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Expression of the microRNA-143/145 cluster is decreased in hepatitis B virus-associated hepatocellular carcinoma and may serve as a biomarker for tumorigenesis in patients with chronic hepatitis B

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Abstract. The aims of the present study were to identify the expression profile of microRNA (miR)-143/145 in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC), explore its association with prognosis and investigate whether the serum miR-143/145 expression levels may serve as a diagnostic indicator of HBV-associated HCC. The microRNA (miRNA) chromatin immunoprecipitation dataset was obtained from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus databases, and analyzed using the Wilcoxon signed-rank test. It was observed that the expression of miR-143 and miR-145 was decreased 1.5-fold in HBV-associated HCC samples compared with non-tumor tissue in the TCGA and the GSE22058 datasets ($P<0.01$). Using the reverse transcription-quantitative polymerase chain reaction, it was further confirmed that miR-143/145 and their host gene MIR143HG were downregulated in HBV-associated HCC tissues compared with corresponding distal non-tumor tissues. The lower level of miR-143 and miR-145 expression was associated with tumor differentiation, and may thus be responsible for a poor prognosis of patients with HBV-associated HCC. The receiver-operating characteristic (ROC) curves were used to explore the potential value of miR-143 and miR-145 as biomarkers for predicting HBV-associated HCC tumorigenesis. In serum, miR-143/145 were identified to be significantly decreased in patients with HBV-associated HCC compared with negative control patients,

and their associated areas under the ROC curves were calculated at 0.813 and 0.852 ($P<0.05$), with each having a sensitivity and a specificity close to 0.80. These results indicated that the decreased expression of the miR-143/145 cluster and their host gene MIR143HG in HBV-associated HCC tissue was associated with prognosis, and each of these miRNAs may serve as a valuable diagnostic biomarker for predicting HBV-associated HCC tumorigenesis.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer mortality worldwide. A variety of risk factors may promote HCC genesis, including hepatitis B virus (HBV) infection, hepatitis C virus infection, heavy alcohol consumption and non-alcoholic fatty liver disease (1,2). In the last decade, a marked increase in the incidence of HBV-associated HCC has been observed, particularly in China. Since the prognosis of patients with HCC is markedly dependent on the stage of the disease, strategies for early detection of HCC have been investigated.

MicroRNAs (miRNAs) are short 20-22-nucleotide single-stranded RNA molecules, which have been associated with epigenetic regulation in a range of diseases, including tumorigenesis (3-5). miRNAs regulate gene expression by altering the stability or the translational efficiency of target mRNAs. A previous study demonstrated that miRNAs are detectable and stable in serum (6); therefore, research has focused on the possibility of using miRNAs as biomarkers for predicting the diagnosis and prognosis of several diseases.

Two human miRNAs, miRNA-143 (miR-143) and miR-145 have been investigated as biomarkers for several types of cancer. miR-143 and miR-145 are stably expressed homologous miRNAs located within the same host gene, MIR143HG (7). The first study to examine the miR-143/145 cluster focused on HBV-associated HCC. This study demonstrated that miR-143 expression was increased in HBV-associated HCC tumors and

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was associated with invasive and metastatic behavior of liver tumor cells (8). Conversely, evaluation of the miR-143/145 cluster expression in other types of tumor, including colonic carcinoma, pulmonary carcinoma, esophageal carcinoma and prostatic carcinoma (9-12), demonstrated that the miR-143/145 cluster expression level was decreased. This raises the question of why the miR-143/145 cluster is overexpressed in HBV-associated HCC-derived tumors, but is underexpressed in other types of tumor. However, previous studies have produced contradictory results (13-15), demonstrating that the miR-143/145 expression level was decreased in HBV-associated HCC-derived tumors which contradicts the results of another previously mentioned study (8). Furthermore, a previous study demonstrated that the expression of the miR-143/145 cluster was negligible in liver tissues, including normal liver tissue or HBV-associated HCC tissue (7). This controversy was investigated further in the present study.

In the present study, the expression profile of miR-143 and miR-145 in HBV-associated HCC tissues and non-tumor tissues was investigated using chromatin immunoprecipitation (ChIP) data from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) datasets. Their expression in HBV-associated HCC tissue was also validated using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The association between miR-143/145 expression and specific clinical features including tumorigenesis, tumor progression and prognosis was subsequently examined. Finally, the serum expression of miR-143 and miR-145 was determined to evaluate their potential clinical applications. The receiver-operating characteristic (ROC) curves were used to explore their potential value as biomarkers for predicting HBV-associated HCC tumorigenesis.

