An In Vivo Method to Identify microRNA Targets Not Predicted by Computation Algorithms: p21 Targeting by miR-92a in Cancer

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Abstract

microRNA (miRNA) dysregulation is involved in the development and progression of various human cancers, including hepatocellular carcinoma (HCC). However, how to identify the miRNAs targeting specific mRNA in cells is a significant challenge because of the interaction complexity and the limited knowledge of rules governing these processes. Some miRNAs are not predictable by current computer algorithms available. Here, using p21 mRNA as target, we established a new method, called miRNA in vivo precipitation (miRIP), to identify which kind of miRNAs can actually bind to the specific mRNA in cells. Several unpredictable miRNAs that bound p21 mRNA in HepG2 and PC-3 cells were identified by the miRIP method. Among these miRNAs identified by miRIP, miR-92a was found and confirmed to interact robustly with p21 mRNA, both in HepG2 and PC-3 cells. miR-92a was found to be remarkably increased in HCC tissues, and higher expression of miR-92a significantly correlated with lower expression of p21, which is related to poor survival of HCC patients. Moreover, inhibition of miR-92a could significantly suppress HCC growth in vitro and in vivo by upregulating p21. Together, miR-92a, which is identified by miRIP, is functionally shown to be associated with HCC growth as an oncogenic miRNA by inhibiting expression of targeting gene p21. In addition, several unpredictable miRNAs that target STAT3 mRNA were also identified by the miRIP method in HepG2 cells. Our results demonstrated that the miRIP approach can effectively identify the unpredictable but intracellular existing miRNAs that target specific mRNA in vivo. Cancer Res; 75(14); 1–11. ©2015 AACR.

Introduction

miRNAs (miRNA) are small noncoding RNAs that are involved in diverse biological processes, including development, proliferation, metabolism, apoptosis, and cell motility (1). The dysregulation of miRNAs has been extensively shown to contribute to various human pathological processes such as carcinogenesis (2–5). Identifying the miRNAs that can target important players in the carcinogenesis and progression of cancer is valuable to understand the characteristics of the cancer types and design new therapeutics. However, how to identify the miRNAs targeting mRNA in cells is a significant challenge because of the interaction complexity and the limited knowledge of rules governing these processes. In the last decades, predictable applications and experimental methods had been established to identify the miRNA–mRNA interaction in cells (6, 7). Although a large number of predicted targets were proved to be false positives, in other cases, many miRNA–target associations identified by experimental methods did not reflect the physiological state (8). Therefore, it is important to establish a simple and reliable experimental protocol to discover and detect miRNA targeting a specific mRNA in vivo.

p21 (also named CDKN1A), a potent cyclin-dependent kinase inhibitor, binds and inhibits the activity of cyclin-CDK2 or -CDK1 complexes, and thus functions as a regulator of cell-cycle progression at G1 (9). Although mutations and deletions of the p21 gene are rare, p21 is found to be frequently downregulated in many human cancers, including hepatocellular carcinoma (HCC; ref. 10). Mice lacking p21 spontaneously develop hematopoietic, endothelial, and epithelial tumors (11). p21 is transcriptionally regulated through p53-dependent/-independent mechanisms (12). Now, translational control has increasingly emerged as a prominent mechanism of p21 regulation, especially through miRNAs targeting. Multiple of miRNAs were identified to target p21 by predictable application (e.g., miR-93, miR-106a/b, miR-20a/b, miR-17, miR-372, and miR-519d, etc.) and experimental methods (e.g., miR-296-5p; refs. 11 and 13–15). However, whether there are other miRNAs targeting p21 in human cancers and how quickly and accurately to identify these miRNAs need to be further investigated.

In this paper, with biotin-tagged p21 mRNA antisense oligonucleotides (p21 probe), we established an efficient protocol, named as miRNA in vivo precipitation (miRIP), to find the miRNAs targeting p21 mRNA in cancer cells. HCC is one of the
most common human malignant tumors in the world and the second leading cause of cancer-related death in China (16). Although clinical treatments have been developed to manage HCC, uncontrolled metastasis and high recurrence always lead to poor prognosis of HCC patients (17). Therefore, it is urgent to explore the molecular mechanisms underlying carcinogenesis and progression of HCC and identify the potential targets for the design of new therapeutics. Therefore, we selected HCC cells HepG2 as the cell model to confirm the effectiveness of miRIP we developed. Several unpredictable miRNAs targeting p21 mRNA were discovered. Among these miRNAs, miR-92a was found to target p21 mRNA in not only HCC cells HepG2 but also prostate cancer cells PC-3. miR-92a was shown to be functionally associated with HCC growth as an oncogenic miRNA by inhibiting expression of the target p21 both in vitro and in vivo. In addition, several unpredictable miRNAs that could target STAT3 mRNA were also discovered by using an miRIP method in HepG2 cells. Therefore, the miRIP method we developed can efficiently identify the unpredictable miRNAs targeting specific mRNA in vivo.

