells are removed from plasma by centrifugation for 10 minutes at 2,000 x g using a refrigerated centrifuge. The plasma was transferred into microcentrifuge tubes followed by a second centrifugation at 12,0006 x g for 10 min at 4℃ to completely remove cellular components and debris as final plasma samples. Up to 5 mL of urine from each participants were collected in a 50-mL centrifuge tube. In order to eliminate the miRNA expression influences of exfoliated cells and debris from the urinary tract, the urine samples were centrifuged at 3,000 x g for 15 min at 4℃ to spin down exfoliated cells and debris, and the supernatant was transferred into microcentrifuge tubes followed by a second centrifugation at 12,000 x g for 10 min at 4℃ to completely remove cellular components as urinary supernatant samples. All samples were stored in the − 80°C lab freezers until use.

Ethics committees from our hospitals approved the study protocol. All participants provided written informed consent for their information and samples to be stored in the hospital database and used for research.

# MiRNAs isolation and quantification

The following procedures were finished with seven days after sampling. Total RNAs were isolated from frozen liver tissues using TRIzol (Thermo Fisher Scientific, USA), and total RNAs in 1mL of plasma or urine were extracted by the mirVana PARIS Kit (Thermo Fisher Scientific, USA) following manufacturer's protocols. The measurements of miRNAs by quantitative polymerase chain reaction (qPCR) were carried out as previously described [14] Each qPCR reaction contained negative controls included no template control, no reverse transcriptase control, and no amplification control. All reactions including controls were perform in triplicate. U6 snRNA is the most frequently used reference gene for miRNA RT-qPCR expression analysis. Several studies indicate that U6 snRNA may be a reliable housekeeping gene for normalization of miRNA RT-qPCR expression analysis for tissue [15], plasma [16] and urine [17]. Hence,

the expression levels of miR-93-5p in tissue, plasma and urine were normalized to corresponding expression levels of U6 snRNA. All expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [18].

## Statistical Analysis

MiR-93-5p expression levels were compared using the Mann Whitney U test or Kruskal-Wallis H test. The differences of the miR-93-5p before and after surgery were analyzed by Wilcoxon signed-rank test. Receiver operating characteristic (ROC) curves were used to evaluate the diagnostic performance of miR-93-5p for differentiating between two groups. The correlation between two groups was analyzed using the Spearman's correlation test. The statistical analyses above were performed using the SPSS software (ver. 13.0). The differences of discriminatory powers between plasmatic and urinary miR-93-5p were compared with the method of Delong using the MedCalc (version 19.4.1) [19, 29]. A two-tailed P value < 0.05 was considered statistically significant.

# Results

## Patient Characteristics

The characteristics of the enrolled participants were presented in Table 1. The patients with HBV-related HCC were divided into two groups: Patients' Group 1 (64 cases) was staged Ia, Ib, IIa with good liver function reserve and eligible for curative hepatectomy according to the Chinese guideline; Patients' Group 2 (66 cases) was staged IV with contraindication to hepatectomy and the presence of distant metastases. Cancer staging is based on China's medical systems [11]. There was no significant difference in the distribution of age and sex among the three groups (Healthy controls, early HBV-related HCC, and advanced HBV-related HCC). Serum alpha feto-protein (AFP) level was well balanced between the two patients' groups. 15.6% of early HBV-related HCC had high serum levels of AFP (> 400 ng/mL), and serum AFP concentration of > 400 ng/mL was seen in 16.7% of advanced HBV-related HCC. And over half of patients from the two groups of patients showed normal AFP levels (< 20 ng/mL) with the proportion of 59.4% and 53.0%, respectively. Totally, 56.2% of HBV-related HCC patients had normal AFP concentration regardless of the tumor resectability.