Materials and methods

TCGA miRNA dataset. The miRNA expression profile of 372 patients with HCC and 50 non-tumor distal liver samples was obtained from the TCGA dataset (cancergenome.nih.gov). According to the clinical information, 102 patients with HBV infection were selected as negative control.

GEO dataset. The GEO database (16) was accessed to obtain the normalized miRNA microarray dataset GSE22058 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22058), which represents the miRNA expression profile of tumor and adjacent non-tumor tissues of 96 patients with HCC. From these patients, ~88% were confirmed positive for the HBV antigen (17).

Identification of differentially expressed miRNAs. For each dataset, the Wilcoxon signed-rank test was used to identify the differentially expressed genes between tumor and non-tumor samples. The null hypothesis was that a gene was not differentially expressed between the two groups and rejection of this hypothesis indicated that the gene was differentially expressed. A miRNA was considered upregulated if $P < 0.01$ and its fold increase was ≥ 1.5 . Downregulated miRNAs were similarly defined.

Clinical samples. HBV-associated HCC tissues, matched distal non-cancerous liver tissues and 5 ml venous peripheral blood

were obtained from 85 patients with HBV-associated HCC from February 2012 to January 2013 at the Provincial Hospital Affiliated to Shandong University (Jinan, China). The mean patient age was 53.2 ± 9.3 years (range, 25-75 years), and 70.6% of the patients were male. Ten tissue samples from patients with hepatic hemangioma and 5 ml venous peripheral blood from 50 patients with chronic hepatitis B were collected as negative controls. All tissue samples were histologically confirmed by a pathologist using hematoxylin and eosin staining of cryosectioned specimens. The criteria used for distal non-cancerous liver tissues were absence of tumor cells and 5 cm distance from the tumors. Blood samples were collected in Vacutainer® serum separating tubes (BD Biosciences, Franklin Lakes, NJ, USA). Serum was collected by centrifugation at $2,000 \times g$ for 10 min at 4°C after allowing the blood to clot for 30 min. Written informed consent was obtained from each patient, and the consent procedure and study protocol were approved by the Medical Institutional Ethical Committee of the Provincial Hospital Affiliated to Shandong University.

Clinical features. Clinical features including histological grade, metastasis, cirrhosis, and complete tumor capsule were reported by pathologists following post-surgical pathological examination of HBV-associated HCC samples.

miRNA/mRNA extraction and RT-qPCR. The mRNAiso Plus and microRNAiso Plus kits (Takara Biotechnology Co., Ltd., Dalian, China) were used for mRNA and miRNA extraction from liver tissue, respectively. The MiRCURY RNA kit (Exiqon A/S, Vedbaek, Denmark), a specific kit for extracting microRNA from the serum, was used to extract miRNA from serum samples in order to guarantee the validation of the concentration of microRNAs. The PrimeScript™ RT Reagent kit and SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) were used for first-strand cDNA synthesis and RT-qPCR, respectively, according to the manufacturer's protocols. β -actin was used for mRNA normalization. β -actin forward, 3'-GGCACCACACCTTCTACAATG-5' and reverse, 3'-TAGCACAGCCTGGATAGCAAC-5'. RT-qPCR for microRNAs was performed on a 480 PCR system (Roche Diagnostics, Basel, Switzerland), at 95°C for 1 min followed by 45 cycles of 95°C for 5 sec, 65°C for 30 sec and 72°C for 30 sec. The expression of miRNAs in serum samples was calculated using the comparison $2^{-\Delta\Delta C_q}$ method (18) relative to RNU6B (U6). The following specific forward and reverse primers were used in pairs when amplifying miRNA: miR-143 forward, 3'-GTG CAGTGCTGCATCTCTGGT-5'; 143HG forward, 3'-GTG AAGGCAGAGGACACACCT-5' and reverse, 3'-AAAACC GTGCATTTGGCTG-5'. The forward primers for miR-145 and U6 and the reverse primers for all microRNAs were purchased as finished products from Takara Biotechnology Co., Ltd. (cat. nos. SJD14025 and SJD14078). PCR product sizes were validated by electrophoresis using 2% agarose and 12% polyacrylamide, 8 M urea gel (19). All kits were used according to the manufacturer's protocol. All the experiments were performed >3 times generating similar results and error bars represent the standard error of the mean of 6 repeats.