Materials and Methods

Cell cultures and treatments

HepG2, QGY-7703, and PC-3 cell lines were obtained from the ATCC. HepG2 and QGY-7703 cells were cultured in Eagle’s Minimum Essential Medium containing 10% fetal bovine serum (FBS). PC-3 was cultured in Ham’s F12K media containing 10% FBS. Cells were maintained at 37 °C and 5% CO2.

Transfection of in vitro growing cell lines with p21 siRNA, miR-92a mimics and inhibitors were conducted as described in the Supplementary Materials.

miRNAs in vivo precipitation

All the following experiments were performed at room temperature except for special mentions. The used probes were listed in Supplementary Table S11.

Buffers. Lysis buffer contained 20 mmol/L Tris, pH 8.0, 500 mmol/L NaCl, 2.5 mmol/L MgCl2, 2% SDS, Superase-In (Ambion), and protease inhibitors (Roche). Wash buffer A contained 10 mmol/L Tris, pH 8.0, 1 M NaCl, 40 mmol/L EDTA, Superase-In, and protease inhibitors. Wash buffer B contained 10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 40 mmol/L EDTA, 1% SDS, Superase-In, and protease inhibitors. Superase-In and protease inhibitors were freshly added into solution immediately prior to use.

Cell culture. Cells were seeded in a 10-cm dish at the density that allowed them to grow for 48 hours without reaching complete confluency. Cells were transfected by the biotin-tagged–specific probe or control probe at a final concentration 200 nmol/L and incubated in 37 °C, 5% CO2 incubator.

Beads preparation. One hundred microliters of Streptavidin Dynabeads (M-280, Invitogen) slurry for each sample was thoroughly homogenized by shaking in 1.5 mL Eppendorf tube, which was washed twice with Solution A (0.1 mol/L NaOH, 0.05 mol/L NaCl) and once with Solution B (0.1 mol/L NaCl). To prevent nonspecific binding of RNA and proteins, the beads were coated with RNase-free BSA and yeast tRNA (both from Ambion). One hundred microliters of bead solution supplemented with 10 μL BSA (10 mg/mL) and 10 μL tRNA (10 mg/mL) was mixed by rotation for 3 hours. After that, the beads mixture were washed twice with 500 μL lysis buffer and resuspended in 1,000 μL lysis buffer ready to be used.

RNA purification. Cells were harvested after transfection 24 hours. Cells were cross-linked by 1% formaldehyde for 10 minutes, equilibrated in glycine buffer for 5 minutes, washed with cold PBS three times, scraped with 1 mL lysis buffer and incubated for 10 minutes. Cell samples were sonicated (VCX 130, SONICS, and MATERIALS) using the following parameters: 50% amplitude, 30 seconds constant pulse, and 30 seconds pause for 10 minutes; and then the samples were centrifuged at 10,000 × g for 10 minutes. The supernatant was transferred to a 2-mL tube and separately saved 50 μL as input analysis. The supernatant lysate was incubated with 1 mL prepared M-280 beads for 1 hours with rotating. The beads–sample mixture was washed twice with wash buffers A and B, and finally incubated with 200 μL lysis buffer for 2 hours to reverse the formaldehyde cross-links. Subsequently, beads–sample mixture was added with 300 μL of TRIzol (Sigma), shaking for two to three times for 10 seconds, and standing for 5 minutes. After 100 μL chloroform added, the solution was shook vigorously for 30 seconds and spun at 12,000 × g at 4 °C for 15 minutes. The supernatant was transferred to a fresh tube. RNAs were precipitated with 1 μL of DNAmate (TaKaRa), 36 μL 3 M NaCl, and 360 μL isopropyl alcohol for at least 1 hour at ~80 °C before pelleting by centrifuging (12,000 × g, 30 minutes, 4 °C). The supernatant was removed, and the pellet was washed with 500 μL 70% ethanol in RNase-free water. The ethanol was carefully removed and allowed the pellet to dry in air. RNAs were dissolved in 50 μL RNase-free water. After DNase I treatment, RNAs were purified by TRIzol again.

