MicroRNA-331-3p Promotes Proliferation and Metastasis of Hepatocellular Carcinoma by Targeting PH Domain and Leucine-Rich Repeat Protein Phosphatase

Rui-Min Chang, Hao Yang, Feng Fang, Jiang-Feng Xu, and Lian-Yue Yang

Hepatocellular carcinoma (HCC) is a highly invasive tumor with frequent intrahepatic or pulmonary metastasis, which is the main reason for high recurrence and poor survival of HCC after liver resection. However, the mechanisms for metastasis remain incompletely clear. Given that microRNAs (miRNAs) are implicated in HCC progression, we explored a potential role of miRNAs in metastasis by performing miRNA expression profiling in three subtypes of HCC with different metastatic potentials. We discovered miR-331-3p as one of most significantly overexpressed miRNAs and highly associated with metastasis of HCC. Increased expression of miR-331-3p was correlated with poor long-term survival of HCC. We provided both in vivo and in vitro evidence demonstrating that miR-331-3p promoted proliferation and metastasis of HCC cells. Using an integrated approach, we uncovered that PH domain and leucine-rich repeat protein phosphatase (PHLPP) was a novel target of miR-331-3p. Indeed, the miR-331-3p-mediated effects were antagonized by reexpression of PHLPP or mimicked by silencing of PHLPP. We further showed that miR-331-3p-mediated inhibition of PHLPP resulted in stimulation of protein kinase B (AKT) and subsequent epithelial mesenchymal transition (EMT). Finally, inhibition of miR-331-3p through a jetPEI-mediated delivery of anti-miR-331-3p vector resulted in marked inhibition of proliferation and metastasis of HCC in xenograft mice. Conclusion: miR-331-3p promotes proliferation and EMT-mediated metastasis of HCC through suppression of PHLPP-mediated dephosphorylation of AKT. Our work implicates miR-331-3p as a potential prognostic biomarker and a novel therapeutic target. (HEPATOLOGY 2014;60:1251-1263)

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor worldwide and the second-leading cause of cancer-induced death. Although numerous therapeutic strategies have been improved and utilized in recent years, liver resection is still the best way to treat HCC, with a 5-year survival rate in approximately 30%. It has been well known that high recurrence and metastasis rates have become the major obstacle to improve long-term survival of HCC. Recent studies have demonstrated...
that many oncogenes or antioncogenes are associated with metastasis of HCC. However, the mechanism for the development of HCC remains unclear. There is an urgent need to determine the exact mechanisms and improve the current therapeutic strategies of HCC.

MicroRNAs (miRNAs) are a class of short (19-22 nucleotides), noncoding RNA sequences. It has been recognized that aberrantly expressed miRNAs play essential roles in a variety of biological processes. Recently, it has been reported that miR-199a/b-3p, miR-26a, miR-29b, and miR-195 function as tumor suppressors in HCC, whereas, on the contrary, miR-216a/217 and miR-657 function as oncogenes. Nevertheless, the relationship between HCC metastasis and miRNAs remains elusive.

To identify key miRNAs associated with metastasis of HCC, we previously examined 840 miRNAs expression in three subtypes of HCC with different metastatic potentialities, including small HCC (SHCC; tumor diameter ≤ 5.0 cm), solitary large HCC (SLHCC; tumor diameter > 5.0 cm; node number = 1), and nodular HCC (NHCC; node number ≥ 2). Among the three subtypes, SLHCC is a specific subtype of HCC with relatively better prognosis than that of NHCC. The metastatic potentiality of SLHCC is significantly lower than that of NHCC and similar to that of SHCC. Our miRNA expression profiles showed that miR-331-3p was one of the miRNAs significantly up-regulated in HCC tissues, especially in NHCC. The expression level of miR-331-3p in HCC was increased more than 3-fold, compared to adjacent nontumorous liver tissues (ANLTs). It has been reported that miR-331-3p is expressed abnormally in different tumors and is associated with proliferation and migration of cancers. However, to date, it remains unclear whether miR-331-3p plays a role in human HCC.

In this study, we present evidence that overexpression of miR-331-3p predicts poor postoperative prognosis in HCC patients. miR-331-3p promotes the proliferation and epithelial mesenchymal transition (EMT)-mediated metastasis of HCC by directly suppressing PH domain and leucine-rich repeat protein phosphatase (PHLPPL)-induced dephosphorylation of protein kinase B (AKT).

