MicroRNA-7 Inhibits Tumor Growth and Metastasis by Targeting the Phosphoinositide 3-Kinase/Akt Pathway in Hepatocellular Carcinoma

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MicroRNAs (miRNAs) are known to be involved in carcinogenesis and tumor progression in hepatocellular carcinoma (HCC). Recently, microRNA-7 (miR-7) has been proven to play a substantial role in glioblastoma and breast cancer, but its functions in the context of HCC remain unknown. Here, we demonstrate that miR-7 inhibits HCC cell growth and metastasis in vitro and in vivo. We first screened and identified a novel miR-7 target, phosphoinositide 3-kinase catalytic subunit delta (PIK3CD). Overexpression of miR-7 would specifically and markedly down-regulate its expression. miR-7-overexpressing subclones showed significant cell growth inhibition by G0/G1 phase cell-cycle arrest and significant impairment of cell migration in vitro. To identify the mechanisms, we investigated the phosphoinositide 3-kinase (PI3K)/Akt pathway and found that Akt, mammalian target of rapamycin (mTOR), and p70S6K were down-regulated, whereas 4EBP1 was up-regulated in miR-7-overexpressing subclones. We also identified two novel, putative miR-7 target genes, mTOR and p70S6K, which further suggests that miR-7 may be a key regulator of the PI3K/Akt pathway. In xenograft animal experiments, we found that overexpressed miR-7 effectively repressed tumor growth (3.5-fold decrease in mean tumor volume; n = 5) and abolished extrahepatic migration from liver to lung in a nude mouse model of metastasis (n = 5). The number of visible nodules on the lung surface was reduced by 32-fold. A correlation between miR-7 and PIK3CD expression was also confirmed in clinical samples of HCC. Conclusion: These findings indicate that miR-7 functions as a tumor suppressor and plays a substantial role in inhibiting the tumorigenesis and reversing the metastasis of HCC through the PI3K/Akt/mTOR-signaling pathway in vitro and in vivo. By targeting PIK3CD, mTOR, and p70S6K, miR-7 efficiently regulates the PI3K/Akt pathway. Given these results, miR-7 may be a potential therapeutic or diagnostic/prognostic target for treating HCC. (HEPATOLOGY 2012;55:1852-1862)

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide.1 Despite the clinical implementation of numerous therapeutic strategies, HCC has a global mortality rate of 94%.2 Given this statistic, there is an urgent need to develop novel strategies for the diagnosis, treatment, and prognosis of HCC.

Recent studies indicate that microRNAs (miRNAs) function to regulate tumor growth and metastasis and are considered promising targets for the diagnosis and prognosis of a number of cancers.3 miRNA-expression profiling has been characterized in a variety of cancers, including breast cancer, lung cancer, ovarian cancer, and HCC.3-5 Previous studies have demonstrated that certain miRNAs are correlated with the proliferation and survival of HCC, including miR-1995 and miR-26a.6

Webster et al.7 recently found that miRNA-7 (miR-7) can regulate epidermal growth factor receptor (EGFR)
expression, which is usually overexpressed in epithelial tumors, such as HCC. microRNA-7 (miR-7) was first reported to inhibit glioblastoma growth by regulating insulin receptor substrate 2 (IRS2) through the EGFR/IRS2/Akt pathway. Reddy et al. also discovered an upstream activator of miR-7 and a novel miR-7 target, p21-activated kinase 1 (Pak1), which is involved in the metastasis of breast cancer. These findings suggest that miR-7 may be associated with HCC progression through the regulation of EGFR expression and other key components of the EGFR pathway. Additionally, the phosphoinositide 3-kinase (PI3K)/AKT/mTOR (mammalian target of rapamycin) pathway, an important pathway downstream of EGFR, is known to be associated with cell proliferation, survival, and motility/metastasis. Based on previous studies indicating that the overexpression of miR-7 inhibits the Akt pathway in glioblastoma, we tested whether miR-7 could regulate HCC tumor growth and metastasis through interactions with the PI3K/AKT/mTOR pathway.

In our study, we screened and identified a novel miR-7 target within phosphoinositide 3-kinase catalytic subunit delta (PIK3CD; also known as p110δ), which functions as an integral component of the PI3K pathway. p110δ is often highly expressed in leukocytic cancers, and this overexpression results in PI3K dysfunction. It is also involved in the neoplasia and tumor progression of neuroblastomas and breast cancers, but its role in HCC carcinogenesis remains unknown.