Exiqon multiplex miRNA arrays assays

Expression analysis of miRNAs was used the Exiqon multiplex miRNA arrays according to the manufacturer’s instructions. Initial data analysis was performed using the software supplied with the real-time PCR instrument to obtain raw Cq values (Cp or Ct, depending on PCR instrument). We recommend performing normalization and further data analysis with the GenExq PCR analysis software (www.exiqon.com/mirna-pcranalysis).

RNA quantification

Total RNA were purified by TRIzol (Sigma). Purified mRNA and miRNAs were detected by qRT-PCR assay using All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia). The used primers were listed in Supplementary Table S11.

Vector construction and luciferase reporter assay

The vectors used in this study were constructed as described in Supplementary Materials, and luciferase reporter assay was performed as described previously (18).

Cell-proliferation assay

Cell proliferation was measured using the CCK-8 Assay Kit (Dojindo Corp.). A total of 2,500 cells were plated into each well of a 96-well plate and transfected. On the day of culture harvest, 10 μL CCK-8 was added to 90 μL of culture medium. The cells were subsequently incubated for 2 hours at 37 °C and the optical density was measured at 450 nm. Three independent experiments were performed to ensure the statistical significance.
EdU incorporation assay

Cell proliferation was assessed by Cell-Light EdU DNA Cell Proliferation Kit (RiboBio), according to the manufacturer’s instructions. A total of 2 × 10^6 cells were seeded in 6-well plates. Forty-eight hours after transfection, 5-ethyl-2’-deoxyuridine (EdU; 50 μmol/L) was added and the cells were cultured for an additional 2 hours. The cells were then harvested and stained according to the following protocol: discard the EdU medium mixture, digest the cells and add 4% paraformaldehyde to fix cells at R for 30 minutes, wash with glycine (2 mg/mL) for 5 minutes, centrifuge and discard the supernatant, add click reaction buffer (Tris-HCl, pH 8.5, 100 mmol/L; CuSO4, 1 mmol/L; Apollo 550 fluorescent azide, 100 mmol/L; ascorbic acid, 100 mmol/L) for 30 minutes while protecting from light, wash with 0.5% Triton X-100 for three times, and, finally, add 500 μL PBS. Flow cytometry was performed with a FACS Calibur Flow Cytometer to detect the EdU-positive cells.

Cell-cycle analysis

The cell cycle was analyzed by flow cytometry. Cells were plated 24 hours prior to transfection in 6-well plates. Nocodazole (100 ng/mL; Sigma-Aldrich) was added 24 hours after transfection for another 16 hours, then the supernatant was replaced by fresh medium supplemented with 10% FBS for 6 hours. Cells were harvested and washed in PBS, then fixed in 75% alcohol for 60 minutes at 4°C. After washed with cold PBS for three times, cells were resuspended in 1 mL of PBS solution with 40 μg of PI and 100 μg of RNase A (Sigma) for 30 minutes at 37°C. Samples were then analyzed for their sequence content by FACS Calibur.

HCC-bearing nude mouse model and in vivo treatment

All animal experiments were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai. Experiments were prepared and treated as previously described (19). The HCC-bearing male nude mice were prepared from subcutaneous passage of SMMC-LTNM mice. 0.2 mL grinded SMMC-LTNM tumor tissue was subcutaneously injected to the back of the mice and inoculated for about 2 weeks. When tumors reached 5 to 15 mm³, the animals were randomized in two groups (n = 5 mice per group) and treated with cholesterol-conjugated miR-92a inhibitors for 2 weeks. For in vivo delivery of cholesterol-conjugated miR-92a inhibitors (Ribobio), 10 nmol RNA in 0.1 mL saline buffer was locally injected into the tumor mass once every 3 days for 2 weeks. Tumor size was measured and serum AFP was detected using ELISA (AutoBio).

For survival analysis, when tumors reached 5 to 15 mm³, the animals were randomized in two groups (n = 9 mice per group) and treated with cholesterol-conjugated miR-92a inhibitors as described above for 2 weeks. Tumors were measured every 3 days and volume was calculated by the formula (length × width^2)/2. Animals were sacrificed when the tumor volume reached 1,000 mm³, which was also the end-point of the overall survival analysis.