Table 1
The brief characteristics of all enrolled participants.

| | Healthy controls | Early HCC | Advanced HCC | Total HCC |
|---|---|---|---|---|
| Variable | | | | |
| Age,years | | | | |
| Mean | 54 | 55 | 58 | 56 |
| SD | 13 | 15 | 15 | 15 |
| Sex(*n*) | | | | |
| Female | 18 | 19 | 21 | 40 |
| Male | 47 | 45 | 45 | 90 |
| Serum AFP(*n*) | | | | |
| < 20 ng/mL | 65 | 38 | 35 | 73 |
| = 20–400 ng/mL | 0 | 16 | 20 | 36 |
| > 400 ng/mL | 0 | 10 | 11 | 21 |

# Discriminatory Expression of the candidate microRNA biomarker in tissue, plasma and urine

In the course of seeking candidate microRNA biomarkers, datasets of microarray-based miRNA profiles related to comparing the miRNA expression in HBV-related HCC tissue with normal liver tissue were searched, downloaded from the Gene Expression Omnibus (GEO) and analyzed. Detailed selection methods were described in the following Section of Materials and methods. Accordingly, two miRNA profiles met the criteria. The miRNA profile of GSE69580 was assessed in five HBV-related HCC tissues, and five normal liver tissues. While GSE10694 revealed the profile HBV-related HCC tissues, and 10 normal liver tissues. MiR-93-5p was upregulated significantly in HCC tissues of both datasets. Accordingly, it is chosen as a candidate microRNA biomarker for HBV-related HCC.

First, the expression levels of miR-93-5p were quantified in 64 tissues of HBV-related HCC and 13 normal liver tissues. MiR-93-5p was significantly elevated 4.0 times in HCC tissues ($P < 0.001$). Moreover, miR-93-5p levels in plasma and urine were also measured three groups of participants, including 65 healthy controls, 64 patients with early HBV-related HCC and 66 patients with patients with advanced HBV-related HCC. The results showed that compared with healthy controls, plasma miR-93-5p were significantly overexpressed 2.8 times in patients with early HBV-related HCC and 2.9 times in patients with patients with advanced HBV-related HCC ($p < 0.001$). But the plasma levels of miR-93-5p had no significant difference between the two groups of patients regardless of the HCC resectability (P = 0.976). Plasmatic

miR-93-5p in all HBV-related patients were also significantly elevated 2.8 times compared with normal controls. Finally, urinary miR-93-5p concentration was also tested in 65 healthy controls, 64 patients with early HBV-related HCC and 66 patients with patients with advanced HBV-related HCC. The results displayed that compared with healthy controls, urinary miR-93-5p significantly increased 3.7 times in patients with early HBV-related HCC and 3.6 times in patients with advanced HBV-related HCC ($p < 0.001$). But the two groups of patients exhibited statistically similar miR-93-5p levels in urine (P = 0.963). In addition, regardless of the cancer resectability, urine miR-93-5p in all HBV-related patients also significantly increased 3.6 times than healthy controls (Fig. 1).

# The miR-93-5p expression correlation between any two kinds of samples of tissue, plasma and urine

64 cancer tissue, plasma and urine samples were collected from the same patients with early HBV-related HCC. The correlation between any two groups of samples was analyzed using the Spearman's correlation test. The results indicated that significantly positive correlation of miR-93-5p levels was observed between tissue and plasma, plasma and urine, tissue and urine ($p < 0.001$) with coefficients of 0.550, 0.431, 0.481, respectively (Fig. 2).

# The levels of plasma and urine miR-93-5p changed after curative hepatectomy

In order to investigate whether plasma and urine miR-93-5p derived from HCC, 64 paired plasma and 64 urine samples were serially collected from the same group of patients with early HBV-relate HCC one month after primary curative hepatectomy. CT or/and MRI did not indicate cancer recurrence in them after one month of the surgery. The results showed that miR-93-5p in plasma and urine was significant reduced 4 times after the surgery (P < 0.001). Meanwhile, the expression levels of plasma and urine miR-93-5p after surgery were compared with the miR-93-5p levels in plasma and urine of healthy controls. The results reveal that both plasma and urine miR-93-5p levels had no significant differences between post-operational HCC and healthy controls (P = 0.264, 0.207, respectively) (Fig. 3).

