Downregulation of microRNA-132 indicates progression in hepatocellular carcinoma

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Abstract. Although miR-132 has been studied in various human tumors, few studies have investigated the role of miR-132 in hepatocellular carcinoma (HCC). The present study aimed to evaluate the associations between miR-132 and clinicopathological parameters, including recurrence, in patients with HCC. Reverse transcription-quantitative polymerase chain reaction analysis was used to detect the expression levels of miR-132 in 95 cases of HCC and their corresponding non-cancerous liver tissues. Th e associations between miR-132 expression levels and clinicopathological characteristics, including recurrence, were investigated in patients with HCC. miR-132 expression levels were significantly reduced in HCC tissues, as compared with adjacent non-cancerous tissues (1.9245±0.7564 vs. 2.7326±1.1475; P<0.001). The area under curve (AUC) of receiver operating characteristic (ROC) used to distinguish cancerous and non-cancerous tissues was 0.711 for miR-132 expression (95% confidence interval, 0.637-0.785; P<0.001) and the optimal cut-off value was 2.25. Expression levels of miR-132 were significantly reduced in the distant metastasis (P=0.031), advanced clinical TNM stage (P=0.022), hepatitis B virus-positive (P<0.001), NM23-expressed (P=0.034), high Ki-67 labeling index (LI; P=0.005) and tumor infiltration or no capsule groups (P=0.026). Spearman correlation analysis demonstrated that miR-132 was significantly correlated with hepatitis B virus infection (r=-0.351; P<0.001), NM23 (r=-0.220; P=0.032), Ki-67 LI (r=-0.264; P=0.010) and tumor capsule (r=-0.207; P=0.044). Kaplan-Meier analysis with the log-rank test indicated an approximate difference of 8 months, although miR-132 may exhibit inferior values for the prediction of recurrence in HCC patients (50.95 vs. 58.68 months; P=0.512). Therefore, the findings of the present study indicated that miR-132 is downregulated in HCC and may serve as a tumor suppressor in its progression.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women worldwide, and it ranks third among the total number of deaths from cancer (1). HCC is particularly prevalent in Africa and eastern/south eastern Asia. Surgical resection and liver transplantation remains the optimal therapeutic strategy for the treatment of HCC. However, due to poor liver conditions, such as cirrhosis and liver dysfunction, only a minority of HCC patients are eligible for surgical intervention, and access to transplantation is limited by the scarcity of donor organs (2,3). Furthermore, the majority of HCC patients are diagnosed during the late or end stages, thus missing the best opportunity for surgical resection. In addition, recurrence and metastasis are frequently detected in patients who undergo surgical resection and the postoperative 5-year survival rate remains low at 30-40% (4). Therefore, it is of great importance that the potential initial molecular mechanisms of HCC progression are investigated in order to identify biomarkers that may be used to evaluate whether HCC patients are at high risk of metastasis or recurrence.

MicroRNAs (miRs) are a family of single-stranded, highly conserved, non-coding functional RNAs (5,6). As >50% of human miRs are located at fragile sites or in cancer-associated genomic regions (7), they may be used as novel biomarkers for the assessment of cancer and potential therapeutic targets. It has previously been reported that miRs act as tumor suppressive genes and oncogenes (8). Various studies have been performed to investigate the association between miRs and human tumors (9-11). Several metastasis-associated miRs have been detected in HCC, including miR-338, miR-19a and miR-122a (12). miR-132, which is located on human chromosome 17p13.3, has been associated with various human cancers including osteosarcoma, colorectal cancer (13,14), breast cancer (15,16), pancreatic cancer (17-20), prostate cancer (21), gastric cancer (22) and glioma (23,24).

Furthermore, Wei *et al* (25) also investigated the association between miR-132 and hepatitis B virus (HBV)-associated HCC. However, this study only included 20 paired samples and did not investigate the association between miR-132 and clinicopathological factors or recurrence in patients with HCC. To the best of our knowledge, there has been no investigation of the association between miR-132 levels and recurrence in any previous study to date. Therefore, a large cohort is required to validate the clinical significance of miR-132 in patients with HCC.

