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miR-129 suppresses tumor cell growth and invasion by targeting PAK5 in hepatocellular carcinoma

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ABSTRACT

Emerging evidence suggests that microRNAs (miRNAs) play important roles in regulating HCC development and progression; however, the mechanisms by which their specific functions and mechanisms remained to be further explored. miR-129 has been reported in gastric cancers, lung cancer and colon cancer. In this study, we disclosed a new tumor suppresser function of miR-129 in HCC. We also found the downregulation of miR-129 occurred in nearly 3/4 of the tumors examined (56/76) compared with adjacent nontumorous tissues, which was more importantly, correlated to the advanced stage and vascular invasion. We then demonstrated that miR-129 overexpression attenuated HCC cells proliferation and invasion, inducing apoptosis *in vitro*. Moreover, we used miR-129 antagonist and found that anti-miR-129 promoted HCC cells malignant phenotypes. Mechanistically, our further investigations revealed that miR-129 suppressed cell proliferation and invasion by targeting the 3'-untranslated region of PAK5, as well as miR-129 silencing up-regulated PAK5 expression. Moreover, miR-129 expression was inversely correlated with PAK5 expression in 76 cases of HCC samples. RNA interference of PAK5 attenuated anti-miR-129 mediated cell proliferation and invasion in HCC cells. Taken together, these results demonstrated that miR-129 suppressed tumorigenesis and progression by directly targeting PAK5, defining miR-129 as a potential treatment target for HCC.

1. Introduction

Hepatocellular carcinoma is one of the five most frequent cancers and the third leading cause of cancer death globally, posing a serious threat to human health [1,2]. Though the treatment has seen improvements and the life of patients have been extended due to progress in liver transplantation and the related clinical studies, the insensitiveness of the chemotherapeutic drugs and recurrence and metastasis still contribute to a poor prognosis [3,4]. Metastasis is a major marker of tumor, but clear studies on its mechanism are yet to emerge [5]. Therefore it is necessary to find the proper molecular markers and potential therapeutic targets to predict the prognosis.

MicroRNAs (miRNAs) have extensive roles in the development of cancer, especially in decreasing the mRNA level of target genes and post-transcriptional regulation of gene expression, through binding to the 3′-untranslated regions of the target gene [6,7]. miRNAs have deregulated expressions in tumor tissue, and those are implicated in a number of cellular biological pathways, including cell proliferation, cell cycle, invasion and metastasis. But the underlying mechanisms still remain unknown. Some miRNAs, for instance miR-30d [8], miR-210 [9] and miR-155 [10], have been proved to contribute to the growth and metastasis of hepatocellular carcinoma.

The p21-activated kinases (PAKs) are serine/threonine protein kinases whose activities are stimulated by the binding of active Rac and Cdc42 GTPases. PAKs are implicated in cell motility, existence, and the regulation of gene transcription [11]. It also plays an important role in the formation and stability of microtubule. Recently, research has also found that PAK5 is overexpressed in ovarian cancer, regulating the paclitaxel-chemoresistance [12]. PAK5 regulates MMP-2 in the mediation of tumor invasion and

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metastasis in glioma cells [13]. But its role in hepatocellular carcinoma needs to be further revealed, although several reports indicated that PAK5 involved in enhanced proliferation and tumorigenicity [14].

This research intends to reveal the role of miR-129 in the development of hepatocellular carcinoma, predict the downstream target genes through TargetScan/RNAhybrid algorithms, and verify it at the histological and cytological level. The research finds that miRNA-129 has a low expression in tumor tissue sample, and that the forced overexpression of it will induce cell-cycle arrest, suppress cell proliferation, as well as reduce the expression of PAK5, and hence suggesting its vital role in the growth of hepatocellular carcinoma cell.

2. Materials and methods

2.1. Patients and cell culture

76 pairs of human primary HCC and matched adjacent non-tumorous liver tissues were collected from 2012 to 2014 at the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University. Clinical pathological features including sex, age, virus infection status, tumor stages and AFP level are mentioned in Table 1. All human tissues were acquired with informed consent and approved by the Ethical Review Committee of the WHO of the Collaborating Center for Research in Human Production. Human tissues were stored at −80 °C.