**Patients and Methods**

**Patients and Tissue Specimens.** From January 2002 to December 2008, a total of 457 pairs of HCC and ANLTs were gathered from patients who had liver resection performed at Department of Surgery, Xiangya Hospital of Central South University (Changsha, China). The training cohort contained 120 randomly selected cases in 241 patients from July 2005 to December 2008. The validation cohort contained 108 randomly selected cases in 216 patients from January 2002 to June 2005 (Supporting Fig. 1). The samples were snap-frozen in liquid nitrogen and stored at −80°C for subsequent RNA extraction or formalin-fixed and paraffin embedded for immunohistochemistry (IHC) or immunofluorescence (IF). Histopathology was evaluated by two certified pathologists in the Department of Pathology at Xiangya Hospital of Central South University. The clinical and pathological features of these patients were described in Supporting Table 1. All research protocols strictly complied with REMARK guidelines for reporting prognostic biomarkers in cancer. All human materials were obtained with informed consent and approved by the ethics committee of Xiangya Hospital of Central South University.

**Luciferase Reporter Assay.** Wild-type (WT) or mutant of 3’ untranslated region (UTR) sequences of PHLPPL or PH domain and leucine-rich repeat protein phosphatase-like (PHLPPL) were inserted into the Xba I and Fse I sites of the pGL3 vector (GeneChem, Shanghai, China). HEK293T cells infected with anti-miR-331-3p lentivirus or negative control (NC) lentivirus were seeded into 96-well plates. pGL3 vector (50 ng) with the above sequence was cotransfected with 10 ng of pRL-TK vector into cells by Lipofectamine LTX (Invitrogen, Carlsbad, CA). Twenty-four hours later, cells were harvested according to the manufacturer’s protocol (Promega, Madison, WI) and firefly and Renilla luciferase activity was detected using Dual-luciferase Reporter Assay System Kits (Promega) with a Victor X machine (PerkinElmer, Boston, MA).

More details are described in the Supporting Materials and Methods.
Results

miR-331-3p Is Up-Regulated in Human HCC Tissue. miRNA expression profiles in SHCC, SLHCC, and NHCC showed that miR-331-3p was highly expressed in HCC, especially in NHCC (Fig. 1A). To validate this result, we performed quantitative real-time polymerase chain reaction (qRT-PCR) in 120 cases of HCC tissues and ANLTs in the training cohort. Compared with the corresponding ANLTs, miR-331-3p was significantly up-regulated (more than 2-fold; i.e., log₂ [fold change] > 1) in 79 HCC cases (65.8%; Fig. 1B). The result from in situ hybridization also showed that miR-331-3p was up-regulated in HCC (Supporting Fig. 2). These data suggest that aberrant expression of miR-331-3p is a frequent event in HCC. Consistent with the miRNA array data, the median level of miR-331-3p expression in NHCC was significantly higher than that in SLHCC and SHCC. But, the median level of miR-331-3p expression in SHCC was similar to that of SLHCC (Fig. 1C).

High miR-331-3p Expression in HCC Tissue Is Associated With Poor Clinicopathologic Features and Low Postoperative Survival Rate. To examine the relationship between miR-331-3p expression and clinicopathological features, we analyzed their correlations in the training cohort. The results showed that miR-331-3p expression was significantly associated with poor clinicopathological features, including tumor size \( (P = 0.041) \), tumor nodular number \( (P = 0.031) \), capsular formation \( (P = 0.034) \), and venous invasion \( (P = 0.007; \) Table 1). We next analyzed the relationship between miR-331-3p expression and patients' prognosis. Patients (120 cases) were divided into two groups based on levels of miR-331-3p expression that were either increased (79 cases) or not (41 cases). The tumor nodular number \( (P = 0.044) \), venous invasion \( (P = 0.033) \), and miR-331-3p \( (P = 0.030) \) were recognized as independent risk factors for overall survival (OS) by the univariate analysis and subsequent multivariate survival analysis (Supporting Table 2). Furthermore, venous invasion \( (P = 0.035) \) and miR-331-3p expression \( (P = 0.038) \) were verified as independent factors for DFS.
risk factors for disease-free survival (DFS) by univariate analysis and multivariate survival analysis (Supporting Table 3).