# The diagnostic accuracy of plasma and urine miR-93-5p for early HBV-relate HCC

Receiver Operating Characteristic (ROC) curves were constructed and used to evaluate the diagnostic power of plasma and urine miR-93-5p for detecting early HBV-relate HCC. Accordingly, plasma miR-93-5p differentiated early HBV-relate HCC from healthy control with sensitivity of 85.9%, and specificity of 95.4%. And, urine miR-93-5p showed 87.5%, and specificity of 97.4% in the detection of early HBV-relate HCC (Fig. 4). There is no significant difference between plasma and urine miR-93-5p for detecting early HBV-relate HCC ($P = 0.7458$), advanced HCC ($P = 0.4953$), and total HCC cases ($P = 0.7629$) according to the Medcal software to compare the two diagnostic performances.

# Discussion

To the best our knowledge, this is the first literature that reports plasma and urine miRNA can serve desirable biomarkers for detecting early HBV-relative HCC. miR-93-5p was found to be significantly upregulated in cancer tissue of HBV-relative HCC in agreement with the two datasets of GSE69580 and GSE10694 from GEO. Notably, miR-93-5p in plasma and urine was also significantly increased in patients with early, advanced and total HBV-relative HCC cases. The expression of miR-93-5p was significantly and positively correlated between any two kinds of samples (tissue vs. plasma; tissue vs. urine, plasma vs. urine). Moreover, miR-93-5p in plasma and urine reduced significantly after over one month of the surgery and returned to normal levels. Take into consideration the results mentioned above, miR-93-5p was probably derived from HCC, enter the circulation, and secreted into urine at the end. Finally, ROC analysis showed both plasmatic and urinary miR-93-5p could serve as a novel biomarker for early HBV-relative HCC with desirable diagnostic accuracy. And their discriminatory power was similar.

Despite major efforts and advancing science and technology, a large proportion of HCC patients without specific symptoms are still diagnosed at advanced stage when curative treatments are lacking. Diagnosis of HCC usually depends on imaging abdominal ultrasonography, imagnetic resonance imaging (MRI), and contrast-enhanced computed tomography (CT) and serum AFP. But MIR and CT are costly and not easily available in developing countries. Ultrasonography can detect large tumor but fails to detect small lesions, and because this procedure is operator-dependent, the diagnostic performance varies. AFP is the most frequently used biomarkers for HCC. However, the accuracy of AFP is not desirable with sensitivity ranged 46–59% and specificity ranged 87–93%. HCC at early stage are often missed by AFP analysis, and serum AFP levels are also overexpressed in patients with benign liver diseases, such as hepatitis and cirrhosis [12]. In this study, serum AFP still showed low sensitive in detecting HCC. Around 30% of early and advanced HCC showed high AFP levels (> 400 ng/mL). Accordingly, Asia–Pacific clinical practice guidelines [13] and American Association for the Study of Liver Diseases practice guidelines [14] do not recommend AFP as a diagnostic test for HCC. Hence, robust biomarkers are vital for early detection and diagnosis of HCC. In particular, biomarkers for early HCC is low-cost, non-invasive, and readily available.

Numerous microRNAs in tissue and circulation are demonstrated to be biomarkers with good sensitivities and specificities for various disease. Dozens of studies prove that microRNAs in tissue [15], circulation [16, 17], and several body fluids including urine [18] are highly stable and readily detected. They can resist degradation at high and low temperatures, in strong acids and bases, and by RNase digest. Hence any one of microRNAs can be readily detected in any kinds of samples.

The mechanism of how microRNAs exist in urine remains elusive. It is postulated that miRNAs are secreted into the blood both passively after cell apoptosis and necrosis due to tissue damage, and actively through microvesicles [19]. MicroRNAs in the blood enter and pass through the glomerulus and nephrons of kidneys, and then travel via urinary tract to the outside of the body in urine. 1,000–2,000mL of urine are normally produced every day in a human based on fluid intake and kidney function.

Both plasmatic and urinary miR-93-5p showed good diagnostic values outperforming AFP for detecting early HBV-related HCC. And miR-93-5p in both kinds of samples exhibited similar diagnostic performance. In addition, urine sampling is non-invasive, and readily performed in a human, while it is relatively expensive, invasive, and cause discomfort or even bloodborne diseases to individuals by blood drawing. Hence urinary miR-93-5p can outplace plasmatic miR-93-5p to server as a more promising biomarker in the help of diagnosing early HBV-related HCC.