Statistical analysis. SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA) was used to analyze the data. Comparisons

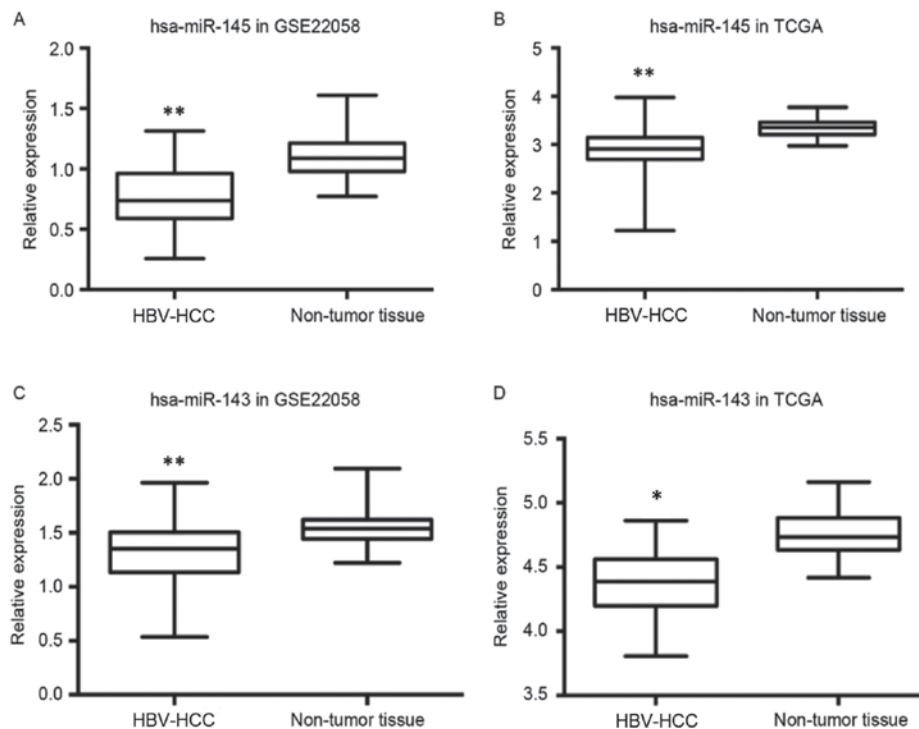


Figure 1. hsa-143/145 expression analysis using miRNA ChIP data from the GEO and the TCGA databases. (A) The expression of miR-145 was decreased in the HBV-associated HCC samples (n=96, 88% positive for HBV antigen) compared with the matched adjacent non-tumor tissues (n=96) in the GSE22058 database (**P<0.001, Wilcoxon signed-rank test). (B) The expression of miR-145 was significantly decreased in the HBV-associated HCC samples (n=372) compared with the non-tumor liver samples (n=50) in the TCGA dataset (**P<0.001; Wilcoxon signed-rank test). (C) The expression of miR-143 was decreased in the HBV-associated HCC samples (n=96, 88% positive for HBV antigen) compared with the matched adjacent non-tumor tissues (n=96) in the GSE22058 database (**P<0.001, Wilcoxon signed-rank test). (D) The expression of miR-143 was decreased in the HBV-associated HCC samples (n=102) compared with the non-tumor liver samples (n=50) in the TCGA dataset (P=0.004, Wilcoxon signed-rank test). *P<0.01. hsa, *Homo sapiens*; miRNA/miR, microRNA; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas; ChIP, chromatin immunoprecipitation; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

between matched HBV-associated HCC tissue and non-tumor tissue was performed by paired Student's t-test and Wilcoxon signed-rank test. Unmatched continuous data were compared using an independent two-tailed t-test. The association between the expression of miR-143, miR-145 and MIR143HG is analyzed by Pearson correlation in HBV-associated HCC tissues. The diagnostic value of serum miR-143/145 expression was assessed by the area under the ROC curves (AUC). The diagnostic accuracy was assessed by sensitivity, specificity, positive predictive value and negative predictive value. P<0.05 was considered to indicate a statistically significant difference.

Results

miRNA expression profile analysis in patients with HBV-associated HCC reveals that miR-143 and miR-145 are among the 63 differentially expressed miRNAs. The HBV-associated HCC and non-tumor tissue miRNA ChIP data were obtained from the TCGA and the GEO datasets (16,17). It was identified that in the HBV-associated HCC samples, 24 miRNAs were increased >1.5-fold (P<0.01) and 39 miRNAs were decreased >1.5-fold compared with the matched adjacent non-tumor samples. miR-143 and miR-145 were among the 39 significantly decreased miRNAs in the HBV-associated HCC samples (Fig. 1). The expression of miR-145 was significantly downregulated in the HBV-associated HCC samples compared with the non-tumor samples from the GSE22058 (P<0.001) and the TCGA datasets (P<0.001). Furthermore, the

expression of miR-143 was significantly downregulated in the HBV-associated HCC samples compared with the non-tumor samples from the GSE22058 (P<0.001) and the TCGA datasets (P=0.004).