SMMC-7721 and Huh7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated at 37 °C in a humidified incubator under 5% CO_2 condition.

2.2. Cell transfection

Cells were seeded into 6 well plates and transfected with miR-129 mimics, NC or miR-129 antagonist (anti-miR-129), using Lipofectamin 2000 (Invitrogen, USA). For inhibiting endogenous PAK5 expression, siRNA targeting PAK5 was purchased from GenePharma.

Table 1
Clinical characteristics of 76 patients with hepatocellular carcinoma.

	No. of cases
Age	
≤50	18
> 50	58
Gender	
Male	61
Female	15
Tumor size (cm)	
≤5	29
> 5	47
Tumor differentiation	
Poor	40
Well	36
Vascular invasion	
Yes	25
No	51
AFP(ng/ml)	
≤400	41
> 400	35
HBsAg	
Positive	62
Negative	14
Tumor stage	
I + II	34
III + IV	42

2.3. RNA extraction and quantitative real-time PCR

Total RNA from 76 pairs of HCC samples and from tumor cells were extracted with TRIzol reagent (Invitrogen, USA). Complementary DNA synthesis was performed by PrimeScript RT reagent kit (Takara, China). Quantitative real-time PCR (qRT-PCR) was carried out using SYBR-Green method. The primers for PAK5 were as followed: forward 5′-TGTTCATGCATTCTAGAGAAAGTGG-3′, and reverse 5′-GCATTTCACCACAGTGTATTTCTGA-3′. The primers for GAPDH which acted as an internal control were as followed: forward 5′-CGCGCCCCCGGTTTCTA-3′, and reverse 5′-GGCTCGGCTGGCGAC-3′. The expression level of miR-129 was determined by TaqMan miRNA assays (Applied Biosystems, USA) according to the provided protocol, and U6 small nuclear RNA was used to normalize the expression.

2.4. Western blot

Total protein from tissue samples and cell lysis were prepared by RIPA buffer. Western blot analysis was performed with standard procedure. Proteins were separated by 10% SDS-PAGE and transferred to membranes. After incubating with primary antibodies, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG as secondary antibodies and using ECL methods to blotting. The primary antibodies were as followed: anti-PAK5 (1:1000; Santa Cruz Biotechnology, USA), anti-GAPDH (1:2000; Santa Cruz Biotechnology, USA).

2.5. Luciferase activity assays

3′-UTR of PAK5 sequences were cloned into the PGL3 basic vector (Promega, USA). For the luciferase assays, 100 ng PGL3-PAK5-3′UTR vector were co-transfected in cells with 100 nM miR-129 mimics or control regent, together with 20 ng Renilla luciferase vector (Promega, USA) as an internal normalized control. Cells were harvested 48 h after transfection and the luciferase activities were assayed according to the manufacturer's protocol. Transfections were performed in duplicate and repeat in three times.

2.6. Flow cytometry

For cell cycle analysis, the cells were harvested 48 h after transfection and fixed in 70% ethanol at 4 °C overnight and resuspended in 300 μl PBS with 25 μl propidium iodide (PI) and 10 μl RNase at 37 °C for 30 min. The DNA content was quantified by FACS Calibur flow cytometer (BD Biosciences, USA). The results were modified using ModFit LT 3.0 software. For apoptosis assay, the cells were cultured in low-serum medium and collected after 48 h transfection. Cells were subsequently stained with Annexin V-FITC (eBioscience, USA) and PI for 30 min as described by the manufacturer. Apoptosis cells were analyzed by FACS.

2.7. Transwell invasion assay

To assess cell invasion *in vitro*, cells (10^4 in serum-free medium) were placed into the top chamber of transwell coated with 150 μg Matrigel (BD Biosciences, USA). After 48 h of incubation at 37 °C, cells adhering to the lower membrane were fixed and stained with 0.1% crystal violet and imaged using microscope (Olympus).

2.8. Cell viability assay

Cells were seeded in the 96 well plates 24 h after transfection at a density of 1500 cells per well. The cell viability assay was performed using Cell Counting Kit-8 (CCK8; Dojindo) according to the manufacturer's protocol. The absorbance at 450 nm were measured. Experiments were performed at three times.