Western blot

Total protein was extracted by lysing cells in RIPA buffer containing protease inhibitor. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% nonfat milk in TBS-T, membranes were incubated with the primary antibody. The following antibodies were used: mouse anti-p21 McAb are from Technology and anti-β-actin and goat-anti-rabbit IgG conjugated to horseradish peroxidase (Technology), which was used as the secondary antibody. Protein was detected with image acquisition using Chemiluminescent Western Blot Scanner (Gene Company).

IHC

IHC was conducted as described previously (20). p21 levels in HCC and adjacent nontumor tissues were evaluated by IHC using anti-p21 mAb (Technology) on commercial tissue arrays (Shanghai Superchip Biotech). The array contained 90 HCC and 90 adjacent nontumor specimens. Staining intensity of each sample was given a modified histochemical score (MH-score), considering both the intensity and the percentage of cells stained at each intensity. All samples were divided into no/weak staining or strong staining according to the average MH score.

Formalin-fixed, paraffin-embedded tissues were cut into 4-μm sections. Following deparaffinization, sections were rehydrated and subjected to antigen retrieval by microwaving in 0.01 M sodium citrate (pH 6) for 10 minutes. Sections were incubated at 4°C overnight with monoclonal antibodies against PCNA and Ki-67 (Technology). Immunostaining was performed using ChemMate DAKO EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (code K 5007; DakoCytomation) according to the manufacturer’s instructions. Subsequently, sections were counterstained with hematoxylin (Dako) and mounted in dimethyl benzene.

In situ hybridization

In situ hybridization (ISH) was conducted as described previously (21). miR-92a level in HCC and adjacent nontumor tissues were evaluated by ISH using specific digoxin labeled miR-92a probe on commercial tissue arrays (Shanghai Superchip Biotech). The sequence of the miR-92a probe was shown in Supplementary Table S11. Staining intensity of each sample was given a modified histochemical score (MH score), considering both the intensity and the percentage of cells stained at each intensity. All samples were divided into no/weak staining or strong staining according to the average MH score.

Sequence alignment analysis

Sequences of mRNA and reverse complemented of miRNAs were aligned by VetoR NTI. Sequences of miRNAs were aligned by ClustalX.

Accession number

Data have been deposited at GEO under accession number GSE68281.

Statistical analysis

All data are presented as the mean ± SD. The Student t test was used to analyze the difference between two experimental groups and a two-tailed P < 0.05 was taken to indicate statistical significance. For analyzing survival of HCC patients, log-rank test in SPSS 15.0 was used with the P values indicated. Analysis of univariate Cox proportional hazards regression was also conducted using SPSS 15.0 with the hazard ratios and P values indicated. Survival analysis was performed by using a Kaplan–Meier plot, and significance assessed by the log rank test.
Results

Affinity purification of target mRNA by the specific probe

miRIP is used to purify miRNAs in vivo from an endogenous segment of mRNA using nucleic acid hybridization. Briefly, the cells were transfected with a specifically designed probe and then cross-linked by formaldehyde. After mRNA was solubilized, the hybridized mRNA complex was captured by magnetic beads, washed, and eluted. The associated miRNAs were identified by multiplex miRNA array (Fig. 1A).

The target p21 mRNA and HCC cells HepG2 were used to develop this method. Probe, reagents, and experimental conditions are key factors. The length and sequence of the probe are two important factors to determine the effectiveness of probe. We optimized the length of the probe (ca. 20, 30, 40, 50, and 60 nt) and found 50 nt was the optimal probe length (data not shown). For the sequence design, miRNAs perform their functions by binding Argonaute (AGO) 1 to 4 proteins. StarBase (http://starbase.sysu.edu.cn/index.php) provides the information of the binding sites between AGO1 to AGO4 and a specific mRNA (22). Theoretically, miRNAs are possibly enriched in such sites. Thus, designing probe sequence complementarily near to such sites is believed to be more efficiently to fish miRNA targets. Therefore, our probes used in this study are designed on the basis of the mRNA-AGO analysis by StarBase. Furthermore, we applied locked nucleic acid (LNA) to modify the probe to increase the stability of the probe–mRNA interactions. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting 2'-oxygen and 4'-carbon. Such bridge locked ribose conformation,