miR-143 and miR-145 are downregulated in HBV-associated HCC samples and their expression pattern is consistent with the expression pattern of the host gene, MIR143HG. To validate the expression profiles of miR-143 and miR-145 and investigate the association with their host gene MIR143HG (Fig. 2A), the expression of the miRNAs and the host gene MIR143HG long non-coding RNA (lncRNA) were examined using RT-qPCR in HCC and normal tissues. As presented in Fig. 2B, miR-143 expression was significantly decreased in HCC tissue compared with matched distal non-cancerous liver tissue (NT) (HCC=0.075 vs. NT=0.205; P<0.05, Student's t-test). Furthermore, miR-145 expression was significantly decreased in HCC tissue when compared with NT (HCC=0.055 vs. NT=0.232; P<0.01, Student's t-test). No significant differences in miR-143 and miR-145 expression were identified between NT and negative control samples (NC). These results suggest that decreased miR-143 and miR-145 expression may be an indicator of HBV-associated HCC tumorigenesis.

To investigate the association between miR-143, miR-145 and their host gene MIR143HG, MIR143HG expression was quantitatively assessed. As presented in Fig. 2B, MIR143HG expression was decreased in HCC tissue compared with NT (HCC=0.43). Linear regression analysis indicated that the

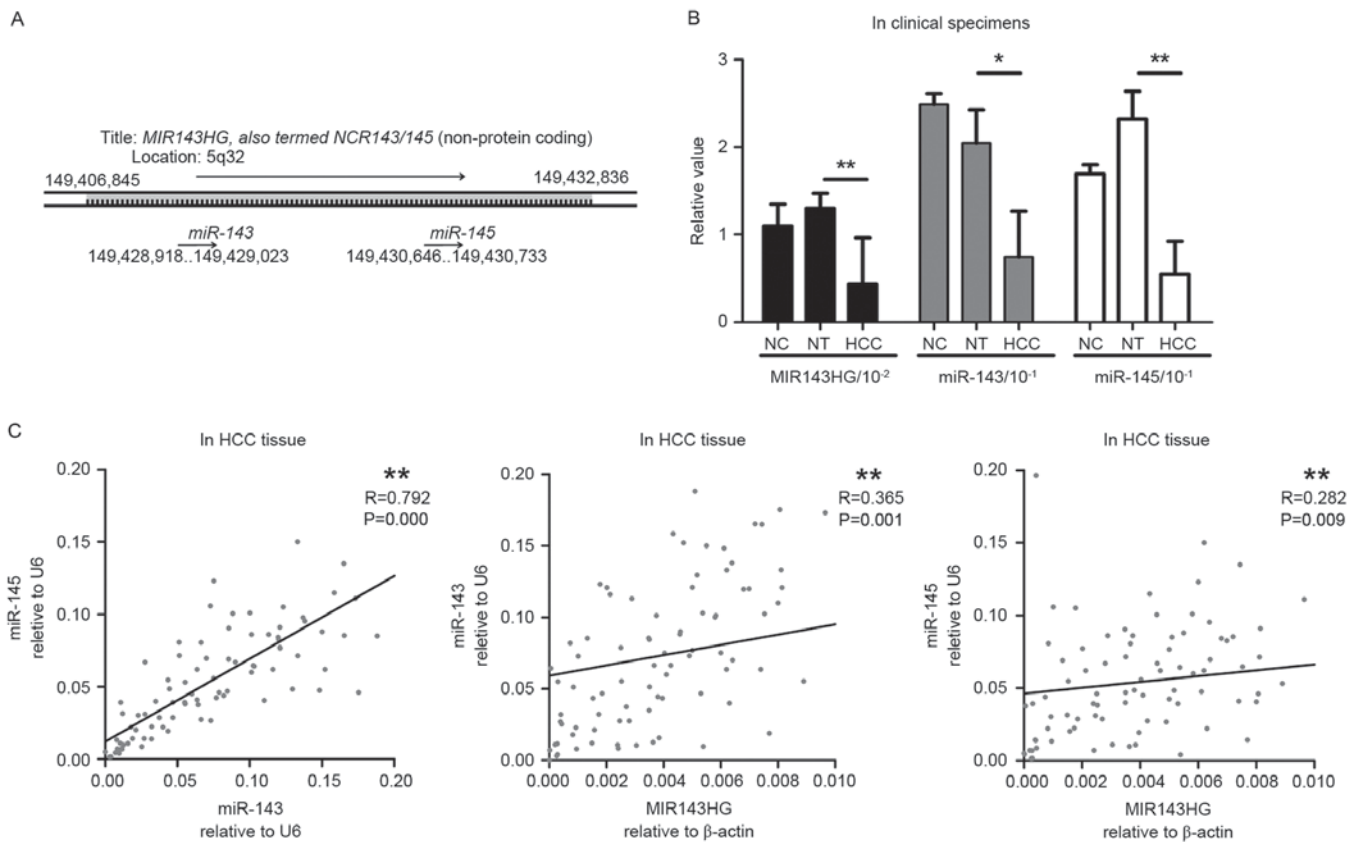


Figure 2. Expression patterns of MIR143HG, miR-143 and miR-145 in HBV-associated HCC tissue. (A) The genomic location of MIR143HG, which encodes miR-143 and miR-145 (7). (B) The expression patterns of MIR143HG, miR-143 and miR-145 in HBV-associated HCC tissue (n=85), NT (n=85) and NC (n=10). Expression levels were determined using RT-qPCR. U6 snRNA was used for miRNA normalization. Results are presented as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$, Student's t-test. (C) Pearson correlation between miR-143, miR-145 and MIR143HG expression in HBV-associated HCC tissue. ** $P < 0.01$. miRNA/miR, microRNA; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NT, non-cancerous liver tissue; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

expression patterns of miR-143 and miR-145 were consistent with that of their host gene, MIR143HG (Fig. 2C) (miR-145 vs. miR-143; $P < 0.001$, miR-143 vs. MIR143HG; $P = 0.001$, miR-145 vs. MIR143HG; $P = 0.009$).