In this study, we hypothesized that miR-7, acting as a tumor suppressor, could repress tumor growth and inhibit the metastasis of HCC by interacting with the PI3K/AKT/mTOR pathway and targeted regulating of PIK3CD expression.

Materials and Methods

Transfection and Luciferase Assay. The human HCC cell line, QGY-7703, was seeded on 24-well plates and transfected 24 hours later using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. The miR-7 precursor molecules (20 nM/well; product ID: PM10047; Applied Biosystems, Foster City, CA) were cotransfected with 100 ng/well of luciferase reporter plasmids (Supporting Materials and Methods) and 2 ng/well of plasmid cytomegalovirus promoter-driven Renilla luciferase, which served as internal controls for the relative luciferase activity assay by using a dual-luciferase assay reporter system (Promega, Shanghai, China).

Cell-Cycle and Cell-Proliferation Analysis. After trypsinization, cells were suspended and stained with propidium iodide (PI). Cell-cycle assay was performed using an Epics Altra Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA) and was analyzed using EXPO32 Multicomp and EXPO32 v1.2 Analysis (Beckman Coulter) software. For cell-proliferation analysis, cells were seeded onto 24-well plates at 5 × 10^3 cells/well and the cell numbers were determined daily for 1 week. Cell density was photographed on day 4.

In Vitro Migration and Invasion Assay. Migration and invasion assays were performed using the 24-well Cell Migration and Invasion Assay kit (Cell Biolabs, Inc., San Diego, CA), according to the manufacturer’s instructions. Briefly, 2.5-5 × 10^5 cells (for the migration assays) or 1.25 × 10^5 cells (for the invasion assays) were resuspended in serum-free medium and plated in the top chamber. Medium with 10% fetal bovine serum was added to the lower chamber as a chemoattractant. After 24-hour incubation, cells on the lower surface of the membrane were stained, photographed, and counted under a microscope in five fields.

Tumor Xenograft Animal Model. Male athymic nude mice were housed and manipulated according to the protocols approved by the Shanghai Medical Experimental Animal Care Commission. For each mouse, 5 × 10^6 “QGY-null” and “QGY-miR-7” subclone cells were injected subcutaneously (SC) into the right and left scapulas, respectively, in 100 µL of serum-free medium. Tumor growth in the nude mice was measured every 5 days for 30 days. After the mice were sacrificed, total RNA and protein were extracted from tumor tissues to detect miR-7, PIK3CD, and PI3K/Akt-pathway components.

In Vivo Metastasis Assay. Male athymic nude mice were randomly divided into two groups (QGY-null and QGY-miR-7; 5 mice per group), and 5 × 10^5 cells were injected intravenously (IV) via the tail vein. Tumor growth and metastasis were analyzed in situ at week 7-8 after injection by green fluorescent protein.
(GFP) fluorescence imaging (LB981NC10D; Berthold Technologies, Oak Ridge, TN). All mice were euthanized at 9 weeks after the initial injection, and the livers and lungs were excised to examine extrahepatic metastasis from the liver to the lungs.15 All of the organs that were excised were embedded in paraffin for sectioning (5 μm) and staining with hematoxylin and eosin (H&E). The metastatic nodules on the lung and partial liver tissues were snap-frozen for RNA and protein extraction. All treatments were performed according to the protocols described above.

**Clinical HCC Sample Analysis.** Ten surgical specimens (both tumor and adjacent normal tissue) were obtained from patients in Shanghai First Peoples’ Hospital and were snap-frozen in liquid nitrogen and stored at −80°C for later RNA and protein extraction. All HCC patients gave written informed consent for the use of clinical specimens in medical research. The studies using human tissues were reviewed and approved by the Committee for Ethical Review of Research Involving Human Subjects at Fudan University. The clinical and pathological features of these patients are described in the Supporting Table 2.

**Statistical Analysis.** Independent Student’s t tests and analysis of variance were used to compare differences between the two groups. The correlation between miR-7 and PIK3CD messenger RNA (mRNA) expression was measured using Pearson’s chi-square test. Statistical significance was determined by the log-rank test. A P value of less than 0.01 was considered to be statistically significant. Error bars represent standard error (SE), unless otherwise indicated.