In the present study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to assess the expression levels of miR-132 in HCC patients. Subsequently, the associations between miR-132 expression levels, clinical parameters and recurrence were investigated in patients with HCC.

Materials and methods

Patients and tissue samples. A total of 95 formalin-fixed, paraffin embedded (FFPE) HCC tissues and their matched adjacent non-cancerous liver tissues were obtained from patients who underwent surgery in the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Guangxi Medical University (Nanning, China) between March 2010 and December 2011. The mean age of the enrolled patients was 52 years (range, 29-82 years), and the mean size of the tumors was 6.4 cm (range, 1-11 cm). Pathologic diagnosis was independently performed by two experienced pathologists. A total of 75 men and 20 women were enrolled in the present study. The characteristics of the patients are shown in Table I. None of the patients had previously received preoperative treatments and the present hepatectomy was the first for each patient. The study protocol was approved by the Research Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was obtained from each patient.

RNA isolation and RT-qPCR. Total RNA was extracted from FFPE cancer ($OD_{260/280}$, 1.84-2.06) and adjacent non-cancerous liver tissues ($OD_{260/230}$, 1.90-2.04) using miRNeasy FFPE kit (73504; Qiagen AB, Sollentuna, Sweden), according to previous reports (26-29). Expression levels of miR-132 were evaluated using RT and qPCR kits. In order to remove genomic DNA, the following mixture with a total volume of 10.0 μl was used: 2.0 μl 5X gDNA Eraser buffer, 1.0 μl gDNA Eraser, and trace amount of total RNA with extra RNase-free water. The mixture was maintained at 42˚C for 2 min and then at 4˚C until further use. Reverse transcription of total RNA into cDNA was performed with the TaqMan MicroRNA Reverse Transcription kit (4366596; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a total volume of 20.0 μl, including 10.0 μl from the previous step (removal of genomic DNA), 4.0 μl 5X PrimeScript Buffer 2, 1.0 μl PrimeScript RTEnzyme Mix I, 1.0 μl RT Primer Mix, and 4.0 μl RNase-free water. qPCR analysis of miRNA was performed using a PCR7900 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). RNU6B and RNU48 were selected as endogenous controls. Primers were purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.) and the respective sequences were as follows: miR-132, UAACAGUCUACAGCCAUGGUCG; RNU6B, CGCAAGGAUGACACGCAAAUUCGUGAAGCG UUCCAUAUUUUU; and RNU48, GAUGACCCCAGGUAA CUCUGAGUGUGUCGCUGAUGCCAUCACCGCAGC GCUCUGACC. NormFinder (MOMA, Aarhus, Denmark) and geNorm (genorm.cmgg.be) were used to select RNU6B and RNU48 as endogenous controls. PCR primers for miR-132, RNU6B and RNU48 were included in the TaqMan MicroRNA assay kit (4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.). A PCR reaction system with a total volume of 20.0 μl was employed, including 10.0 μl LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany), 0.8 μl PCR forward primer (10 μM), 0.8 μl PCR reverse primer (10 μM), 1.0 μl cDNA template (<100 ng) and 7.4 μl RNase-free water. A LightCycler 480 (Roche Diagnostics GmbH) was used to perform PCR under the following conditions: Pre-denaturation at 95˚C for 5 min; 40 cycles of 95˚C for 10 sec, 60˚C for 10 sec, and 72˚C for 10 sec; analysis of solubility curve at 95˚C for 5 sec and 65˚C for 1 min); and then cooling at 40˚C for 30 sec. Each reaction was performed in triplicate. Relative mRNA expression levels of miR-132 were calculated using the $2^{-\Delta\Delta Cq}$ method (30).

Statistical analysis. Statistical analysis was conducted using SPSS 20.0 (IBM SPSS, Armonk, NY, USA) for Windows. Independent samples t-test and one-way analysis of variance were used to determine the differences between the groups. Data were presented as the mean ±standard deviation. Receiver operating characteristic (ROC) curve was used to identify the predictive power of miR-132. Spearman correlation analysis was performed to investigate the association between miR-132 expression levels and clinicopathological parameters. Kaplan-Meier and log-rank tests were performed to assess the association between the expression levels of miR-132 and recurrence in patients with HCC. All reported P-values were two tailed, and P<0.05 was considered to indicate a statistically significant difference.

