MiR-219-5p inhibits hepatocellular carcinoma cell proliferation by targeting glypican-3

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1. Introduction

Hepatocellular carcinoma (HCC), a common primary liver cancer with high incidences of tumor recurrence and metastasis, is ranked as the third most common cause of cancer-related death worldwide [1]. As with other cancers, the development of HCC is a multistep process with a variety of changes in gene expression and structure. In recent years, a new class of regulatory RNAs, the microRNAs (miRNAs), has been discovered. Aberrant microRNA expression has been linked to the molecular pathogenesis of HCC [2].

MiRNAs negatively regulate gene expression by binding to the 3’ untranslated region (3’UTR) of corresponding target messenger RNAs (mRNAs) [3]. Emerging evidence has recently shown that several miRNAs play an important role in various biological processes, such as the proliferation, differentiation, and apoptosis of human cancers [3,4]. The alteration of miRNA expression is observed in HCCs and has been linked to the molecular pathogenesis of HCCs through their ability to impact the expression of crucial mRNAs. Depending on the target gene, miRNAs can function as tumor suppressor genes or oncogenes. For instance, it has been found that miR-22, miR-29c, miR-101, miR-124, miR-125b, miR-142-3p, miR-519d, and miR-637, which regulate the expression of many genes, are downregulated during HCC carcinogenesis and progression [5–13]. MiR-375 targets AEG-1 and inhibits HCC cell growth, and miR-519d suppresses HCC cell growth by targeting the MKi67 [12,14]. In addition, miR-122 is frequently downregulated in HCC and sensitizes HCC cells to adriamycin and vincristine by modulating the expression of multidrug resistance and inducing cell cycle arrest [15]. In contrast, other miRNAs, such as miR-29a, miR-191, miR-221, and miR-199a-3p, are often upregulated and found to function as oncogenes in HCC [16–18]. It has been found that inhibition of miR-191 can decrease cell proliferation and induced apoptosis [17]. MiR-221 can stimulate tumor growth via p27 and DDIT4 down-regulation [19]. In summary, dysregulation of miRNAs is involved in hepatocarcinogenesis. Therefore, identification of cancer-specific miRNAs and their targets is critical to understand their role in tumorigenesis and might be important for defining novel therapeutic targets [20,21]. Recently, miR-219 was found to be downregulated in squamous cell
2. Materials and methods

2.1. Patients and tissue samples

Eighty-three pairs of primary HCC and corresponding non-tumor adjacent tissue samples from patients were obtained from Nanfang Hospital with patients’ informed consent. The study was approved by our Research Ethics Committee.

2.2. Cell lines and transfection

Human HCC cell lines Huh-7, MHCC-97H, MHCC-97L, HepG2, HCCLM6 and the human hepatocyte cell line HL-7702 were propagated in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum. The cells were maintained at 37 °C in an atmosphere of humidified air with 5% CO₂.

Transfection was performed with Lipofectamine 2000 Reagent (Invitrogen, CA, U.S.) according to the manufacturer instructions. miR-219-5p mimics, mimics negative controls (m-NCs), miR-219-5p inhibitor and inhibitor negative controls (i-NCs) were purchased from Ribobio (Guangzhou, China). A final concentration of 50 nM of mimics or 100 nM of inhibitor and their respective negative controls were used for each transfection in proliferation, cell cycle and apoptosis experiments.