**Results**

**PIK3CD Is a Novel Target of miR-7.** Given the observation that EGFR and its downstream components, Pak1 and IRS-2, are targets of miR-7,7,9,10 we wondered whether other miR-7 targets existed in the EGFR downstream pathways. Using TargetScan (www.targetscan.org),16 we identified PIK3CD as being a likely target of miR-7 because it contains four putative miR-7 target sites in its 3’UTR (untranslated region) (Fig. 1A). We generated a series of luciferase reporter vectors (Fig. 1B; Supporting Materials and Methods), containing oligonucleotides that were fully complementary to either the intact mature miR-7 sequence or the full-length wild-type (WT) PIK3CD 3’UTR. To determine the major targets, each putative target site or its relevant mutant was cloned into an identical reporter vector (Fig. 1C).

Pre-human (Homo sapiens)-miR-7 RNAs or nonfunctional control miR-NC (negative control) RNAs were cotransfected with the above-mentioned reporter vectors into the HCC cell line, QGY-7703, which overexpresses p110δ, to assess relative luciferase activity. Our results indicate that miR-7 targets and full-length WT PIK3CD 3’UTRs reduced relative luciferase activity only when miR-7 was present (Fig. 1D). When evaluating the relative contribution of each putative miR-7 target site, we observed that relative luciferase activity was reduced to 56% ± 6% (34 ± 3.5 versus 61 ± 5.3), 42% ± 4% (26 ± 2.5 versus 62 ± 2.3), or 39% ± 6% (24 ± 3.6 versus 62 ± 6.2) when the reporter vectors harbored the putative mir-7 target sites A, B, or C, respectively, but not when the corresponding mutant was introduced with miR-7 (Fig. 1E). Additionally, putative target site D only reduced relative luciferase activity to 76% ± 4% (48 ± 2.6 versus 63 ± 3.8). When the putative target sites A, B, and C were integrated into a new artificial target E, we found that relative luciferase activity was reduced to 42% ± 2% (25 ± 1.2 versus 60 ± 4.2), which was similar to what was observed with the WT PIK3CD 3’UTR (Fig. 1E). These results indicate that PIK3CD mRNA is a specific target of miR-7 and demonstrate that the miR-7 target sites A, B, and C are major sites for interaction with miR-7.

**miR-7 Inhibits HCC Cell Proliferation by Repressing p110δ Expression.** Based on the findings described above, we hypothesized that miR-7 might reduce HCC cell proliferation and arrest cell-cycle progression by repressing p110δ expression. We transiently transfected QGY-7703 with either miR-7 or miR-NC precursors or PIK3CD short interfering RNA (siRNAs) (Supporting Materials and Methods) and found that both miR-7 precursors and PIK3CD siRNAs repressed p110δ expression at both the transcriptional and translational levels (Supporting Fig. 1A). We then measured cell-cycle progression every 4 hours for 48-72 hours after transfection. Our results indicate that the majority of cells were arrested in G0/G1 phase (70%-73%) for 24 hours when transfected with miR-7, whereas no obvious G0/G1-phase arrest was observed when transfected with miR-NC or mock (Fig. 2A; Supporting Fig. 1B, top). By comparing the proportion of cells in S phase (Supporting Fig. 1B, middle) and G2/M phase (Fig. 1B, bottom), we found that cells transfected with miR-7 exhibited a delay in cell-cycle progression for almost 16 hours after transfection (Supporting Fig. 1B). When cells were transfected with PIK3CD siRNA#3, we observed results similar to those obtained in miR-7-transfected cells.
Up to 72 hours after transfection, no obvious apoptosis was observed, as evidenced by the absence of a sub-G1 cell population by PI staining and flow-cytometry analysis (Supporting Fig. 2). These results indicate that miR-7 may arrest cell-cycle progression by repressing p110δ expression.

To verify our observations, we established relevant stable subclones in QGY-7703, which were named QGY-null (mock), QGY-miR-NC (noneffective control), and QGY-miR-7, respectively. Ectopic expression of miR-7 was elevated by approximately 7-fold (Supporting Fig. 3A), which resulted in a 0.24-fold reduction of PIK3CD mRNA (Fig. 2B). Western blotting analysis showed that miR-7 specifically repressed p110δ protein expression (Fig. 2B), but did not affect the expression of the other two p110 catalytic subunits (p110α and p110β) or their corresponding regulatory subunit, p85 (Supporting Fig. 3B).
investigated the effect of the stable expression of miR-7 on HCC cell growth in vitro. Using the cell-proliferation assay, we observed a significant decrease in cell number in QGY-miR-7 cells (538.8 ± 39.0 × 10^3, n = 3; P < 0.01) versus QGY-null cells (1,164 ± 34.1 × 10^3, n = 3; P < 0.01) or QGY-miR-NC cells (949 ± 48.1 × 10^3, n = 3; P < 0.01) on day 7 (Fig. 2C). No apoptosis was observed on day 4 (Supporting Fig. 3C) when miR-7 was stably expressed, indicating that the decrease in cell numbers might be caused by the arrest of cell-cycle progression (Fig. 2C). A similar inhibition in cell proliferation was observed in the PIK3CD siRNA#3 group, but not in the control siRNAs (Supporting Fig. 4).