Although the functional experiments of miR-93-5p were not performed in our study, some studies have found miR-93-5p increases significantly compared with adjacent non-caner tissue and act as an oncomir. Consistent with our study, Ohta et al. finds that miR-93-5p expression is enhanced in HCC tissue. And it increases proliferation, migration and invasion of HCC cells via activating c-Met/PI3K/Akt pathway activity [20]. Ji et al. finds miR-93 is significantly upregulated in HCC tissues, enhances HCC invasion and metastasis by EMT through targeting PDCD4 [21]. Work carried out by Xue et al. showed that Exosomal miR-93-5p inhanced proliferation and invasion in HCC via directly inhibiting TIMP2/TP53INP1/CDKN1A [22]. Shi et al. identified that miR-93-5p was overexpressed in HCC specimens and cell lines, leads to poor outcomes in HCC patients, and promotes proliferation, migration via a microRNA-93-5p/MAP3K2/c-Jun positive feedback circuit [23]. In contrast, downregulation of miR-93-5p expression results in reducing cell proliferation, migration, and clonogenicity of HCC cells [24]. Taken together, because miRNAs act as key molecule in HCC development, it provides the rationale for its use as also promising target for new HCC therapies.

A few limitations exist in this study. First, miRNA microarray was not conducted in this work. So other potential miRNA biomarkers may not be found. Second, the low number of patient samples of HBV-related HCC represents a limitation in interpreting our results and evaluating the methods used. But we believe that the study power found in this work will be largely increased when more HCC cases are enrolled. Third, the patients were limited to a Chinese Han population. The ability of miR-93-5p to detect early HCC not associated with HBV and in multi-center worldwide with other ethnicities merits investigation.

# Conclusions

In summary, this work demonstrated that plasmatic and urinary miR-93-5p have significant diagnostic values for early HBV-related HCC diagnosis with good sensitivity and specificity. Due to simple, non-invasive, and readily sampling, urinary miR-93-5p has high potential as a promising and robust biomarker for detecting early HCC. This study provides new evidence and prospect for the early detection of diseases using a noninvasive screening method of urine sampling, and urinary microRNAs can be non-invasive biomarkers for facilitating the diagnosis and prognosis of various human diseases.

# Declarations

### Acknowledgments

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### Authors' contributions

Conceptualization, GZ and QZ; Data curation, YZ, YL,SG and LB; Formal analysis, GZ and YJ; Funding acquisition, GZ and QZ; Methodology, QZ; Writing – original draft, YZ, YL and SG; Writing –review & editing, GZ and QZ. All authors read and approved the final manuscript.

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### Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research ethics committee of The Fifth People's Hospital of Ganzhou and The First Affiliated Hospital of Gannan Medical University, Ganzhou, China, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

### Consent for publication

Not applicable.

### Competing interests

All authors declare no conflicts of interest.