2.3. RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissue samples and cell lines using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For the detection of mature miRNA, we performed a stem-loop qRT-PCR assay. One microgram RNA was resuspended in 12 μl RNase-free ddH₂O and incubated at 85 °C for 5 min and then chilled on ice. Reverse transcription was carried out by adding 1 μl specific stem-loop RT primers (Applied Land, Guangzhou, China) and 7 μl M-MLV RT reagent containing 2 μl of 10 mM dNTP Mix (Promega, Madison, WI, U.S.), 0.5 μl RNase inhibitor (Promega), 4 μl 5 × buffer (Promega), and 0.5 μl M-MLV (Promega). The final volume was 20 μl in total. The reaction mixture was incubated at 42 °C for 60 min, at 85 °C for 10 min and then held at 4 °C. The cDNA was amplified using SYBR® Green PCR Master Mix (Toyobo, Osaka, Japan). The 20 μl PCR reaction mixture included 5 μl cDNA (1:20 dilution), 10 μl 2 × SYBR® Green PCR Master Mix (Toyobo), 0.5 μl forward primer, 0.5 μl reverse primer, and 4 μl RNase-free ddH₂O. Primers are listed in Supplementary Table 1. PCR reactions were performed under the following conditions: an initial denaturation step (95 °C for 5 min), 40 cycles of 95 °C for 15 s, 65 °C for 15 s and 72 °C 32 s. In brief, detection of the relative transcript levels of GPC3 was performed using qRT-PCR, an All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia™, Guangzhou, China), and an All-in-One™ qPCR Mix (GeneCopoeia™) according to the manufacturers’ instructions. Human GPC3 primer and GAPDH primer were acquired from GeneCopoeia™. The PCR cycles were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 15 s. The mean cycle threshold (Ct) was determined by triplicate PCR runs, and the relative expression was normalized to that of internal control snRNA U6 or GAPDH and calculated using 2⁻^ΔΔCt method [26]. Changes in the expression of HCC were found to be relative to the non-tumorous controls (HL-7702 cells and non-tumorous tissue adjacent to tumors). Therefore, the value of the relative expression ratio < 1.0 was taken to indicate downregulation in cancer relative to the non-tumorous control, and the ratio > 1.0 was considered as normal/upregulation [27].

2.4. Cell proliferation assay

The cell proliferation was determined by WST-8 staining with a Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China) according to the manufacturer’s instructions. 5000 cells were seeded in 96-well plates and transfected with miR-219-5p mimics or m-NCs the next day. CCK-8 solution was used to measure cell viability at 48 h after transfection. The absorbance of each well was measured with a microplate reader set at 450 nM. All experiments were performed in triplicate.

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Table 1

| Features                  | miR-219-5p Downregulation (%) | Normal/upregulation (%) | P       |
|--------------------------|-------------------------------|-------------------------|---------|
| Gender                   |                               |                         |         |
| Male                     | 45 (60.8)                     | 29 (39.2)               | 1.000*  |
| Female                   | 6 (66.7)                      | 3 (33.3)                |         |
| Age (years)              |                               |                         |         |
| <60                      | 41 (65.1)                     | 22 (34.9)               | 0.227   |
| ≥60                      | 10 (50.0)                     | 10 (50.0)               |         |
| Serum HBsAg              |                               |                         |         |
| Normal                   | 8 (57.1)                      | 6 (42.9)                | 0.717   |
| Overexpression           | 43 (62.3)                     | 26 (37.7)               |         |
| Serum AFP                |                               |                         |         |
| Negative                 | 17 (60.7)                     | 11 (39.3)               | 0.922   |
| Positive                 | 34 (61.8)                     | 21 (38.2)               |         |
| Tumor size-diameter of the biggest nodule |                 |                         |         |
| <5 cm                    | 12 (44.4)                     | 15 (55.6)               | 0.027*  |
| ≥5 cm                    | 39 (69.6)                     | 17 (30.4)               |         |
| Histological differentiation |                           |                         |         |
| Well/moderately          | 35 (54.7)                     | 29 (45.3)               | 0.030 a |
| Poorly                   | 16 (84.2)                     | 3 (15.8)                |         |
| Hepatic cirrhosis        |                               |                         |         |
| No                       | 12 (54.5)                     | 10 (45.5)               | 0.438   |
| Yes                      | 39 (63.9)                     | 22 (36.1)               |         |
| TNM staging              |                               |                         |         |
| I-II                     | 28 (54.9)                     | 23 (45.1)               | 0.122   |
| III-IV                   | 23 (71.9)                     | 9 (28.1)                |         |

* P < 0.05.

a Two-sided Fisher’s exact test.
2.5. Colony formation assay

Two hundred cells were counted and plated onto 12-well plates after transfection. Fresh culture medium was replaced every 3 days, and colonies were counted 14 days after plating. The cells were stained with crystal violet. The number of colonies containing more than 50 cells was determined under a microscope, as previously described [28]. All experiments were performed in triplicate.