To further validate our results, we assayed for alterations in cell-cycle progression every 2 hours for 24 hours after 30 hours of serum starvation (Fig. 2D). A G_0/G_1 cell-cycle arrest that was detected in QGY-miR-7 cells was associated with miR-7 overexpression. It took QGY-miR-7 cells 8-9 hours to recover after serum starvation (G_0/G_1 ≤60%), whereas the controls recovered in approximately 5 hours, and the percentage of cells in the G_0/G_1 phase remained over 50% and had no significantly periodic change when miR-7 was stably expressed, which was obviously higher than those in S or G_2/M phase (Fig. 2D, top). By analyzing changes in the cell proportion in S or G_2/M phase, we found that QGY-miR-7 required 14 hours to complete a cell cycle after serum recovery, compared to approximately 12 hours for control cells (Fig. 2D, middle and bottom). All the results were consistent with those observed in transient transfection experiments. These data strongly suggest that miR-7 inhibits HCC cell growth by G_0/G_1 arrest, but not by triggering apoptosis.
miR-7 Decreases HCC Cell Invasion and Migration In Vitro. We further investigated whether overexpression of miR-7 could weaken the invasiveness and migratory capabilities in HCC. Using the wound-healing assay (Supporting Materials and Methods), we found that ectopic expression of miR-7 decreased cell motility in QGY-miR-7 cells, compared to QGY-null and QGY-miR-NC cells (Supporting Fig. 5). Invasive capability was slightly, but not significantly, lower in QGY-miR-7 cells (135 ± 3 per field for five fields), compared to QGY-null (150 ± 5 per field for five fields) or QGY-miR-NC cells (152 ± 6 per field for five fields) (Supporting Fig. 6), suggesting that miR-7 may have a relatively smaller effect on regulating the expression of molecules associated with invasion. In contrast, migratory capacity was significantly down-regulated in QGY-miR-7 cells (120 ± 3 per field for five fields; \( P < 0.01 \)) versus QGY-null (180 ± 8 per field for five fields) or QGY-miR-NC cells (170 ± 6 per field for five fields) (Fig. 3). Similar results were observed in both invasion and migration assays when cells were transiently transfected with PIK3CD siRNA#3 (Supporting Fig. S7). These results indicate that miR-7 participates in the regulation of cell proliferation and migration by directly regulating PIK3CD expression.

miR-7 Regulates the Expression of Key Components of the PI3K/Akt-Signaling Pathway. We further evaluated the effects of p110δ-expression inhibition by miR-7 in the PI3K/Akt-signaling pathway. We found that the transcription of AKT, mTOR, and P70S6K, which are major components of the PI3K/Akt pathway, was down-regulated to 0.4-, 0.25-, and 0.3-fold, respectively, in QGY-miR-7 cells (Fig. 4A). The transcription of eIF4E binding protein 1 (4EBP1), which is usually inhibited by mTOR, was up-regulated by 2.7-fold, as assessed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Total and phosphorylated protein levels of all four molecules described above showed the same results (Fig. 4B), indicating that miR-7 may be an important regulator of this signaling pathway. These findings were also validated by using PIK3CD siRNA#3 (Supporting Fig. 8). Based on these results and previous studies that miRNA could regulate multiple and functionally related targets in one pathway, we wondered whether these four genes could be regulated by miR-7. As a result, no miR-7 target sites were in the AKT or 4EBP1 3’UTR, but one was found in the mTOR 3’UTR (Fig. 5A). Using the luciferase reporter assay, we found that relative luciferase activity was reduced to 38% ± 5% (25 ± 3.5 versus 66 ± 5.3) for the reporter plasmid that contained the putative miR-7 target site, but not the corresponding mutant counterpart that was cotransfected with miR-7 (Fig. 5B). Two putative miR-7 target sites were also found in the P70S6K 3’UTR (Fig. 5C). These two target sites repressed luciferase activity by approximately 50%, when combined with ectopic miR-7 expression (Fig. 5D). These data indicate that miR-7 can regulate the expression of mTOR, p70S6K, and PIK3CD by directly binding to target sites within the 3’UTR. For the PIK3CD expression, the mean level of miR-7 expression within the tumors derived from QGY-miR-7 cells was significantly elevated, and the expression level of both PIK3CD and
the downstream components of the pathway were reduced, compared to the controls (Fig. 6B). These data indicate that overexpression of miR-7 may inhibit HCC tumorigenesis by blocking PIK3CD expression.