Survival curves showed that HCC patients with high levels of miR-331-3p expression had shorter OS and DFS time than those with low levels of miR-331-3p expression (Fig. 1D). The 1-, 3-, and 5-year OS rates of patients in the training cohort with high miR-331-3p expression were 64.4%, 31.6%, and 14.0%, respectively, which were significantly lower than those with low miR-331-3p expression (82.1%, 61.1%, and 41.0%, respectively; \( P = 0.003 \)). The 1-, 3-, and 5-year DFS rates of the high miR-331-3p group were 42.3%, 18.3%, and 10.4%, respectively, which were also significantly lower than those of the low miR-331-3p group (77.0%, 46.9%, and 25.2%, respectively; \( P = 0.001 \)). These associations were further validated in another cohort comprised of 108 postoperative HCC patients with longer follow-up. Similarly, shorter OS and DFS in the high miR-331-3p expression group than those in the low expression group were also observed (Fig. 1E). The 1-, 3-, and 5-year OS rates in the high miR-331-3p group were significantly lower than those in the low miR-331-3p group (77.2% vs. 90.1%, 52.6% vs. 82.1%, and 21.4% vs. 57.8%, respectively; \( P = 0.002 \)). The 1-, 3-, and 5-year DFS rates in the high miR-331-3p group were also significantly lower than those in the low miR-331-3p group (53.2% vs. 80.8%, 32.6% vs. 68.1%, and 18.4% vs. 45.8%, respectively; \( P = 0.001 \)). These results together revealed that elevated expression of miR-331-3p is correlated with poor prognosis of HCC, implicating that miRNA participates in HCC progression.

**miR-331-3p Promotes Proliferation and Migration of HCC Cells In Vitro.** Having observed the association of miR-331-3p expression and tumor size and venous invasion in HCC patients (Table 1), we went on functionally characterizing miR-331-3p by focusing on its effect on proliferation and migration of HCC cells. We first compared a primary human hepatocyte and normal liver cell line (L02) with a panel of HCC cell lines (Huh7, HepG2, PLC/PRF/5, Hep3B, SMMC7721, MHCC97-L, MHCC97-H, HCCLM3, and Bel7402) for expression of miR-331-3p (Supporting Fig. 3). Results indicated that miR-331-3p was overexpressed in all HCC cell lines, but not in normal or primary hepatocytes, consistent with the data derived from patient specimens. We selected MHCC97-L, HCCLM3, HepG2, PLC/PRF/5, and Huh7 as representatives for further characterization. Expression of miR-331-3p was overexpressed in all HCC cell lines, but not in normal or primary hepatocytes, consistent with the data derived from patient specimens. We selected MHCC97-L, HCCLM3, HepG2, PLC/PRF/5, and Huh7 as representatives for further characterization. Expression of miR-331-3p in these cell lines was either over or underexpressed by stable infection of miR-331-3p expression lentivirus or anti-miR-331-3p. Expression of miR-331-3p in each cell line was measured with qPCR (Supporting Fig. 4).

Methyl thiazol tetrazolium (MTT) assay and colony formation assay were performed to assess the effect of miR-331-3p on cell proliferation. HCCLM3, HepG2, and Huh7 cells expressing anti-miR-331-3p showed a lower proliferation rate and fewer numbers of colonies than control cells. In contrast, MHCC97-L and PLC/PRF/5 cells expressing miR-331-3p exhibited higher proliferation rate and