Results

miR-132 expression is downregulated in HCC. Following normalization against RNU6B and RNU48 expression levels, the expression levels of miR-132 in HCC tissues was demonstrated to be significantly decreased, as compared with adjacent non-tumorous tissues (1.9245±0.7564 vs. 2.7326±1.1475; P<0.001; Table I). The area under curve (AUC) of ROC used to distinguish cancerous from non-cancerous tissue was 0.711 for miR-132 expression [95% confidence interval (CI), 0.637-0.785; P<0.001; Fig. 1] and the optimal cut-off value was 2.25. Thus, the results indicated that miR-132 expression was downregulated in HCC.

Association of miR-132 expression with clinicopathological features in HCC patients. The associations between miR-132 expression levels and pathological characteristics were analyzed in order to better elucidate the potential role of miR-132 in the development and progression of HCC. The results suggested that the expression levels of miR-132 were significantly decreased in HCC tissues with distant metastasis

Table I. Association between the expression of miR-132 and clinicopathological features in patients with HCC.

		Relative expression of miRNA-132 ($2^{-\Delta\Delta Cq}$)		
Clinicopathological features	N	Mean ± SD	t-value	P-value
Tissue			-5.731	<0.001
Adjacent non-cancerous liver	95	2.7326±1.1475		
HCC	95	1.9245±0.7564		
Age			0.696	0.488
≥50 years	46	1.9804±0.8967		
<50 years	49	1.8720±0.6006		
Gender			0.381	0.704
Male	75	1.9399±0.7858		
Female	20	1.8670±0.6490		
Differentiation			0.272	0.763
High	6	1.9167±0.9745		
Moderate	60	1.8837±0.7898		
Low	29	2.0107±0.6522		
Size			0.007	0.994
<5 cm	8	1.9233±0.8599		
≥5 cm	77	1.9248±0.7363		
Tumor nodes			0.370	0.712
Single	52	1.9508±0.7608		
Multi	43	1.8928±0.7588		
Metastasis			2.193	0.031
Without metastasis	46	2.0967±0.7740		
With metastasis	49	1.7629±0.7096		
Clinical TNM stage			2.323	0.022
I-II	22	2.2455±0.7900		
III-IV	73	1.8278±0.7238		
Portal vein tumor embolus			0.261	0.794
-	63	1.9390±0.8197		
+	32	1.8959±0.6243		
Vaso-invasion			-0.044	0.965
-	59	1.9219±0.8013		
+	36	1.9289±0.6873		
Tumor capsular infiltration			2.264	0.026
With complete capsule	45	2.1058±0.7603		
No capsule or infiltration	50	1.7614±0.7219		
HCV			1.023	0.309
-	63	1.9811±0.7596		
+	32	1.8131±0.7494		
HBV			4.594	<0.001
-	17	2.6176±0.9534		
+	78	1.7735±0.6167		
AFP			-1.803	0.075
-	41	2.0722±0.8840		
+	38	1.7634±0.5986		
Cirrhosis			-1.654	0.101
-	50	2.0452±0.7716		
+	45	1.7904±0.7241		
NM23			2.146	0.034
-	20	2.2410±0.6714		
+	75	1.8401±0.7594		

Table I. Continued.

		Relative expression of miRNA-132 ($2^{-\Delta\Delta Cq}$)		
Clinicopathological features	N	Mean ± SD	t-value	P-value
MTDH1			-0.133	0.895
-	38	1.9055±0.8265		
+/++/+++	51	1.9275±0.7251		
MTDH2			0.187	0.852
-/+	50	1.9316±0.7788		
++/+++	39	1.9008±0.7580		
P53			1.064	0.290
-	40	2.0212±0.6458		
+	55	1.8542±0.8262		
P21			0.201	0.841
-	62	1.9360±0.7050		
+	33	1.9030±0.8558		
VEGF			0.431	0.667
-	25	1.9808±0.7791		
+	70	1.9044±0.7528		
Ki-67 labeling index			2.893	0.005
Low	47	2.1430±0.7889		
High	48	1.7106±0.6637		
MVD			1.229	0.222
Low	47	2.0206±0.7705		
High	48	1.8304±0.7381		

HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; AFP, α-fetoprotein; MTDH, metadherin; VEGF, vascular endothelial growth factor; MVD, microvessel density.