We further investigated the effect of miR-7 overexpression on HCC metastasis in vivo. QGY-miR-7 or QGY-null cells were injected into nude mice (n = 5) by IV tail injections to observe the extrahepatic metastatic nodules that formed in lungs and liver. Inoculated cells expressed GFP, allowing us to employ GFP-fluorescence imaging to detect cancer cell distribution in situ (Supporting Fig. 9C) 8 weeks postinjection. We observed high fluorescence intensity in the breasts and upper venters of the control group, but fluorescence was nearly undetectable in the miR-7-overexpression group. Mice were sacrificed 9 weeks after injection, their lungs and livers were excised, and the number of nodules on the surface of both organs was counted. No obvious nodules were observed on the surface of the liver in either group, yet local inflammation and necrosis was found in 1 sample from the QGY-null group (Supporting Fig. 10). Additionally, large nodules on the surface of the lung were observed in all 5 mice in the QGY-null group, whereas only small nodules were detected in 1 mouse from the QGY-miR-7 group. The mean number of metastatic nodules on the surface of the lung was significantly repressed (32-fold) in the QGY-miR-7 group, compared to the QGY-null group (0.2 ± 0.4 versus 6.4 ± 1.1, P < 0.01; Fig. 6C). We examined the expression of miR-7- and PI3K/Akt-pathway components in both liver (Supporting Fig. 11A) and lung-metastatic nodules (Supporting Fig. 11B) and found that the pathway was inhibited by miR-7. Histological staining showed that the lesions in the lungs were caused by extrahepatic extravasation and subsequent tumor growth in the QGY-null group (Fig. 6D). Although no visible nodules were detected on the surface of the liver in either group, a small quantity of HCC cells was observed in the QGY-null group, but not in the QGY-miR-7 group (Supporting Fig. 11C). These data indicate that overexpression of miR-7 can inhibit the tumorigenesis and metastasis of HCC cells in vivo.

miR-7 Regulates the PI3K/Akt Pathway in Clinical HCC Specimens. To further investigate whether miR-7 is involved in HCC progression through regulation of the PI3K/Akt pathway, the expression of miR-7 and components of the PI3K/Akt pathway were analyzed by TaqMan MicroRNA Assay (Invitrogen), qRT-PCR (Fig. 7), and western blotting (Supporting Fig.
12) in 10 pairs of clinical HCC samples. Compared to paired normal tissues, repression of miR-7 expression was detected in 7 of 10 HCC cases. In accord with miR-7 repression (average, 0.44-fold; range, 0.13- to 0.73-fold), the expression of PIK3CD (average, 2.6-fold; range, 1.4- to 4.6-fold), Akt (average, 2.7-fold; range, 1.3- to 5.5-fold), and mTOR (average, 4.1-fold; range, 1.8- to 7.2-fold) was up-regulated in all 7 HCC cases (Fig. 7). Correlation analysis indicated that PIK3CD expression was reduced, along with miR-7 overexpression, in these 10 pairs of HCC specimens ($r^2 = 0.725$, Pearson’s chi-square test; Supporting Fig. 13).

Discussion

HCC remains one of the top three causes of cancer death in the Asia Pacific region and is also a severe disease worldwide. In addition to conventional therapeutic strategies having promise as potentially curative therapies for patients with early HCC, targeted therapies are currently being developed to interfere with the transduction of key signaling pathways or to inhibit the function of tumor-specific molecules, such as tyrosine kinases, in HCC.

In the last decade, miRNA has emerged as a critical regulator of carcinogenesis and tumor progression. Changes in miRNA profiling are associated with almost all aspects of cancer biology, including cell proliferation and metastasis. Studies have shed light on tumor-targeting therapies using miRNAs as a novel diagnostic and therapeutic tool. In this study, we focused on miR-7, which has been demonstrated to suppress tumor growth in brain cancers and metastasis in breast cancer. Chou et al. recently found that miR-7 was overexpressed in lung cancer and was targeted to Ets2 repressor factor, a tumor suppressor, to enhance the oncogenic properties of lung cancer. These findings indicated that miR-7 might function as an oncogenic miRNA in lung cancer. Given this finding, we sought to determine whether miR-7 plays a role in HCC.