| Clinicopathologic Variable                  | \( n \) | Low Expression | High Expression | \( P \) Value |
|--------------------------------------------|--------|---------------|----------------|-------------|
| Gender                                     |        |               |                |             |
| Female                                     | 18     | 9             | 9              | 0.127       |
| Male                                       | 102    | 32            | 70             |             |
| Age, years                                 |        |               |                |             |
| \( \leq 60 \)                              | 71     | 22            | 49             | 0.435       |
| \( >60 \)                                  | 49     | 19            | 30             |             |
| AFP, ng/mL                                 |        |               |                |             |
| \( <20 \)                                  | 45     | 12            | 33             | 0.234       |
| \( \geq 20 \)                               | 75     | 29            | 46             |             |
| HBsAg                                      |        |               |                |             |
| Negative                                   | 18     | 4             | 14             | 0.293       |
| Positive                                   | 102    | 37            | 65             |             |
| Liver cirrhosis                            |        |               |                |             |
| Absence                                    | 34     | 13            | 21             | 0.558       |
| Presence                                   | 86     | 28            | 58             |             |
| Tumor size, cm                             |        |               |                |             |
| \( \leq 5 \)                                | 40     | 19            | 21             | 0.041       |
| \( >5 \)                                   | 80     | 22            | 58             |             |
| Tumor nodule number                        |        |               |                |             |
| Solitary                                   | 71     | 30            | 41             | 0.031       |
| Multiple (\( \geq 2 \))                    | 49     | 11            | 38             |             |
| Capsular formation                         |        |               |                |             |
| Presence                                   | 59     | 26            | 33             | 0.034       |
| Absence                                    | 61     | 15            | 46             |             |
| Edmondson-Steiner grade                    |        |               |                |             |
| I-II                                      | 64     | 23            | 41             | 0.665       |
| III-IV                                    | 56     | 18            | 38             |             |
| Venous invasion                            |        |               |                |             |
| Absence                                    | 64     | 29            | 35             | 0.007       |
| Presence                                   | 56     | 12            | 44             |             |
| TNM                                        |        |               |                |             |
| I                                          | 42     | 16            | 26             | 0.213       |
| II                                         | 49     | 19            | 30             |             |
| III                                        | 29     | 6             | 23             |             |
| BCLC staging                               |        |               |                |             |
| O-A                                       | 20     | 9             | 11             | 0.060       |
| B                                          | 74     | 27            | 47             |             |
| C                                          | 26     | 5             | 21             |             |
| Liver function                             |        |               |                |             |
| Child-Pugh A                               | 82     | 30            | 52             | 0.416       |
| Child-Pugh B                               | 38     | 11            | 27             |             |

Abbreviations: AFP, alpha-fetoprotein; HBsAg, hepatitis B surface antigen; TNM, tumor node metastasis; BCLC, Barcelona Clinic Liver Cancer.
greater number of colonies than control cells (Fig. 2A, B and Supporting Fig. 5A, B). We also carried out cell-cycle analysis to corroborate data of proliferation. Results showed that reduced expression of miR-331-3p was associated with G1 cell-cycle arrest, as evidenced by the increased percentage of G1 and the reduced percentage of S and G2/M (Fig. 2C and Supporting Fig. 5C).

We next investigated the potential role of miR-331-3p in modulating the ability of HCC cells to invade and migrate. Results of wound-healing assays indicated that lower levels of miR-331-3p expression correlated with slower rates of wound healing and higher levels of miR-331-3p expression with faster healing (Fig. 2D and Supporting Fig. 6A). Similarly, transwell assays with Matrigel revealed that HCCLM3, HepG2, and
Huh7 cells expressing anti-miR-331-3p exhibited significantly reduced rate of invasion, compared to control cells, whereas MHCC97-L and PLC/PRF/5 cells overexpressing miR-331-3p migrated faster, relative to control cells (Fig. 2E and Supporting Fig. 6B). Interestingly, a marked morphological change in miR-331-3p-expressing cells was evident from immunostaining (Fig. 2F and Supporting Fig. 6C). Cells with relatively high miR-331-3p expression displayed a mesenchymal-like phenotype. These data together suggest that miR-331-3p enhances HCC cell proliferation and invasion potentialities.

**PHLPP Is a Novel Target of miR-331-3p.** To reveal the mechanism by which miR-331-3p affects cell proliferation and invasion in HCC, we made an attempt to identify potential target genes of miR-331-3p. Three databases, including TargetScan, PicTar, and miRanda, were searched for potential genes under the control of miR-331-3p. Hundreds of different targets were screened, and we selected genes predicted by all three databases. Among these predicted targets, two members (named PHLPP and PHLPPL) of the serine/threonine phosphatase family seemed to be of particular interest. To further verify whether miR-331-3p would bind directly to the 3' UTR of PHLPP and PHLPPL, protein in HCCLM3 or HepG2 infected with anti-miR-331-3p and MHCC97-L or PLC/PRF/5 infected with miR-331-3p and their corresponding NC. (D) Expression of PHLPP mRNA in 22 pairs of samples of HCC was tested with qRT-PCR. Expression of PHLPP mRNA in tumors (T) was normalized to their own ANLTs. (E) Representative images of RT-PCR and western blotting shows the level of PHLPP mRNA and protein in tumors (T) and adjacent nontumorous liver tissues (ANLT).
miR-331-3p inhibited the luciferase activity of WT 3’ UTR of PHLPP, but not PHLPPL, and there was little activity when miR-331-3p only was present (Fig. 3B). Results were corroborated by western blotting, showing that miR-331-3p expression suppressed expression of PHLPP protein, but not PHLPPL (Fig. 3C), confirming the findings obtained from the dual luciferase report assay. In addition, messenger RNA (mRNA) and protein expressions of PHLPP were downregulated in HCC tissues, when compared with ANLTs (Fig. 3D,E). Thus, our data indicate that miR-331-3p negatively regulates the expression of PHLPP by directly targeting its 3’ UTR.