miR-143 and miR-145 expression is associated with the histological differentiation of HBV-associated HCC tumors. To investigate the role of miR-143/145 in HBV-associated HCC, the expression level of the miRNAs was associated with key clinical features including tumor progression and disease prognosis. miR-143/145 expression was not associated with the histological grade of the HBV-associated HCC samples. However, as presented in Fig. 3A, the moderately differentiated group had significantly increased miR-143 expression compared with the well-differentiated group ($P = 0.026$). Similarly, miR-145 expression in the moderately differentiated group was increased compared with the well-differentiated group ($P = 0.012$). miR-143 and miR-145 were significantly overexpressed in the well-differentiated group compared with the negative control ($P = 0.004$ and $P = 0.016$, respectively). The expression of the miRNAs was also associated with the presence of metastasis, cirrhosis and complete tumor capsule (Fig. 3B-D); however, no statistically significant association was detected. Similarly, no statistically significant association was identified with age or sex (data not shown). These results demonstrate that decreased miR-143 and miR-145 expression

is associated with tumor differentiation and may be a useful indicator of poor prognosis for patients with HBV-associated HCC.

Serum levels of miR-143 and miR-145 are decreased in patients with HBV-associated HCC and may serve as a potential diagnostic biomarker for HBV-associated HCC. The results of the present study confirmed that miR-143 and miR-145 were decreased in HBV-associated HCC tissue. To evaluate their use in clinical applications, their expression was quantified in serum prior to and following surgical tumor excision. As presented in Fig. 4A, miR-143 expression was identified to be significantly decreased in the serum of patients with HBV-associated HCC compared with NC (HCC=0.06 vs. NC=0.22; $P < 0.01$, independent Student's t-test). The miR-145 expression was also identified to be significantly decreased in the serum of patients with HBV-associated HCC compared with NC (HCC=0.13 vs. NC 0.27; $P < 0.01$; Fig 4B).

Subsequently, the serum miR-143 and miR-145 RT-qPCR data were used to generate ROC curves for each miRNA and determine the respective AUC. As presented in Fig. 4C and D, the AUC of the miR-143 ROC curve was 0.813 ± 0.04 (mean \pm standard error of the mean; 95% confidence interval, 0.735-0.892; $P < 0.01$) and that of the miR-145 ROC curve was 0.852 ± 0.039 (mean \pm standard error of the mean; 95% confidence interval, 0.776-0.928; $P < 0.05$).