In this study, we demonstrated that miR-7 inhibits tumorigenesis and cancer metastasis in HCC both in vitro and in vivo by blocking a novel miR-7 target, PIK3CD. This molecule is a major component of the
PI3K pathway, which functions downstream of EGFR, and transduces signals through the PI3K/Akt pathway. Our results indicate that the growth of HCC cells is repressed by cell-cycle arrest, but not by inducing apoptosis, when miR-7 is overexpressed. We also found that overexpression of miR-7 significantly repressed the migratory capability of HCC cells and inhibited invasiveness. In vivo, tumor volume decreased by approximately 3.5-fold when QGY-miR-7 subclone cells were injected into mice, compared to injection with control cells. Additionally, a significant inhibition of extrahepatic metastasis from the liver to the lungs was observed after overexpression of miR-7. We further investigated the function of miR-7 in the PI3K/Akt pathway to elucidate mechanisms underlying tumorigenesis, progression, and metastasis. We found that mTOR and p70S6K, which are key downstream signals of PI3K, also contained miR-7 target sites in their 3’UTR. Using PIK3CD siRNAs (Fig. 2A; Supporting Figs. 2, 3B, 7, and 8) and mTOR siRNAs (Supporting Figs. 14 and 15) as positive controls, we concluded that miR-7 regulates the PI3K/Akt/mTOR-signaling pathway. The repression of both mTOR and p70S6K by miR-7 and the upstream regulator, PIK3CD, interfered with the transcription of various proteins, including cell-cycle–associated proteins, providing a possible basis for the observed cell-cycle delay.

It was recently revealed that miR-7 is induced by a dysregulation of EGFR signaling and that it acts as an important modulator of EGFR-mediated oncogenesis in lung cancer cells. EGFR is a known target of miR-7. These findings suggest that a negative feedback loop might exist between miR-7 and its targets (Fig. 8, left). We hypothesized that the transcription factors associated with miR-7, such as c-myc and HoxD10, might be activated by the PI3K/Akt/mTOR pathway through an unknown mechanism and...
induce miR-7 expression, resulting in the suppression of miR-7's molecular targets. This homeostasis could be dysregulated in cancer cells by a failure to activate the transcriptional factors, inhibition of the transcription of miR-7, or aberrant action of miR-7 without altering its expression (Fig. 8, right). It is established that polymorphisms within miRNA target sites can impact the interaction between miRNA and target mRNAs, a process that is associated with neoplasia, disease, and organismal development. In addition to miRNA target-site mutations, chromosomal translocations, which separate the oncogene open reading frame (ORF) from its 3'UTR, containing related miRNA target site(s), and alternative splicing events may also lead to the loss of miRNA function in the post-transcriptional regulation.

We explored miR-7 function in the context of HCC. We previously compared the endogenous expression of miR-7, PIK3CD mRNA, and p110δ proteins in QGY-7703 with L-02, a normal liver cell line (Supporting Fig. 16A). We found that both PIK3CD mRNA and p110δ protein were overexpressed in QGY-7703, although they had a similar level of miR-7 expression. We then aligned PIK3CD 3'UTRs cloned from QGY-7703 and L-02, but no mutations in the miR-7 target regions were found (Supporting Fig. 16B). It has not been reported that chromosomal translocation or alternative splicing events occur at the PIK3CD gene locus in HCC. We demonstrated that overexpression of miR-7 markedly down-regulated the reporter luciferase activity, indicating that luciferase expression was suppressed when PIK3CD 3'UTR was cloned into the 3' terminal region of the luciferase ORF. When miR-7 was transiently transfected into cells, both PIK3CD mRNA and...
p110δ expression was repressed, compared to the controls (Supporting Fig. 1A). Our data allow us to conclude that no alteration of miR-7 action occurred in HCC, and we speculate that the reason that p110δ overexpression did not stimulate miR-7 expression in response to HCC was likely the result of a failure to activate the transcriptional regulators of miR-7 and/or a failure to promote miR-7 expression. Further studies of the mechanisms underlying this process are required.

In conclusion, we found that PIK3CD is a novel target of miR-7. As a tumor suppressor in HCC, overexpression of miR-7 arrests cell-cycle progression and impairs cancer cell migration both in vitro and in vivo. Our results revealed that miR-7 regulates cell proliferation and metastasis through the PI3K/Akt/mTOR pathway and indicates that exogenous overexpression of miR-7 may prove to be a promising strategy for targeted HCC therapies.

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