miR-331-3p Exerts Its Function by Suppressing PHLPP Expression. To examine whether miR-331-3p exerts its function through PHLPP, we induced or silenced PHLPP expression in previous HCC cells. MTT and cell colony assays showed that expression of PHLPP blocked miR-331-3p-mediated proliferation of HCC cells. Conversely, silencing of PHLPP mimicked the function of miR-331-3p, resulting in increased proliferation (Fig. 4A and Supporting Fig. 7A). Similarly, flow cytometry (FCM) analysis showed that PHLPP blocked the activity of miR-331-3p in promoting the entry of HCC cell in G1 phase into S phase or G2/M phase (Fig. 4B and Supporting Fig. 7B). Furthermore, the wound-healing and transwell assays also confirmed that ectopic expression of PHLPP blocked the role of miR-331-3p in the invasion of HCC cells (Fig. 4C,D and Supporting Fig. 7C,D). Interestingly, IF staining showed a marked alteration of HCC cell morphology after transfection with PHLPP or short hairpin (sh)PHLPP.
vector. Upon PHLPP expression, HCC cells displayed an epithelial-like shape, whereas the shPHLPP expression was associated with mesenchymal-like morphology. Again, the effect of miR-331-3p on cell morphology was blocked by PHLPP (Supporting Fig. 8).

It was reported that PHLPP exerts its roles by an AKT- or protein kinase C (PKC)-independent pathway or a mitogen-activated protein kinase (MAPK) pathway in other diseases. Thus, we examined the expression of AKT, extracellular signal-regulated kinase (ERK), and PKC by western blotting. miR-331-3p induced phosphorylation on Ser473 of AKT, but not on Thr308 of AKT or of ERK or expression of PKC (Fig. 4E and Supporting Fig. 9). Effects of miR-331-3p were antagonized or mimicked by ectopic expression or silencing of PHLPP, respectively. Collectively, these findings indicate that miR-331-3p promotes HCC cell proliferation and metastasis by suppressing PHLPP-mediated activation of AKT signaling.

**miR-331-3p Promotes Growth and Metastasis of HCC In Vivo.** To determine the function of miR-331-3p in vivo, HCC mouse models were developed with the HCC cell lines and the development of tumor was monitored. After 6 weeks, mean tumor volume of the anti-miR-331-3p group of HCCLM3, HepG2, or Huh7 cells was significantly smaller than their control group. On the other hand, mean volumes of tumors generated by MHCC97-L and PLC/PRF/5 cells expressing miR-331-3p were greater than those of the control groups (Fig. 5A and Supporting Fig. 10A). Hematoxylin and eosin (H&E) staining of tumor sections showed that tumors of the control group had a more invasive edge than those of the anti-miR-331-3p group (Fig. 5B). To further examine intrahepatic metastasis and pulmonary metastasis, serial sections of liver and lung stained with H&E were used to identify metastasis nodules (Fig. 5C and Supporting Fig. 10B). Incidences of intrahepatic metastasis and pulmonary metastasis decreased in HCCLM3, HepG2, or Huh7 cells expressing anti-miR-331-3p, but increased in MHCC97-L or PLC/PRF/5 cells expressing miR-331-3p (Fig. 5D and Supporting Fig. 10C). In situ hybridization of tumor sections showed that miR-331-3p expression was inhibited in the HCCLM3anti-miR-331-3p group and overexpressed in the PLC/PRF/5mir-331-3p group (Fig. 5E). IF staining showed that expressions of PHLPP and phosphorylated AKT (p-AKT) (p-AKT; S473) were reversely correlated in HCC tissues. In HCC tissues with higher miR-331-3p expression, lower PHLPP expression correlated with stronger staining of p-AKT (S473; Fig. 5E). Taken together, these results indicate that miR-331-3p promotes the growth and metastasis of HCC in vivo by suppressing PHLPP and increasing Ser473 phosphorylation of AKT.