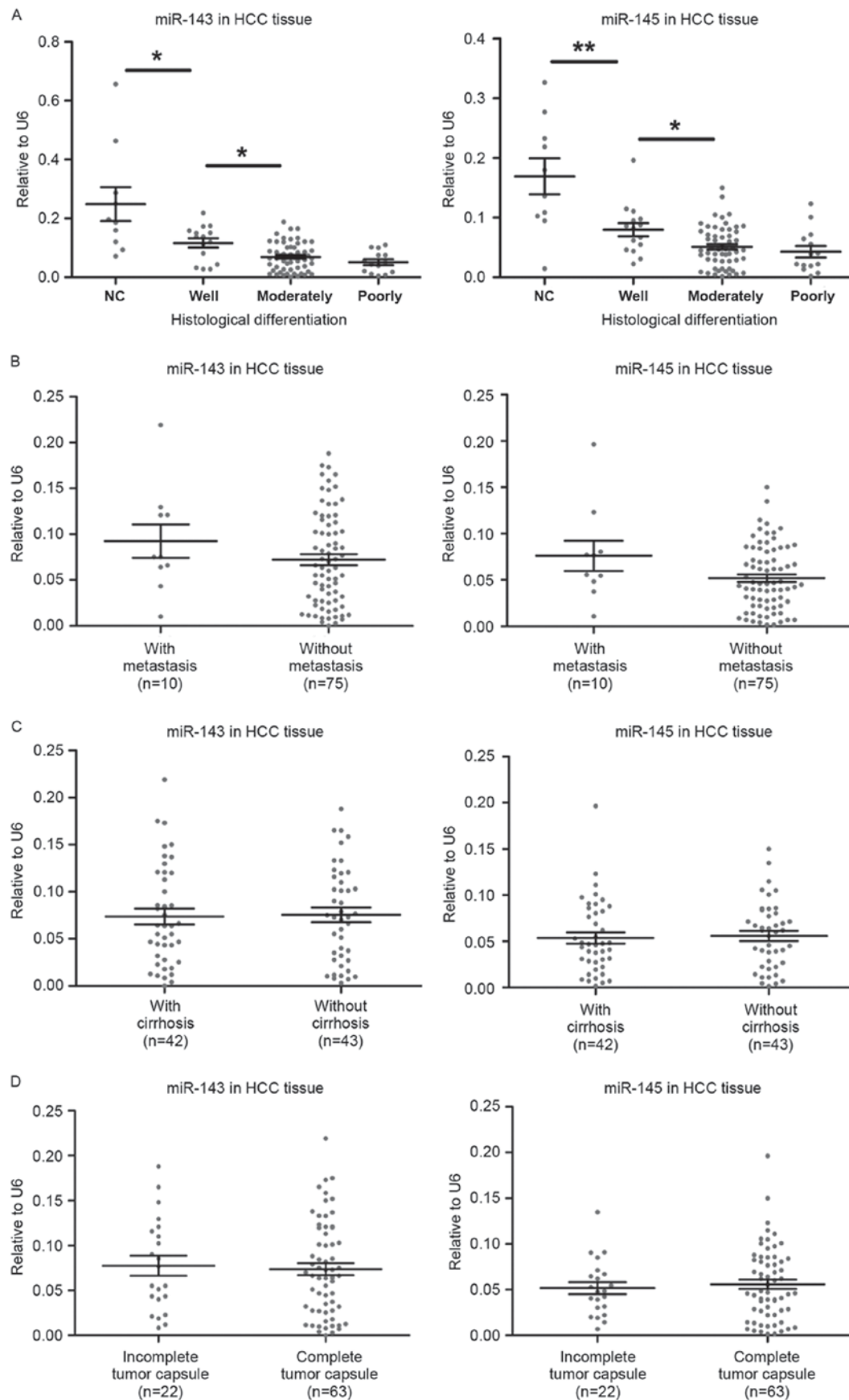


Figure 3. Association of the miRNA expression patterns with several clinical features. (A) Tumors were characterized by pathological analysis as well, moderately or poorly differentiated. The expression of miR-143 and miR-145 was determined using RT-qPCR. U6 snRNA was used for miRNA normalization. Results are presented as the mean \pm standard error of the mean. * $P < 0.05$, ** $P < 0.01$, Student's t-test. The HBV-associated HCC samples were also grouped according to the following clinical features: (B) (+/-) Metastasis, (C) (+/-) cirrhosis or (D) (+/-) complete tumor capsule. No statistically significant association was identified between these clinical features and the expression of miR-143 and miR-145. miRNA/miR, microRNA; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