2.6. Cell cycle analysis and apoptosis assay

For cell cycle analysis, 48 h after transfection, the adhered cells were obtained and fixed into 70% ethanol at 4 °C. After 12 h, propidium iodide (PI) and RNase A were added to the cells according to manufacturers’ instructions (Beyotime, Shanghai, China). Samples were analyzed 30 min after staining with use of flow cytometry-BD FACSCalibur (BD Bioscience, MA, U.S.). Results were analyzed using CellQuest software (BD Biosciences). For the apoptosis assay, 48 h after transfection, cells were resuspended in binding buffer containing annexin V-FITC and PI according to the manufacturers’ instructions (BD Bioscience). Samples were assessed using flow cytometry as described above. Each sample was run in triplicate.

2.7. Dual-luciferase reporter assay

The dual-luciferase reporter plasmid psi-CHECK2-w-GPC3 (containing the wild-type GPC3 putative 3′UTR binding site) and psi-CHECK2-mGPC3 (containing mutant GPC3 3′UTR) was constructed by LAND. HEK-293T cells were plated into 24-well plates with 50–60% confluence 24 h before transfection. A mixture of 50 nM miR-219-5p mimics and 0.5 μg psi-CHECK2 report plasmid was cotransfected into cells using Lipofectamine 2000 reagent. After 48 h, luciferase activity levels were measured using a dual-luciferase reporter assay system (Promega) according to the manufacturers’ instructions. Each transfection was performed in triplicate.

2.8. Western blot

Whole cell protein extracts were quantified by BCA assay and then resolved on a 6% SDS polyacrylamide gel (Invitrogen). The bands were then transferred onto a nitrocellulose membranes (Bio-Rad) and then blocked in 5% skim milk in TBST, incubated with anti-GPC3 antibody (Abcam, HongKong) or anti-GAPDH antibody overnight at 4 °C. Signals were revealed after incubation with anti-rabbit IgG secondary antibody coupled to peroxidase by using ECL.

2.9. Immunohistochemical (IHC) and staining evaluation

IHC was performed in 45 HCC tissues samples according to the methods that had been used in previous studies [29].

2.10. Statistical analysis

All statistical analyses were performed using SPSS13.0 for Windows (SPSS Inc., Chicago, IL, U.S.). P < 0.05 was considered statistically significant.

3. Results

3.1. Expression of miR-219-5p and its correlation with clinicopathological characteristics of HCC

In this study, we sought to confirm that miR-219-5p was downregulated in primary HCC. Using qRT-PCR, the expression levels of miR-219-5p in all 83 (100%) pairs of HCC tissues, their matched non-tumor adjacent tissues, and the HCC cell lines were determined and normalized against an endogenous control, snRNA U6. We found miR-219-5p to be significantly downregulated in HCC samples (P < 0.01). The median miR-219-5p expression level in HCC tissues was four times lower than that of the non-tumor adjacent tissues (median expression 0.088 and 0.384, respectively) (Fig. 1A). Among the 83 patients with primary HCC, 51 (61.45%) showed a reduction in the miR-219-5p level of over 50% relative to their matched non-tumor adjacent tissues (Fig. 1B). We then studied the correlation between miR-219-5p expression and the clinicopathological characteristics of HCC. The patients with lower levels of miR-219-5p expression tended to have larger tumor sizes (≥5 cm; P = 0.027) and poor differentiation (P = 0.030; Table 1). Kaplan–Meier analysis also showed that downregulation of miR-219-5p was associated with decreased survival (Fig. 1C). Specifically, the median overall survival time was 13.0 and 30.0 months in miR-219-5p downregulation and normal/upregulation groups, respectively (P = 0.005, log-rank test).