**Metastasis Is Enhanced by miR-331-3p by Promoting EMT.** IF analysis of cytoskeleton (Fig. 2F and Supporting Figs. 6C and 8) had showed that inhibition of miR-331-3p changed HCC cells into a mesenchymal-like morphology, suggesting that miR-331-3p may alter the properties of EMT of HCC cells. To examine whether miR-331-3p promotes EMT, IF analysis of cells and IHC analysis of HCC tissues were performed using E-cadherin and vimentin as the epithelial and the mesenchymal marker, respectively. IF analysis revealed that inhibition of miR-331-3p in HCCLM3 decreased the expression of vimentin and increased the expression of E-cadherin in HCC cells, whereas overexpression of miR-331-3p in PLC/PRF/5 increased the expression of vimentin, but decreased the expression of E-cadherin (Fig. 6A). To determine whether miR-331-3p had a similar role in human HCC tissues, we examined the expression of E-cadherin and vimentin as well as the expression of miR-331-3p in one HCC sample with low miR-331-3p expression (D128) and another with high miR-331-3p expression (D149). In situ hybridization showed that miR-331-3p expression in tumor was higher in D149 than in D128 (Fig. 6B). There was relatively higher vimentin expression, but lower E-cadherin expression, in tumor of D149 than those in tumor of D128 (Fig. 6B). Together, the data from the HCC cells and patients’ samples indicate that miR-331-3p promotes EMT in HCC. Given that AKT/glycogen synthase kinase 3 beta (GSK-3β)/Snail signaling is important for EMT,14,26 western blotting was performed to examine whether miR-331-3p-mediated suppression of PHLPP stimulates the activity of AKT/GSK-3β/Snail signaling to facilitate EMT. Results showed that inhibition of miR-331-3p was associated with decreased expression of GSK-3β and Snail; in contrast, ectopic expression of miR-331-3p resulted in increased expressions of these two proteins (Fig. 6C). Taken together, these data support the notion that miR-331-3p promotes EMT by suppressing PHLPP and subsequently activating downstream AKT/GSK-3β/Snail signaling.

**The Polyethylenimine/Anti-miR-331-3p Complex Decreases HCC Growth and Metastasis in Vivo.** Having shown that miR-331-3p increased frequently in HCC and played a critical role in promoting the proliferation and metastasis of HCC, we investigated the potential value of miR-331-3p inhibitor in HCC therapy. To achieve this goal, we constructed anti-miR-331-3p vector as well as its control vector. The vectors were embedded with an in vivo/jetPEI reagent to
establish an \textit{in vivo} drug delivery system named polyethylenimine (PEI)/anti-miR-331-3p or PEI/NC, respectively. Among the five HCC cell lines, HCCLM3 was chosen to create the HCC nude model because of its high metastatic potential.\textsuperscript{27} The PEI/anti-miR-331-3p, PEI/NC, and their solvents (5%...
glucose) were injected into the HCC xenograft model through the tail vein every week, respectively. Tumor volume was monitored, and mice were sacrificed 6 weeks later. Results showed that the tumor sizes of mice injected with PEI/anti-miR-331-3p were significantly smaller than those of mice injected with PEI/NC or 5% glucose (Fig. 7A,B), and no difference was observed between the PEI/NC group and the 5% glucose group. Serial sections with H&E staining demonstrated that mice in the PEI/anti-miR-331-3p group...
had less intrahepatic metastasis (Fig. 7C) and pulmonary metastasis (Fig. 7D) than those of the other two groups. To validate the inhibitory efficiency of PEI/anti-miR-331-3p complex in vivo, in situ hybridization was carried out and results showed that miR-331-3p was significantly inhibited by PEI/anti-miR-331-3p, compared to the PEI/NC and 5% control glucose groups (Fig. 7E). Thus, these results showed that systemic administration of PEI/anti-miR-331-3p complex effectively inhibited miR-331-3p expression in tumor and decreased HCC growth and metastasis in nude mice.