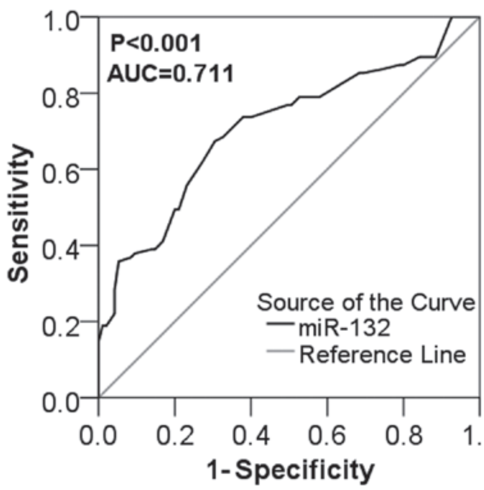

Figure 1. Receiver operating characteristic (ROC) curve of miR-132 was used to distinguish hepatocellular carcinoma. The AUC of the ROC of miR-132 for HCC was 0.711 (95% confidence interval, 0.637-0.785; P<0.001). AUC, area under the curve.

(P=0.031), advanced clinical TNM stage (P=0.022) and tumor infiltration or no capsule (P=0.026), as compared with the adjacent non-cancerous tissues (Table I; Fig. 2). Similar outcomes were observed in the HBV-positive group (P<0.001), NM23-expressed group (P=0.034), and high Ki-67 labeling index (LI) group (P=0.005) (Table I; Fig. 2). No significant differences were detected in the expression levels of miR-132 and age, gender, histological differentiation, tumor size, tumor nodes, portal vein tumor embolus, micro-vascular invasion, hepatitis C virus, α-fetoprotein, para-carcinoma cirrhosis, metadherin, p53, p21, vascular endothelial growth factor, or microvessel density (Table I). Spearman correlation analysis was performed to investigate these associations. Positive results were detected between the expression levels of miR-132 and capsules (r=-0.207; P=0.044), HBV (r=-0.351; P<0.001), NM23 (r=-0.220; P=0.032), and Ki-67 LI (r=-0.264; P=0.010). The cut-off value for miR-132 was 2.15. False positive and false negative rates were 0.22 and 0.556, respectively. Therefore, the results revealed that miR-132 expression was associated with several clinical parameters in HCC.

Recurrence analysis. Median duration of follow-up was 32.78±1.43 months (range, 2.68-68.00 months) for the patients who were successfully followed up. Among the 70 HCC patients with recurrence data included in the present study, 59 exhibited recurrent tumors and data from 11 patients were not included due to subsequent mortality, withdrawal or loss to follow-up. The median level of miR-132 expression among the 70 patients followed up was 1.89. Accordingly, 1.89 was used

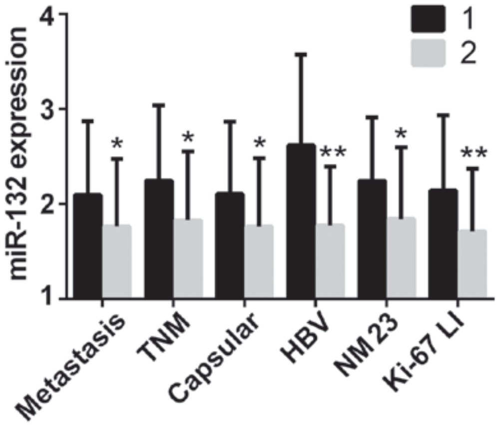

Figure 2. Statistically significant correlations between miR-132 expression levels and clinicopathological parameters. miR-132 expression levels were significantly downregulated in hepatocellular carcinoma tissues with distant metastasis (P=0.031), advanced clinical TNM stage (P=0.022) and tumor infiltration or no capsule (P=0.026), as well as the hepatitis B virus (HBV)-positive group (P<0.001), the NM23-expressed group (P=0.034), and the high Ki-67 labeling index (LI) group (P=0.005). *P<0.05 and **P<0.01, vs. group 1. Metastasis: 1, without metastasis; 2, with metastasis. TNM: 1, I-II; 2, III-IV. Capsular: 1, with complete capsule; 2, no capsule or infiltration. HBV: 1, HBV-negative; 2, HBV-negative. NM23: 1, NM23-negative; 2, NM23-positive. Ki-67 LI: 1, low; 2, high. TNM, tumor, node and metastatis; HBV, hepatitis B virus; NM23