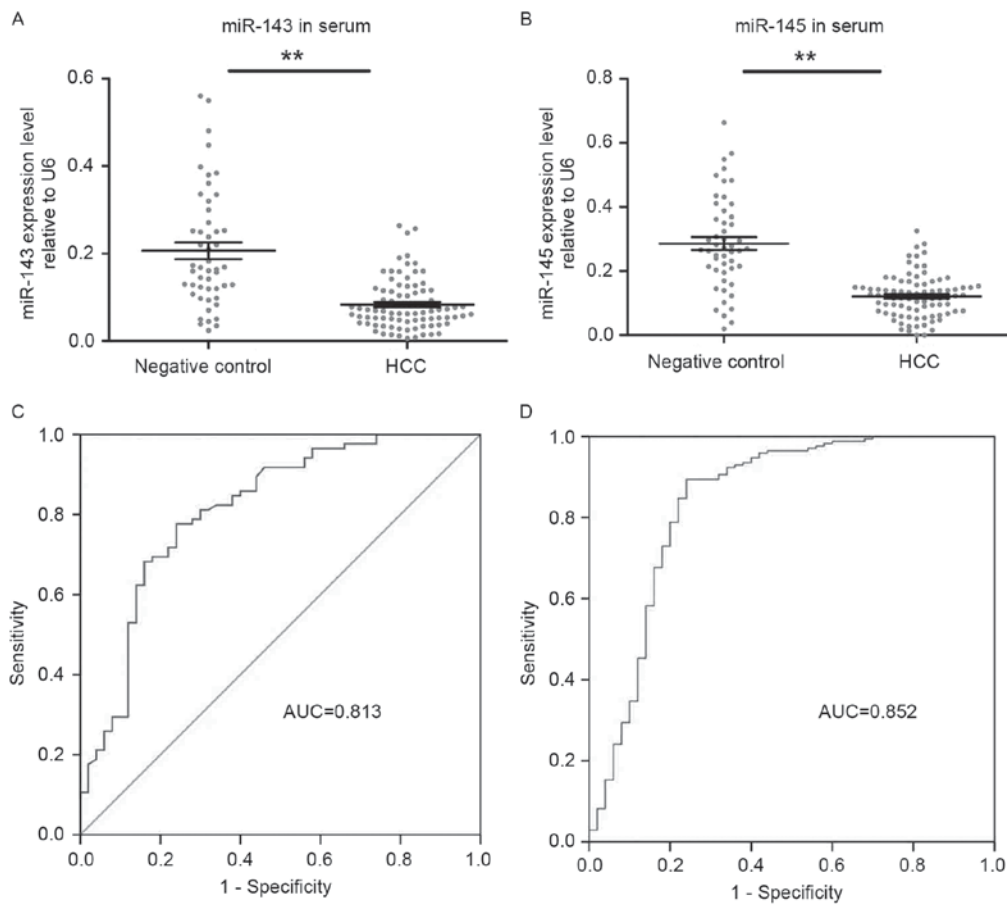


Figure 4. miR-143 and miR-145 serum levels are decreased in patients with HBV-associated HCC and may be used to predict tumorigenesis. RT-qPCR was used to determine the (A) miR-143 and (B) miR-145 expression levels in the serum samples. miR-143 and miR-145 expression was significantly decreased in the HBV-associated HCC samples compared with NC. Results are presented as the mean \pm standard error of the mean. ** $P < 0.01$, Student's t-test. (C) The ROC curve of serum miR-143 expression for predicting HBV-associated HCC tumorigenesis. The AUC was calculated to be 0.813. (D) The ROC curve of serum miR-145 expression for predicting HBV-associated HCC tumorigenesis. The AUC was calculated to be 0.852. miRNA/miR, microRNA; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ROC, receiver-operating characteristic; AUC, area under the ROC curve.

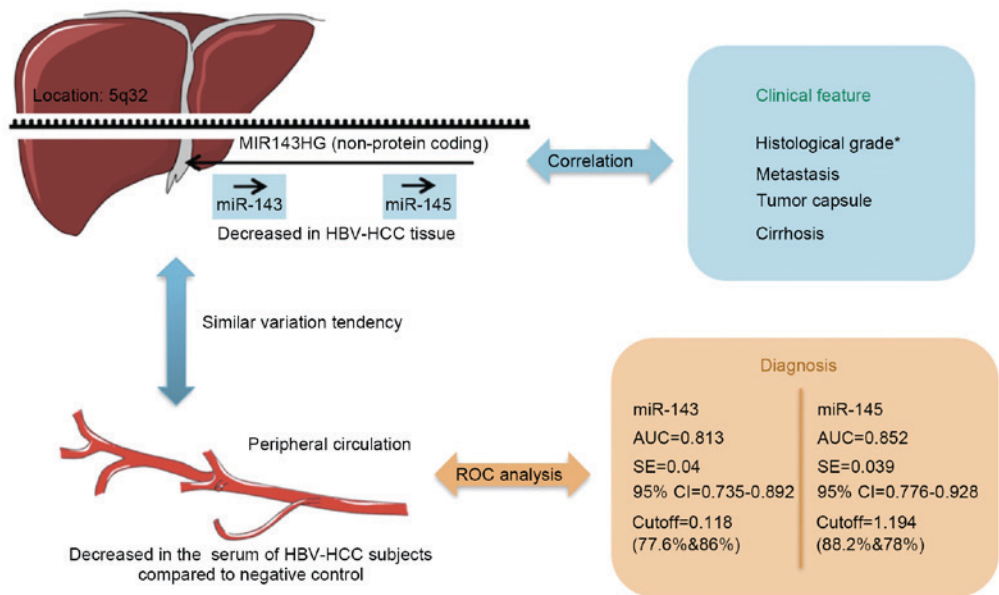


Figure 5. Consolidated model for the role of miR-143 and miR-145 in HBV-associated HCC. In this model, the expression of miR-143, miR-145 and their host gene MIR143HG is decreased in HBV-associated HCC tissue, and their expression is associated with the histological grade of the tumor (* $P < 0.05$). Serum miR-143 and miR-145 are decreased in patients with HBV-associated HCC compared with NC, and may serve as potential diagnostic biomarkers to predict tumorigenesis in patients infected with HBV. miRNA/miR, microRNA; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ROC, receiver-operating characteristic; AUC, area under the ROC curve; SE, standard error; CI, confidence interval.