Figure 1.
Affinity purification of p21 and STAT3 mRNA by special probe in HepG2 and PC-3 cells. A, schematic outline of the affinity purification procedure. B, p21 mRNA and probe binding sites. The green bar, 5'-UTR; the yellow arrow, coding sequence; the blue bar, 3'-UTR; the black bars and the numbers at the bottom denote regions analyzed by qRT-PCR; red bar, the probe binding sites. C, RT-PCR analysis of region “0” of p21 miRNAs from p21 probe and control probe affinity purification in HepG2 and PC-3 cells. D, qRT-PCR analysis of regions of p21 miRNAs from p21 probe and control probe affinity purification in HepG2 cells and PC-3 cells. E, STAT3 mRNA and probe binding sites. Green bar, 5'-UTR; orange arrow, coding sequence; the blue bar, 3'-UTR; the black bars and the numbers on the bottom denote regions analyzed by qRT-PCR; red bar at the top, the probe binding sites. F, RT-PCR analysis of region “0” of STAT3 miRNAs from STAT3 probe and control probe affinity purification in HepG2 cells. G, qRT-PCR analysis of regions of STAT3 mRNA from STAT3 probe and control probe affinity purification in HepG2 cells. P, p21 probe; S, STAT3 probe; C, control probe.
which significantly increases the hybridization properties (melting temperature) of oligonucleotides (23). As probe secondary structure is another reason to affect the probe–mRNA interaction, the sequence of probe should avoid high guanine and cytosine content. Based on the comprehensive considerations of these factors above and many preliminary experimental trials, we designed the 5′ end biotin-tagged DNA probe for p21 mRNA. The probe binding sites to the corresponding mRNAs are indicated in Fig. 1B. A control probe that cannot hybridize with any miRNAs was also designed.

In addition to the design of probe, we optimized the components of the solutions, the cell growing and transfecting conditions, and numerous parameters of temperature, time, and centrifugation, etc. It is worth mentioning that in order to increase immobilization of beads–probe–mRNA complex, we incubated beads and cell lysate at room temperature rather than the commonly used 4°C (on ice). Unlike strategies of antibody–antigen affinity, nucleic acid hybridization is insensitive to the presence of ionic detergents (24), which allows the use of detergents such as SDS to limit contamination and preserve integrity of RNA at room temperature or high temperature such as 65°C. All final experimental parameters and conditions were determined after numerous experimental trials. In the method summary, the specific experimental protocol had been described in detail.

The probe of p21 mRNA was transfected into HepG2 cells to hybridize p21 mRNA for 24 hours. The associated miRNA of p21 was purified using Streptavidin Dynabeads (M-280) and isolated by TRIzol. RT-PCR analysis of the mRNA extractions demonstrated a specific enrichment for the mRNA of p21 (Fig. 1C). The scope analysis of purified mRNA was also investigated by qRT-PCR using multiple primers in different positions. Because of the supportive evidence in Fig. 1D, the purified mRNA segments were around 1,000 bp and the majority segments were approximately 500 bp around probe binding sites. To validate the efficiency and reliability of this method, we performed miRIP experiments by using the same p21 probe in PC-3 cells and one STAT3 probe in HepG2 cells. As shown in Fig. 1C to G, ~1,000 bp mRNA segments around probe binding sites were also purified by p21 probe in PC-3 cells and by STAT3 probe in HepG2 cells.

Analysis of miRNAs targeting to the specific mRNA loci

Exiqon multiplex miRNA array was used to analyze the sequences of several hundreds of miRNAs simultaneously from a single RT-PCR reaction to discover these associated miRNAs (25). With this array analysis, we found that multiple miRNAs, including miR-296-5p, which was identified to target p21 mRNA in HCC (17), were interacted with p21 mRNA in HepG2 cells (Fig. 2A). Three miRNAs (miR-92a, miR-92b, and miR-296-5p) that most robustly interacted with p21 mRNA were confirmed by RT-PCR (Fig. 2B). In addition, multiple miRNAs, including miR-92a and miR-17-5p, were confirmed to interact with p21 mRNA in PC-3 cells (Fig. 2C and D). The corresponding results for STAT3 indicated that multiple miRNAs, including miR-92b, miR-23a, and miR-17, were enriched in HepG2 cells (Fig. 2E and F).