We also found that, compared to normal liver cell line HL-7702, miR-219-5p was downregulated in all HCC cell lines; (Huh-7, 0.06 ± 0.02-fold; MHCC-97H, 0.57 ± 0.12-fold; MHCC-97L, 0.74 ± 0.07-fold; HepG2, 0.11 ± 0.02-fold; HCCLM6, 0.14 ± 0.02-fold; Fig. 1D).

3.2. Effects of miR-219-5p on HCC cell proliferation in vitro

The downregulated miR-219-5p in HCC prompted us to investigate whether miR-219-5p functions as a tumor suppressor. In HCC cell lines Huh-7 and HepG2, we found miR-219-5p expression was markedly decreased (Fig. 1C), and restoration of miR-219-5p expression by miR-219-5p mimics inhibited cell proliferation in Huh-7 and HepG2 cells (Fig. S1A and Fig. 2A). To provide further evidence that miR-219-5p is involved in HCC cell growth, we selected the HCC cell line MHCC-97L, which has a relatively high level of miR-219-5p (Fig. 1C), and studied the effects of an inhibitor of miR-219-5p. In contrast, proliferation of MHCC-97L cells transfected with miR-219-5p inhibitors was found to be higher than that of the cells transfected with i-NCs (Fig. S1B and Fig. 2B). Colony formation assay showed that enforced expression of miR-219-5p in HepG2 cells (Fig. 2C), Transfection of miR-219-5p inhibitors into MHCC-97L cells significantly increased the number of colonies when relative to i-NCs (Fig. 2D).

3.3. Effects of miR-219-5p on cell cycle in HCC cells

Given that miR-219-5p obviously inhibited HCC cell proliferation in vitro, we next sought to determine whether miR-219-5p would have any impact on cell cycle progression among HCC cells. The cell cycle distribution of HepG2 cells showed that the number of cells in the G1 phase was greater in cells transfected with miR-219-5p mimics than in m-NCs (P < 0.001). The number of cells in the S phase dropped sharply (Fig. 3A). In contrast, the cell cycle distribution analysis of MHCC-97L cells after transfection of miR-219-5p inhibitor showed that inhibition of miR-219-5p could increase the number of cells in the S phase over i-NCs (P < 0.001 (Fig. 3B and C). We also examined the effects of miR-219-5p on apoptosis. Data indicated that miR-219-5p had no effect on apoptosis in HepG2 cells at 48 and 72 h after transfection (Fig. S2).

3.4. GPC3 as a direct downstream target of miR-219-5p

Next, we investigated the molecular mechanisms responsible for the anti-tumor effects of miR-219-5p in the HCC cells observed above. Because miRNA usually exerts its functions by suppressing the expression of target mRNAs, we searched the putative
target genes of miR-219-5p in TargetScan (http://www.targetscan.org/) and miRanda (miCRorna.org and miRBase). GPC3 was predicted to be a potential target of miR-219-5p by miRanda (Fig. 4A). Our previous studies have indicated that GPC3 is commonly overexpressed in human HCC, and it directly contributes to the carcinogenesis and development of HCC by controlling cell proliferation [28,29]. A putative binding site for miR-219-5p was found in the 3'UTR of GPC3, so we chose GPC3 for further study.

To verify whether GPC3 is a direct target of miR-219-5p, we constructed luciferase reporter plasmid containing either wild-type or mutant 3'UTR of GPC3 (Fig. 4B). The wild-type and mutant plasmids were cotransfected into HEK-293T cells with miR-219-5p mimics or m-NCs. Relative luciferase activities were measured by a dual-luciferase reporter assay system and normalized by dividing firefly luciferase activity by that of Renilla luciferase. As shown in Fig. 4C, the relative luciferase activity in cells cotransfected with wild-type GPC3 3'UTR and miR-219-5p mimics was significantly decreased after 48 h (P = 0.004). The reduction in the luciferase activity with mutant GPC3 3'UTR was not as sharp as that observed in the wild-type counterpart (P = 0.515), suggesting that miR-219-5p could directly bind to the 3'UTR of human GPC3. We examined the GPC3 mRNA levels in HepG2 and Huh-7 cells transfected with miR-219-5p mimics or m-NCs by PCR. Forty-eight hours after transfection, we found that mRNA level of GPC3 to have been suppressed by miR-219-5p both in HepG2 and Huh-7 cells (P = 0.016 and 0.002, respectively) (Fig. 4D). We found that miR-219-5p reduced GPC3 protein levels in HepG2 at 96 h post-transfection (Fig. 4E). These data suggest that miR-219-5p negatively regulates GPC3 protein expression through mRNA degradation and translational repression.