as a cut-off value to divide the 70 patients into two respective groups, low expression (<1.89; n=35) and high expression (>1.89; n=35). The overall recurrent period of the whole group was 57.10 months (95% CI, 51.46-62.73). The recurrent period in the high expression group (58.68 months; 95% CI, 51.59-65.76) was longer than the low expression group (50.95 months; 95% CI, 45.71-56.20) despite the limited statistical significance (χ^2=0.430; P=0.512 log-rank test). Thus, the results demonstrated that the high miR-132 expression group showed a longer recurrent period by ~8 months compared with the low expression group.

Discussion

The expression of miR-132 in human cancer has attracted a large amount of research. Its molecular mechanisms have been studied in osteosarcoma (31), colorectal cancer (13), pituitary tumor (32), prostate cancer (21), breast cancer (15), lung cancer (33,34) and pancreatic cancer (17-19). The majority of studies have demonstrated the suppressive role of miR-132 in different classes of cancers via various novel molecule networks. For example by repressing CCNE1 expression (31), targeting zinc finger E-box-binding homeobox 2 (ZEB2) (13), Sox5 (32), hematological and neurological expressed 1 (HN1) (15) or ZEB2 (33) and being methylation-silenced and antimetastatic in PCa controlling cellular adhesion (21), as well as via the induction of acetylcholinesterase-independent apoptosis (31). All of these studies mention identified miR-132 as a tumor suppressor. However, a discrepancy in the data was detected in pancreatic cancer. Luo *et al* (19) demonstrated that stem-cell-like BxPC-3-LN cells expressed lower levels of miR-132 than the parental BxPC-3 cells. Zhang *et al* (17) further supported this finding by demonstrating the downregulation of miR-132 in pancreatic cancer via promoter methylation.

Researchers have also investigated the clinical significance of miR-132 in various tumors, where greater divergence emerged by predominantly focusing on the different expression levels of miR-132 between cancerous tissues and corresponding non-cancerous tissues. miR-132 upregulation was observed in gastric cancer (22), glioma (23,35) and pancreatic cancer (18). Conversely, miR-132 downregulation was detected in osteosarcoma (31,36), colorectal cancer (13), ductal carcinoma *in situ* of the breast (16), pancreatic cancer (17) and breast cancer (15), where lower levels of miR-132 were observed in cancerous tissues, as compared with corresponding non-cancerous tissues.

The clinical application of miR-132 remains a hot topic in associated research. According to Cote *et al* (20), miR-132 expressed in plasma may be used as a diagnostic test for pancreatic ductal adenocarcinoma when in conjunction with other miRNAs; whereas Chung *et al* (37) suggested that the downregulation of miR-132 in serum may be considered as one of the novel biomarkers in serous ovarian cancer. Furthermore, Salendo *et al* (14) demonstrated that miR-132 may be able to identify the chemoradiosensitivity of colorectal cancer cells. As for its utilization in differential diagnosis, Lages *et al* (24) concluded that deregulated miR-132 may facilitate the proper discrimination of oligodendroglioma from glioblastoma.

In the present study, RT-qPCR was performed to detect the expression levels of miR-132 in 95 paired HCC and adjacent non-cancerous liver tissues to explore correlations with clinicopathological features. Only one previous study has investigated miR-132 in HCC, which predominantly focused on the role of miR-132 in the mechanism of HBV-mediated hepatocarcinogenesis (25). The present study focused on the correlations between miR-132 and clinicopathological parameters, and a larger cohort of 95 patients was investigated compared with only 20 in the previous study. Furthermore, recurrent free survival analysis was performed in the present study, which the previous research lacked.