To investigate whether such purified miRNAs could be predicted by computational algorithms, we used five algorithms, TargetScan, PicTar, PITA, miRanda, and RNA22 to predict all possible miRNAs that can target to the given mRNA regions isolated by the probes (about 1,000 bp around probe binding site as in Fig. 1B). However, we found that almost all the robustly interacted miRNAs (>10-fold) we identified could not be predicted by any of five prediction algorithms and almost all predicted miRNAs showed weak interaction with p21 mRNA in HepG2 cells (Fig. 2G; Supplementary Tables S1–S5). Similar results were also found for p21 probe in PC-3 cells (Fig. 2H; Supplementary Tables S1–S5) and for STAT3 probe in HepG2 cells (Fig. 2I; Supplementary Table S6–S10). In addition, no similar sequences were found in the most robustly interacted miRNAs and it was very difficult to summarize a complementary pattern between the most robustly interacted miRNAs and their targeting miRNAs (Supplementary Fig. S1).

miR-92a directly targets p21 mRNA and downregulates p21 protein expression

Because miR-92a was the most robust miRNA, targeting p21 mRNA, which was found and confirmed by miRIP both in HepG2 and PC-3 cells, so miR-92a was selected to determine whether such newly discovered miRNA was capable of regulating expressions and biological functions of its targeting gene. First, luciferase assay was used to test miRNAs regulation of p21. The results indicated that miR-92a negatively regulated p21 (Fig. 3A and B). Furthermore, HepG2 cells were transfected with miR-92a inhibitors or mimics. Using the miRIP method, we found that the affinity purified of miR-92a was significantly decreased in inhibitor-treated cells and increased in mimics-treated cells. Although, the purified p21 mRNA and miR-296-5p did not change both in inhibitor-treated cells and mimics-treated cells (Fig. 3C). Western blotting and RT-PCR were used to prove that the miRNA-mediated regulations were at the protein level but not at mRNA level, that is, p21 mRNA expression was not affected by both miR-92a inhibitors or mimics, whereas p21 protein expression was increased by miR-92a inhibitors and decreased by miR-92a mimics, respectively (Fig. 3D). In addition, similar results were also obtained in PC-3 cells (Supplementary Fig. S2). Therefore, miR-92a-mediated regulation of p21 expression is at posttranscriptional level in human cancer cells.

Higher miR-92a expression and lower p21 protein expression correlate with poor survival rate of HCC patients

Numerous studies demonstrated that p21 was downregulated or miR-92a upregulated in several human cancers, including HCC (12, 26). To investigate the association between miR-92a and p21 in HCC tissues, ISH was performed to detect expression of miR-92a, whereas IHC staining was performed to detect the protein expression of p21 by using HCC tissue arrays. As shown in Fig. 4A and B, expression of miR-92a was significantly increased (P < 0.001), whereas protein level of p21 was significantly decreased in HCC tissues versus adjacent nontumor tissues (P < 0.001). In order to explore the correlation between miR-92a and p21 expression in HCC progression, after normalization to adjacent nontumor tissues, RNA level of miR-92a and protein level of p21 in HCC tissues were analyzed by Pearson correlation coefficient analysis. Markedly, p21 protein level was inversely correlated with miR-92a expression level in HCC tissues (r = −0.6665, P < 0.001; Fig. 4C).

To investigate whether low endogenous p21 or high miR-92a in HCC tissues correlated with prognosis of HCC patients, total of 90 HCC patients who had undergone tumor resection between year 2006 and 2010 were analyzed by the Kaplan–Meier method. As shown in Fig. 4D and Table 1, statistical analysis revealed that patients with low p21 (P = 0.0209) or high miR-92a (P = 0.0025) had a poorer prognosis with shorter survival time.

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Collectively, these results imply that high endogenous miR-92a may downregulate p21 protein level in HCC tissues. miR-92a-mediated p21 decrease may contribute to the progression of HCC and may be a prognosis predictor of HCC patients.