3.5. Inverse correlation between the expression of miR-219-5p and GPC3 protein in HCC tissue samples

We measured GPC3 protein levels in 45 of the previously studied 83 HCC tissues using IHC. These samples had already been verified as expressing miR-219-5p by qRT-PCR. The expression level of GPC3 protein was defined as normal/underexpression (+/−) and overexpression (++/+++). GPC3 was overexpressed in 30 (66.67%) cases, and an obvious inverse correlation was observed between the expression of GPC3 protein and miR-219-5p in HCC tissue samples (P = 0.034) (Table 2). The correlation coefficient was not perfect (r = −0.316).

4. Discussion

Recent studies have demonstrated the importance of miRNAs in human hepatic carcinogenesis [2]. The identification of cancer-specific miRNAs and their targets is critical to understanding their role in tumorigenesis. Our present study confirmed that the downregulation of miR-219-5p in ~60% of HCC tissues. However, it has been reported that miR-219 is downregulated in chronic hepatitis (CH)
Fig. 2. MiR-219-5p suppresses HCC cell proliferation in vitro. (A) Measurement of cell proliferation by CCK-8 assay. After Huh-7 and HepG2 cells were transfected with miR-219-5p mimics or m-NCs, the CCK-8 solution was added to determine the relative cell growth activity at 24, 48, and 72 h post-transfection. (B) CCK-8 assay in MHCC-97L cells. After MHCC-97L cells were transfected with miR-219-5p inhibitors or i-NCs, the CCK-8 assay was used to determine the relative cell growth activity at 24, 48, 72, and 96 h post-transfection. (C, D) Effects of miR-219-5p on cell proliferation as evaluated by a colony formation assay. HepG2 and MHCC-97L cells were transfected with miR-219-5p mimics or inhibitor and their respective negative controls. After 14 days of culture, the cells were stained with crystal violet and the colony formation rate was calculated. Representative pictures of colonies are shown. The histogram shows the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01.
and HCC, relative to normal liver tissues, as determined by microarray analysis. This study also reported no difference between CH and HCC in the expression of miR-219 [25]. Our data showed that downregulation of miR-219-5p was not significantly associated with HBsAg and hepatic cirrhosis. The non-tumorous adjacent samples examined in our study included normal liver, CH, and hepatic cirrhosis tissues. And we found that the expression of miR-219-3p was not significantly different in HCC tissues and non-tumorous adjacent tissues (data not shown). These can perhaps explain the discrepancies.

Moreover, the HCC patients who showed low expression levels of miR-219-5p tended to have larger tumors, poorer differentiation, and shorter survival times, suggesting that miR-219-5p might participate in HCC progression and negatively control HCC cell growth.

Previous studies found that miR-219 inhibited cell proliferation [22,30]. Indeed, our findings showed that miR-219-5p could significantly suppress HCC cell proliferation in vitro. Mechanistic insight into the inhibitory effects of miR-219-5p on cell proliferation indicate that miR-219-5p can induce cell cycle arrest at the G1 to S transition of HCC cells, suggesting that downregulation of miR-219-5p in HCC may cause the cancer cells to divide and grow more quickly. Previous data, in combination with our current findings, raise the possibility that loss of miR-219-5p expression may contribute to the aberrant proliferation observed in HCC.