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# Figures

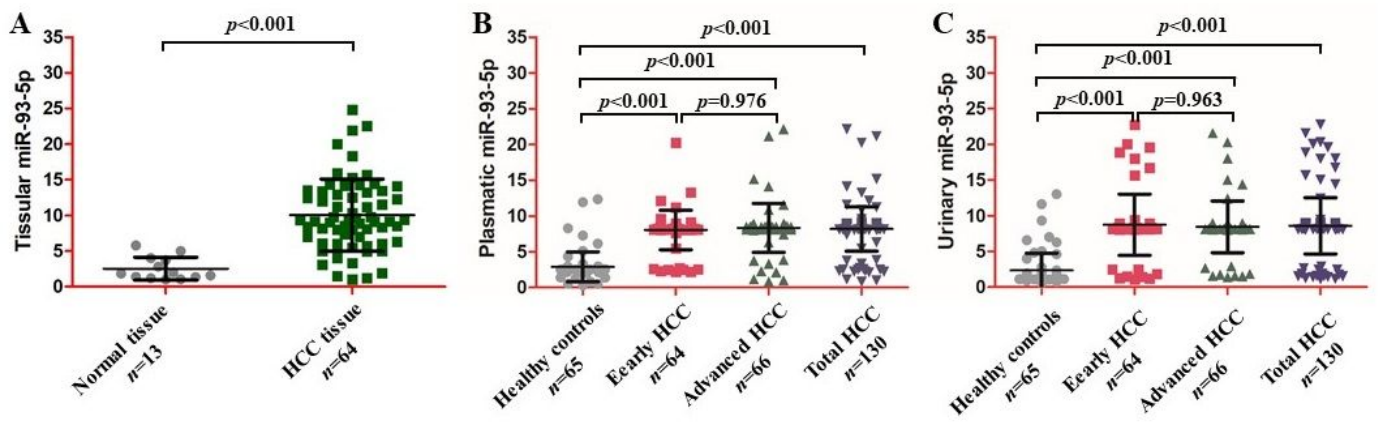



**Figure 1**

The aberrant expression of miR-93-5p in HCC tissue, plasma and urine of HBV-related HCC patients. Compared with normal liver tissue, miR-93-5p was significantly upregulated in HBV-related HCC tissue (A). Compared with healthy controls, plasma (B) and (C) urine miR-93-5p was significantly overexpressed in early, advanced and total HCC patients. And plasma and urine miR-93-5p showed no significant difference between early and advanced HCC patients.