Downregulation of miR-92a inhibits HCC growth in vitro by targeting p21

The above results showed that miR-92a was upregulated in HCC and directly targeted and regulated p21 in cancer cells, and
miR-92a targets and regulates the expression of p21 in HepG2 cells. A, schematic representation of pMIR-firefly luciferase reporter construction. 481 bp of p21 is the majority purified fragment in Fig. 1B; different purified fragments (168 and 320 bp) are also indicated. B, analysis of luciferase activity. HepG2 cells were cotransfected with pMIR-firefly luciferase reporter plasmids, pTK-Renilla luciferase plasmids, together with miR-92a inhibitors (anti-92a) or miR-92a mimics (miR-92a). After 36 hours, firefly luciferase activity was measured and normalized by Renilla luciferase activity. C, qRT-PCR analysis the affinity purification of p21 mRNA, miR-296-5p, and miR-92a in HepG2 cells treated with anti-92a or miR-92a. D, effects of miR-92a on the endogenous p21 levels were analyzed by Western blotting and RT-PCR. Data, mean ± SD of one representative experiment (n = 3). Similar results were obtained in three independent experiments. **, P < 0.01; ***, P < 0.001. NC, negative control oligonucleotides.

Identification of miR-92a Targeting p21 mRNA

Figure 3.

Downregulation of miR-92a inhibits HCC growth in vivo

A human HCC-bearing nude mouse model SMMC-LTNM (28) was employed to identify the in vivo effect of miR-92a on HCC growth. Compared with other human HCC-bearing nude mouse models generated by subcutaneously inoculating with HCC cell lines, SMMC-LTNM is more similar to clinical progression of HCC model generated by subcutaneously inoculating with HCC cell lines, SMMC-LTNM is more similar to clinical progression of HCC. As AFP is detected with high level in sera of SMMC-LTNM model, SMMC-LTNM is more similar to clinical progression of HCC bearing mice. These in vivo observations further confirmed a potential oncogenic effect of miR-92a in HCC and suggested the potential use of miR-92a inhibitors in the treatment of HCC.

The functional studies both in vitro and in vivo indicate that miR-92a is functionally associated with HCC growth as an oncogenic miRNA by inhibiting p21 expression.

Discussion

In this study, we successfully established a new experimental approach (miRIP) to identify the unpredictable miRNAs that can target specific mRNA in cells. Several unpredictable miRNAs targeting p21 mRNA and STAT3 mRNA were discovered through miRIP approach. miR-92a is identified and confirmed to target p21 mRNA both in HepG2 cells and PC-3 cells. miR-92a was also found to be remarkably increased in HCC tissues, and higher expression of miR-92a significantly correlated with lower expression of p21, which was related with poor survival of HCC patients. Moreover, inhibition of miR-92a could suppress HCC growth both in vitro and in vivo through targeting p21. Therefore, our method could be applied universally to discover the unpredictable but intracellular existing miRNAs that target specific mRNA in vivo.

miRNAs play an important role in the regulation of gene expressions in plant and animal (4). The commonly accepted mechanism in animal for miRNA regulation is that the “seed region” (2–8 nt at 5’ end) of miRNA is complementary with 3’ untranslated region (3’-UTR) of mRNA (3, 29). Most of the current target prediction algorithms such as miRanda, TargetScan, and PicTar are programmed based on the common mechanism.
However, the number of predicted targets tends to be large. Some of the predicted targets are proved to be false positives by further experimental validation (30). Moreover, more updated mechanisms of miRNA regulation are being discovered, such as, some mismatches in the "seed region" are allowed; some miRNAs may regulate mRNAs through complementarity with the translated region and 5'-UTR of mRNAs; and miRNAs may use their middle continued 10 to 12 nt to target mRNAs (31, 32). In the meanwhile, more efforts are used to develop an experimental method to unveil the miRNA–mRNA interaction. In the reviews and articles (9, 10, 33), a number of experimental methods are summarized and compared. For example, ectopic expression of one miRNA following by the analysis of altered mRNA or protein abundance was performed to studies the regulatory potency of an individual miRNA (34, 35). Various pull-down strategies were

Table 1. Univariate analysis of factors correlated with overall survival of HCC patients