Bioinformatics analysis has indicated that GPC3 is a putative target of miR-219-5p. Dual-luciferase assay, qRT-PCR and Western blot analysis identified GPC3 as one of the genes directly targeted by miR-219-5p. A decrease in miR-219-5p expression was shown to be inversely correlated with GPC3 protein expression levels in 45 pairs of HCC tissue samples. These results suggest that miR-219-5p interacts with GPC3 and negatively regulates its expression. GPC3 is a cell surface protein that has been implicated as a possible tumor marker for HCC[31]. GPC3 is highly expressed in HCC and at a lesser degree in melanoma, ovarian clear-cell carcinomas, yolk sac tumors, and some other human tumors[31]. Our previous study indicated that GPC3 might be a valuable marker closely related with poor outcomes and post-operative metastasis/recurrence of HCC [29]. GPC3 plays an important role in cell growth, differentiation and metastasis. In 2005, GPC3 was reported to promote proliferation of HCC cells by stimulating the canonical Wnt pathway through facilitating the interaction between the Wnts and their signaling receptors [32]. Our previous study confirmed that knockdown of GPC3 expression significantly inhibited the growth and invasive ability of MHCC-97L cells [28]. We now propose that miR-219-5p may have an effect on HCC proliferation by downregulating GPC3. Although it has been demonstrated that miR-96 downregulated GPC3 expression by targeting its mRNA 3’UTR, the mechanism of GPC3 overexpression in HCC has not been well characterized [33]. In the present study, we found that
overexpression of GPC3 in HCC may be caused by the downregulation of miR-219-5p. Although we found an obvious inverse correlation between the expression of GPC3 and miR-219-5p in tissues samples, the correlation coefficient was not prefect \((r = -0.316)\). It is worth considering whether other mechanisms may be involved in the regulation of miR-219-5p. Dugas et al found that miR-219 targets PDGFR\(\alpha\), Sox6, FoxJ3, and ZFP238 to repress oligodendrocyte precursor cell proliferation and promote oligodendrocyte differentiation [30]. In this way, miR-219-5p may regulated several targets in HCC. This issue merits further investigation.

In conclusion, we have shown that miR-219-5p is downregulated in most cases of HCC and closely associated with larger tumor size, poorer histological differentiation, and shorter overall survival time in HCC patients. MiR-219-5p can suppress HCC cell growth

**Table 2**

| GPC3 | n | MiR-219-5p | Spearman’s correlation |
|------|---|------------|------------------------|
|      |   | Underexpression (No. of cases) | Normal/overexpression (No. of cases) | P | R |
| PR   | 15 | 5 | 10 | 0.034 | -0.316 |
| Overexpression (++/+++) | 30 | 20 | 10 | |

Fig. 4. GPC3 as a direct downstream target of miR-219-5p (A) miRanda predicted that miR-219-5p would target the 3’UTR of GPC3. (B) Construction of binding site of wild-type and mutant GPC3 mRNA 3’UTR. The mutant binding site is underlined. (C) Relative luciferase activity analyses. The psi-CHECK2, psi-CHECK2-wGPC3, or psi-CHECK2-mGPC3 was cotransfected into HEK-293T cells with miR-219-5p mimics, m-NCs. (D) The expression level of GPC3 mRNA was down-regulated by miR-219-5p using qRT-PCR and semi-quantitative PCR assays. (E) Western blot results of GPC3 protein in HepG2 transfected with miR-219-5p mimics or m-NCs at 48 and 96 h. *P < 0.05.
and induce cell cycle arrest at the G1 phase. These tumor-suppressive functions of miR-219-5p are mediated by the target gene GPC3, a new target for HCC immunotherapy. These findings suggest that miR-219-5p may have an important role in tumorigenesis through the regulation of GPC3.

Authors’ contributions
Luo Rongcheng Ph.D and Zheng Dayong Ph.D carried out the conception and design, and given final approval of the version to be published. Huang Na M.D and Lin Jing M.D carried out the design of the study, performed the study and the statistical analysis. Ruan Jian M.D, Su Ning M.D, and Lv Chengwei Ph.D participated in analysis and interpretation of the study and related data. Qing Ruizhai M.D, Liu Feiye M.D and He Benfu Ph.D helped to immunohistochemistry detection. All authors reviewed and approved the final manuscript.

Competing interests
None of the authors of this article declare any competing interests.

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Appendix A Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.02.017.

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