Concerning the expression of miR-132 in HCC, the present findings were consistent with those published by Wei *et al* (25), as the expression of miR-132 in HCC was significantly reduced, as compared with the corresponding normal tissues (P<0.001). Wei *et al* (25) suggested that the downregulation of miR-132 may be modulated by HBx expression via DNA methylation. This hypothesis may help to explain the present results despite the difference that all their cancerous tissues were HBV-associated HCC tissues and various HCC tissues were not included. Meanwhile, the AUC of the expression level of miR-132 was 0.711 (95% CI, 0.637-0.785; P<0.001), which implied that miR-132 may be used as a reference index in the diagnosis of HCC.

In an attempt to further complement the study conducted by Wei *et al* (25), the correlations between miR-132 expression levels and major clinicopathological features in HCC were explored, which was absent in their study. Firstly, decreased expression levels of miR-132 were observed in HCC tissues with distant metastasis (P=0.031), advanced clinical TNM stage (P=0.022) and tumor infiltration or no capsule (P=0.026). These findings inferred a tumor-suppressing role, which suggests that miR-132 may have a role in HCC progression. Secondly, the associations between miR-132 expression and other conventional biomarkers in HCC, including HBV, NM23 and Ki-67, were also investigated. HBV infection is a common risk factor for the development of HCC (38). Nm23 gene is a putative metastatic suppressor gene (39) and Ki-67 LI can be used to indicate cell proliferative activity (40). miR-132 expression levels were significantly reduced in the HBV-positive (P<0.001), NM23-expressed (P=0.034), and high Ki-67 LI (P=0.005) groups. Spearman correlation analysis demonstrated positive results between the expression of miR-132 and HBV (r=-0.351, P<0.001), NM23 (r=-0.220, P=0.032), and Ki-67 LI (r=-0.264, P=0.010). Taken together, these results demonstrated that the expression of miR-132 was elevated in HCC cells with reduced cell proliferation, indicated that miR-132 may be associated with cell proliferation in HCC.

Recurrence analysis was also performed in the present study. Followed-up patients demonstrated a median of follow-up duration of 32.78±1.43 months (range, 2.68-68.00 months). The cohort, which was composed of 59 cases with recurrent tumors and recurrence data as well as 11 censored cases, exhibited an overall recurrent duration of 57.10 months (95% CI, 51.46-62.73). As to the recurrent period, the high expression group (>1.8900, n=35) exhibited a longer duration of 58.68 months (95% CI, 51.59-65.76), as compared with the low expression group (<1.8900; n=35) (50.95 months; 95% CI, 45.71-56.20) in spite of the inferior statistical value (χ^2=0.430; P=0.512 log-rank test).

The findings of the present study, which was the first to include recurrent analysis to investigate the correlations between miR-132 and mainstream clinicopathological characteristics in HCC, indicated that miR-132 may be significantly decreased in HCC and may perform as a tumor suppressive gene in HCC development. Nevertheless, some limitations still exist. Firstly, limited insights were emphasized in terms of the molecular mechanisms. Given the similar results of decreased miR-132 published by Wei *et al* (25), their theory that downregulation of miR-132 may result from the HBx expression via DNA methylation may also apply to the present study. Other targets in previous research into miR-132 in cancer, such as ZEB2 (13), Sox5 (32), HN1 (15) and ZEB2 (33), should not be ignored since consistent suppressive roles of miR-132 were observed. Furthermore, tissue analysis was employed in the present study, which has various disadvantages over non-invasive methods such as serum detection. Future studies should aim to harvest tissue and serum samples of HCC in order to investigate the molecular networks or mechanisms of miR-132 in HCC with a larger cohort.

In conclusion, the present study was the first to investigate the associations between miR-132 and clinicopathological parameters, including recurrence, in patients with HCC. The results demonstrated that miR-132 is downregulated in HCC. These findings strongly supported the hypothesis that miR-132 serves as a tumor suppressor in the development of HCC, and HCC patients with downregulated miR-132 may suffer from poorer outcomes. A subsequent study has been designed to investigate the potential underlying mechanisms between HCC and miR-132 based on the results from the present study.

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