A threshold for each miRNA was selected to predict HCC between patients with HBV infection. The 1.194 miR-145 threshold identified 88.2% of patients with HBV-associated HCC with a comparatively lower specificity of 78%. The 0.118 miR-143 threshold was characterized by 77.6% sensitivity and 86% specificity (Fig. 5). The results of the present study indicate that the two miRNAs may potentially be used to predict HCC in patients infected with HBV, with a sensitivity and specificity of ~0.80, according to the recommendations of the International Mesothelioma Interest Group for MPM biomarkers (20).

Discussion

According to GenBank, miR-143 and miR-145 are encoded by the MIR143HG gene, which is a non-protein coding gene. miR-143 and miR-145 have distinct sequences, and are located in proximity within the MIR143HG gene. Previous studies have demonstrated decreased miR-143/145 expression in colonic carcinoma, pulmonary carcinoma, esophageal carcinoma and prostatic carcinoma (9-12). However, their expression profile in HCC remains controversial. A previous study demonstrated increased expression (8), whereas decreased expression was demonstrated in others (14,15). By contrast, a previous study demonstrated that miR-143/145 expression was markedly decreased in liver tissue (7). To investigate this further, miR-143 and miR-145 expression was examined in tissue and serum samples of patients with HBV-associated HCC. In addition, serum miRNA data were used to evaluate the potential clinical application of miRNAs as biomarkers for HBV-associated HCC.

In the present study, a model was developed to describe the role of the miR-143/145 cluster in HBV-associated HCC. As Fig. 5 indicates, the expression of miR-143 and miR-145 was significantly decreased in tumor tissue of patients with HBV-associated HCC compared with non-tumor distal tissue. This was particularly evident in patients with moderately and poorly differentiated tumors. The miR-143 and miR-145 serum levels were also decreased in patients with HBV-associated HCC in accordance with the expression profile observed in the tumor tissues. By contrast, the expression of MIR143HG in the serum presented no significant alteration in patients with HBV-associated HCC compared with NC, even though MIR143HG was identified to be downregulated in tumor tissue. The ROC curves generated from miR-143 and miR-145 serum expression data indicated that serum miR-143/145 expression may be a promising biomarker for predicting tumorigenesis in patients with HBV infection.

When examining the expression of a miRNA, its expression in tissue samples is first determined. However, when investigating its potential clinical application, examination of serum miRNA levels is required. HBV-associated HCC is a serious condition, characterized by high morbidity and mortality, and early detection is crucial for effective surgical excision. Since the HCC biomarkers used currently, including serum α -fetoprotein and the carcinoembryonic antigen, have limited sensitivity and specificity, novel biomarkers are required to improve the diagnosis and management of patients with liver diseases. It has been reported that tumor-derived miRNAs are frequently released into the peripheral blood circulation and remain stable, protected from RNase (21). The

results of the present study support this hypothesis. It was demonstrated that serum miR-143/145 expression is consistent with the expression in tumor tissue and the expression levels of miR-143/145 in patients with HBV-associated HCC were decreased compared with NC. Taking into consideration the ROC curves as well, the results of the present study support the hypothesis that serum miR-143/145 may be a potential diagnostic biomarker for HBV-associated HCC. Further research is currently underway to validate this hypothesis and elucidate the underlying molecular mechanism of miR-143/145-mediated regulation of tumor biology.

Using miRNA ChIP and RT-qPCR data, the results of the present study confirmed that miR-143/145 and their host gene MIR143HG are downregulated in tumor tissue of patients with HBV-associated HCC. It was also demonstrated that the expression of miR-143/145 was associated with the histological differentiation of HBV-associated HCC tissues. In addition, serum levels of miR-143 and miR-145 were examined and it was demonstrated that the two miRNAs were decreased in patients with HBV-associated HCC compared with NC. On the basis of the ROC curves, the results of the present study suggest that serum miR-143 or miR-145 expression may be a valuable diagnostic biomarker for predicting tumorigenesis in patients with HBV-associated HCC.

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