Discussion

Recurrence and metastasis remain the most common lethal outcomes after curative resection in HCC. Thus, it is critical to gain a better understanding of the mechanisms underlying HCC metastasis. In our previous study, we have defined a specific subtype, called SLHCC, with better OS and DFS than that of NHCC after liver resection, which was also validated in our study (Supporting Fig. 11). In this study, we focused on the functions of a newly identified miRNA (miR-331-3p) in HCC. We found that miR-331-3p expression was correlated with poor clinicopathological characteristics and was one of the independent risk factors of OS and DFS. Furthermore, miR-331-3p accelerated the progression of HCC by promoting proliferation and metastasis.

It has been well documented that miRNAs alter target gene expression at a posttranscriptional level. We employed an integrated approach to investigate the mechanism by which miR-331-3p promotes the progression of HCC. PHLPP was predicted to be a direct target of miR-331-3p by bioinformatics analysis and was confirmed with dual luciferase report assay and western blotting. PHLPP has been reported to be down-regulated in many types of tumors and correlated with the growth, tumorigenesis, and metastasis potential of cancer. However, the role of PHLPP in HCC was still unknown. In the present study, we demonstrated that mRNA and protein of PHLPP were both significantly down-regulated in HCC patient specimens. To demonstrate whether PHLPP is a major target to mediate the activity of miR-331-3p, we used a

![Fig. 7. Antitumor effect of PEI/anti-miR-331-3p complex in HCC mouse models. (A) The HCC mouse model was built with HCCLM3 cells. PEI/anti-miR-331-3p, PEI/NC, and 5% glucose were injected through the tail vein from the second day after tumor inoculation, respectively, and drug administration was repeated once a week until the sixth week. Tumors at the sixth week are shown. (B) Volumes of tumors in each group were calculated with the formula: (length × width²)/2. Percentage of mice in each group with or without metastatic nodules in livers (C) or lungs (D) was calculated. (E) Paraffin sections were stained with the in situ hybridization technique to examine expression of miR-331-3p in tumors of the PEI/anti-miR-331-3p, PEI/NC, and 5% glucose groups. Original magnification: 200×. *P > 0.05; **P < 0.05.]
combined loss- and gain-of-function approach to functionally characterize PHLPP in cellular proliferation and metastasis assays. Results showed that knockdown of PHLPP mimicked the roles of miR-331-3p, whereas overexpression of PHLPP antagonized the functions of miR-331-3p, indicating that PHLPP is the primary functional target of miR-331-3p in HCC. Studies have shown that PHLPP plays its role by regulating AKT, PKC, or MAPK pathways. Our data indicate that miR-331-3p, by down-regulation of PHLPP, activates AKT, but not ERK or PKC. Furthermore, we found that the miR-331-3p/PHLPP/AKT axis contributes to EMT of HCC cells. By examining the expression of epithelial and mesenchymal markers (E-cadherin and vimentin) in vitro and in vivo, we demonstrated that elevated miR-331-3p or inhibited PHLPP promotes EMT, whereas knockdown of miR-331-3p or ectopic expression of PHLPP showed an opposite effect. The results together implicate that miR-331-3p promotes proliferation and EMT-induced metastasis by suppressing PHLPP and activating AKT/GSK3β pathways.

To test whether anti-miR-331-3p could be used as a potential therapeutic drug for HCC, we used a clinically tested PEI-derived in vivo-jetPEI transfection reagent (Polyplus-Transfection, Illkirch, France) to deliver anti-miR-331-3p in HCC xenograft mouse models. No sign of toxicity, such as weight loss, local effects, or other visible impairments of these mice, was found after systemic delivery. miR-331-3p expression was inhibited efficiently in the PEI/anti-miR-331-3p group. Also, tumors of the PEI/anti-miR-331-3p group were significantly smaller than those of the other two. In addition, intrahepatic and pulmonary metastasis rates in the PEI/anti-miR-331-3p group were also lower than the others, implicating that PEI/anti-miR-331-3p can be a potential therapeutic drug for HCC. Further studies on the stability, metabolism, toxicity, dose-effect relationship, and fatal dose of PEI/anti-miR-331-3p complex are needed to validate its therapeutic usage.

In conclusion, miR-331-3p is overexpressed in HCC and is significantly correlated with poor prognosis of patients. Our data indicate that miR-331-3p promotes the proliferation and EMT-induced metastasis of HCC by suppressing PHLPP-mediated dephosphorylation of AKT(S473). PEI-based delivery of miR-331-3p through systemic administration may have important therapeutic potential for HCC treatment.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website.