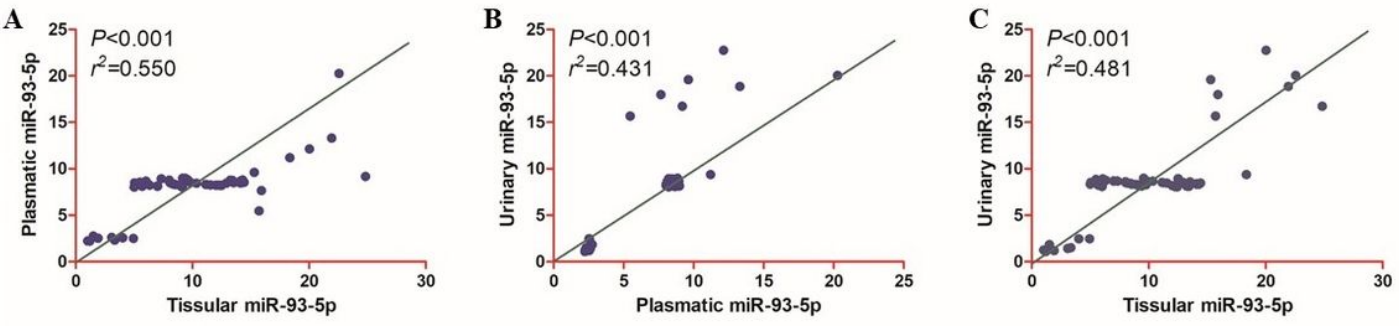
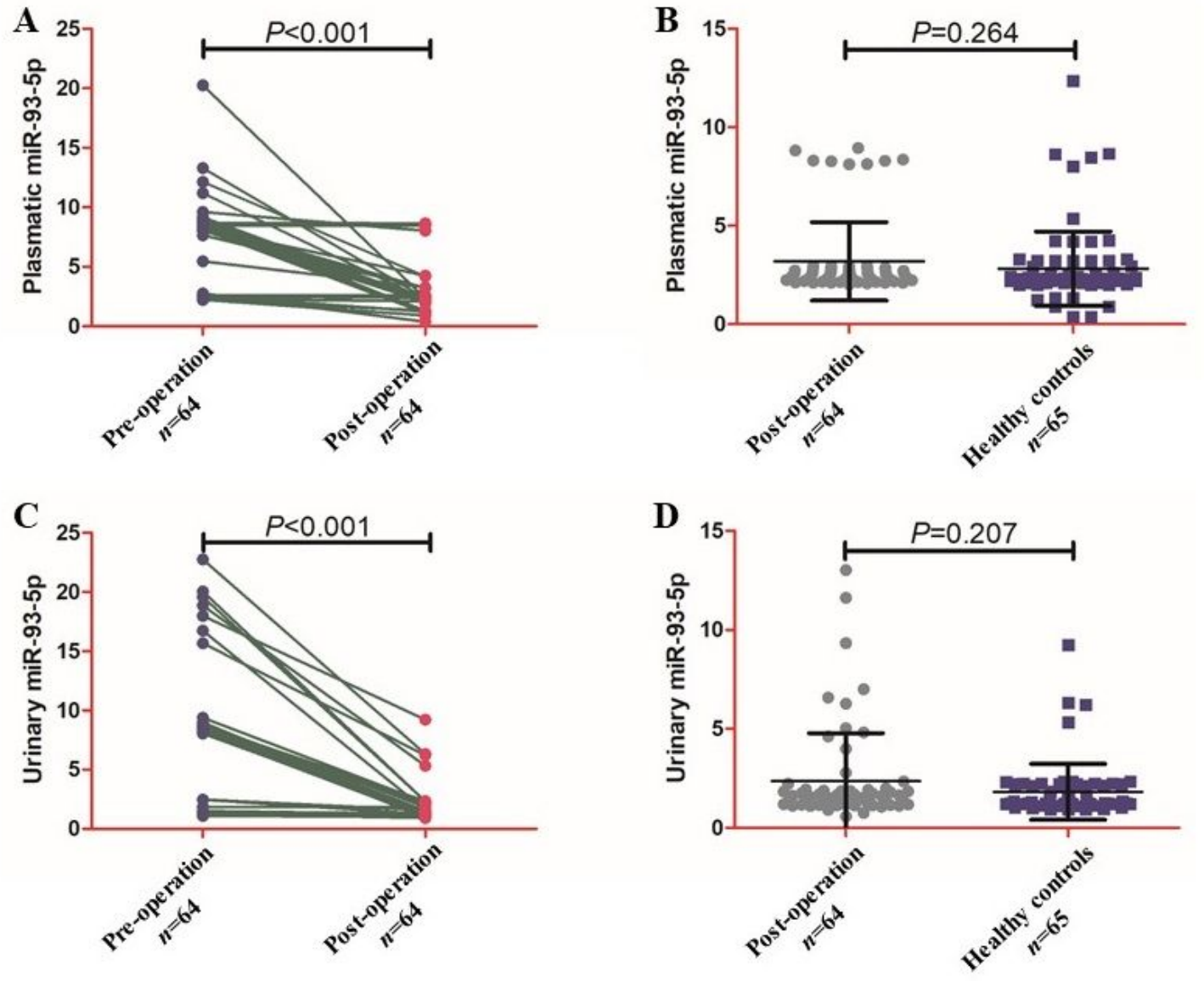