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>Case number</th>
<th>HR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-92a (lower vs. higher)</td>
<td>45/45</td>
<td>2.49 (1.37–4.51)</td>
<td>0.0025</td>
</tr>
<tr>
<td>p21 (lower vs. higher)</td>
<td>45/45</td>
<td>0.49 (0.27–0.89)</td>
<td>0.0209</td>
</tr>
<tr>
<td>Sex (male vs. female)</td>
<td>77/13</td>
<td>1.05 (0.45–2.49)</td>
<td>0.9040</td>
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<tr>
<td>Age (&gt;55 years vs. ≤55 years)</td>
<td>42/48</td>
<td>0.99 (0.55–1.80)</td>
<td>0.9910</td>
</tr>
<tr>
<td>Cirrhosis (yes vs. no)</td>
<td>32/58</td>
<td>0.78 (0.42–1.47)</td>
<td>0.4460</td>
</tr>
<tr>
<td>Tumor size (&gt;5 cm vs. ≤5 cm)</td>
<td>54/36</td>
<td>2.09 (0.93–4.42)</td>
<td>0.0261</td>
</tr>
<tr>
<td>Tumor number (&gt;1 vs. 1)</td>
<td>9/81</td>
<td>1.97 (0.88–4.42)</td>
<td>0.0998</td>
</tr>
<tr>
<td>TNM stage (III/IV vs. I/II)</td>
<td>47/43</td>
<td>2.65 (1.40–5.01)</td>
<td>0.0029</td>
</tr>
<tr>
<td>Histological grade (&gt;II vs. ≤II)</td>
<td>36/54</td>
<td>1.28 (0.75–2.19)</td>
<td>0.3670</td>
</tr>
</tbody>
</table>

NOTE: Bold, P < 0.05 indicate statistical significance. Abbreviations: CI, confidence interval; TNM, tumor-node-metastasis staging system.
established, including the immunopurification of ectopically expressed RISC components or the affinity purification of synthetic miRNAs transfected into cells (36–41). Many approaches aiming at the target-specific identification of regulatory miRNAs are based on the copurification of miRNAs with either endogenous or exogenously expressed mRNAs (10, 42–44). However, some of these experimental methods failed to discriminate between primary and secondary miRNA targets, whereas in other cases, the identified miRNA–target associations did not reflect the physiological state (10). Thus, it is important to establish a simple and reliable experimental protocol to discover and detect the existing miRNAs that can target the specific mRNA in vivo.

In this study, we established a new experimental approach (miRIP) that can identify and detect the unpredictable miRNAs, which target specific mRNA in cells. For the miRNAs regulating p21 expression, we found different miRNA candidates in HepG2 cells and PC-3 cells by miRIP. miR-106b and miR-93 had been reported to downregulate p21 expression in Snu-16 cell line (45). However, miR-106b showed very low affinity interaction with p21 mRNA in HepG2 cells and PC-3 cells, and miR-93 was only 5-fold enrichment in HepG2 cells and 0.7-fold enrichment in PC-3 cells. Such results indicated that a gene may be regulated by different miRNAs in different cell types to maintain various cell functions. Moreover, we detected total miRNA expressions using the same multiplex
miRNA array (data not shown) in HepG2 cells. We found that total miR-92a and miR-93 showed high expression while miR-92b, miR-296-5p, and miR-106b showed low expressions (Supplementary Fig. S3). Our miRIP data showed that high expression of miRNA did not result in high interaction with p21 mRNA (e.g., miR-93), whereas low expression of miRNAs could result in high interaction (e.g., miR-92b and miR-296-5p). Our data firstly revealed the intracellular existing miR-92a that can target p21 mRNA both in HepG2 cells and PC-3 cells. However, CLASH data, which was considered as a comprehensive investigation of miRNA–mRNA interaction in one cell, had not indicated miR-92a targeting p21 mRNA in HEK 293 cells (41). Therefore, our results might indicate other important unknown factors might possibly exist to affect the miRNA–mRNA interaction selection in vivo, as described previously (46).

In addition to find new miRNAs targeting the specific mRNA, miRIP could also be used to detect the interaction changes between mRNA and miRNA (Fig. 3C; Supplementary Fig. S2B), proposing a new method to investigate the regulation mechanism of gene expression and the experimental basis to develop new ChIP screening at mRNA level.

In summary, we established an miRIP approach that can identify the unpredictable miRNAs targeting the specific mRNA, and demonstrated with this method that miR-92a is functionally associated with HCC growth as an oncogenic miRNA by inhibiting p21 expression. We anticipate that miRIP is a useful tool for studying miRNAs targeting a given mRNA, which may provide an alternative way to study the function of miRNAs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X. Su, M. Yang, N. Li, X. Cao
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Su, H. Wang, M. Yang, J. Hou, N. Li, X. Cao
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An *In Vivo* Method to Identify microRNA Targets Not Predicted by Computation Algorithms: p21 Targeting by miR-92a in Cancer

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