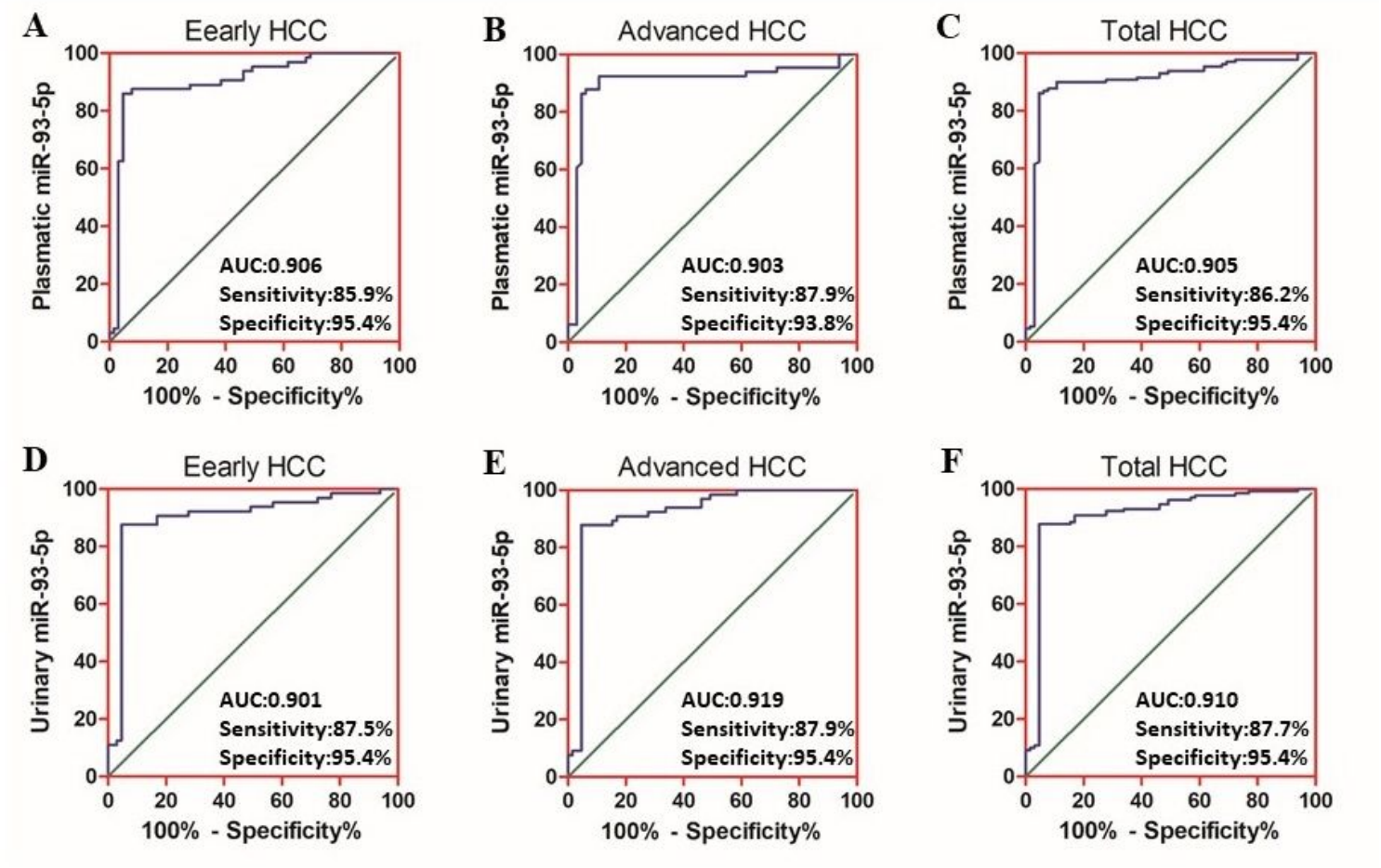

**Figure 2**

The miR-93-5p expression correlation between any two kinds of samples of tissue, plasma and urine. The expression of tissular miR-93-5p was significantly and positively correlated with plasmatic miR-93-5p (A). The levels of plasmatic miR-93-5p showed significantly positive correlation with urinary miR-93-5p (B). The concentration of tissular miR-93-5p significantly and positively correlated with urinary miR-93-5p (C).




## Figure 3

The expression changes of plasmatic and urinary miR-93-5p after curative hepatectomy. Over one month after the curative hepatectomy, plasmatic (A) and urinary (C) miR-93-5p were significantly reduced. And the post-operational levels of plasmatic (B) and urinary (D) miR-93-5p showed no significantly differences with healthy controls.




## Figure 4

The ROC curves of plasmatic and urinary miR-93-5p for detecting HBV-related HCC. Plasmatic miR-93-5p showed 85.9% of sensitivity and specificity of 95.4% with the area under the curve (AUC) of 0.906 for detecting early HBV-related HCC (A). It had 87.9% of sensitivity and 93.8% of specificity with AUC of 0.903 in the detection of apced HCC (B). It exhibited 86.2% of sensitivity and 95.4% of specificity with AUC of 0.905 in the help of diagnosis of total HCC cases (C). Urinary miR-93-5p showed 87.5%, 87.9%, 87.7% of sensitivities, and all of 95.4% of specificities for detecting early HBV-related HCC with AUC of 0.901(D), advanced HCC with AUC of 0.919 (E), and total HCC cases with 0.910 (F).