2.9. Immunohistochemical staining

The paraffin-embedded 76 pairs of HCC tissues undergoing 5 μm section were used to assess the expression of PAK5. Immunohistochemical staining was carried out. In brief, tissue slides were incubated at 4 °C overnight with anti-PAK5 antibody (1:200) and then incubated with HRP-labeled anti-IgG for 30 min. The sections were stained using diaminobenzidine substrate chromogen and counterstained with hematoxylin. All these were performed under microscope to control the reactions and PBS was performed instead of primary antibody as the negative controls.

Scoring was determined by the cell cytoplasm staining pattern of tumor and nontumorous tissues as described: no staining = 0; <15% = 1; 15–50% = 2; 50–75% = 3; >75% = 4. Furthermore, according to the percentage of tumor cell over the sections (<15% = 1; 15–50% = 2; 50–75% = 3; >75% = 4), the scores was determined as intensity × percentage to produce a final score of 0–16.

2.10. Statistical analysis

Statistical analyses were performed with SPSS 21.0 software (IBM, USA). The statistical significance of miRNA and PAK5 expression between tumor and nontumorous tissues were determined by Wilcoxon matched pairs signed-rank test. The relevance of miR-129 and PAK5 and the statistical significance were assessed using Spearman analysis. All other experiments were assessed by Student's t test. Data were expressed as mean ± SEM. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. miR-129 is downregulated in HCC and associated with advanced stage and metastasis

miR-129 has been reported to be downregulated in various cancers including gastric cancer, lung cancer and colorectal carcinoma [15–17]. However, the expression of miR-129 in HCC has not been determined yet. To analyze the expression of miR-129 in HCC, the mature miR-129 was detected in 76 pairs of HCC and adjacent nontumorous liver samples using qRT-PCR. The results showed that the expression of miR-129 was significantly downregulated in HCC when compared to the nontumorous tissues (Fig. 1A). Further investigation showed that miR-129 expression was declined in about 3/4 of HCC samples and only 9% of HCC samples showed miR-129 overexpression (Fig. 1B), indicating that downregulation of miR-129 was predominant in HCC progression. Moreover, we decided to understand the relationship between miR-129 expression and HCC clinical pathological features. We found that miR-129 expression was lower in stage III and IV HCC samples compared with stage I and II HCC samples (Fig. 1C). In addition, the expression of miR-129 was also lower in HCC samples with metastasis than that in HCC samples without metastasis (Fig. 1D). Taken together, these results implied miR-129 had potentially crucial roles in carcinogenesis and progression of HCC and led us to hypothesize that miR-129 overexpression may inhibit tumor cell growth and invasion.

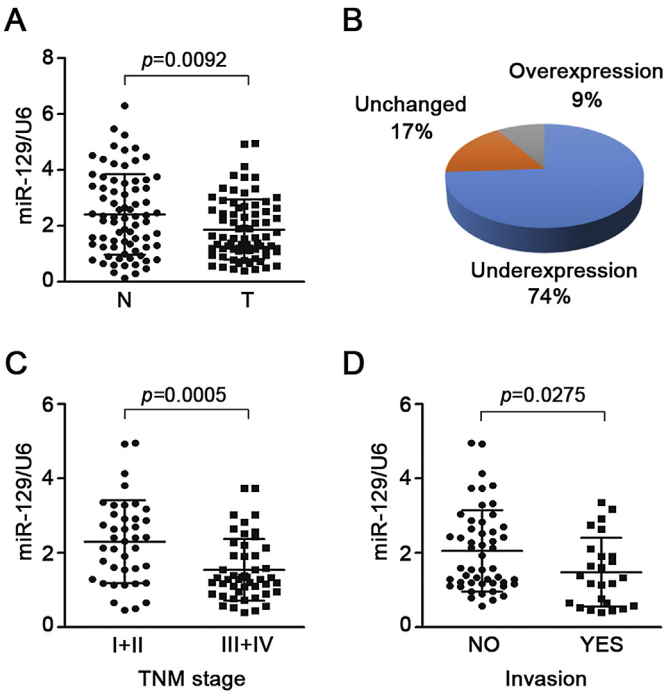
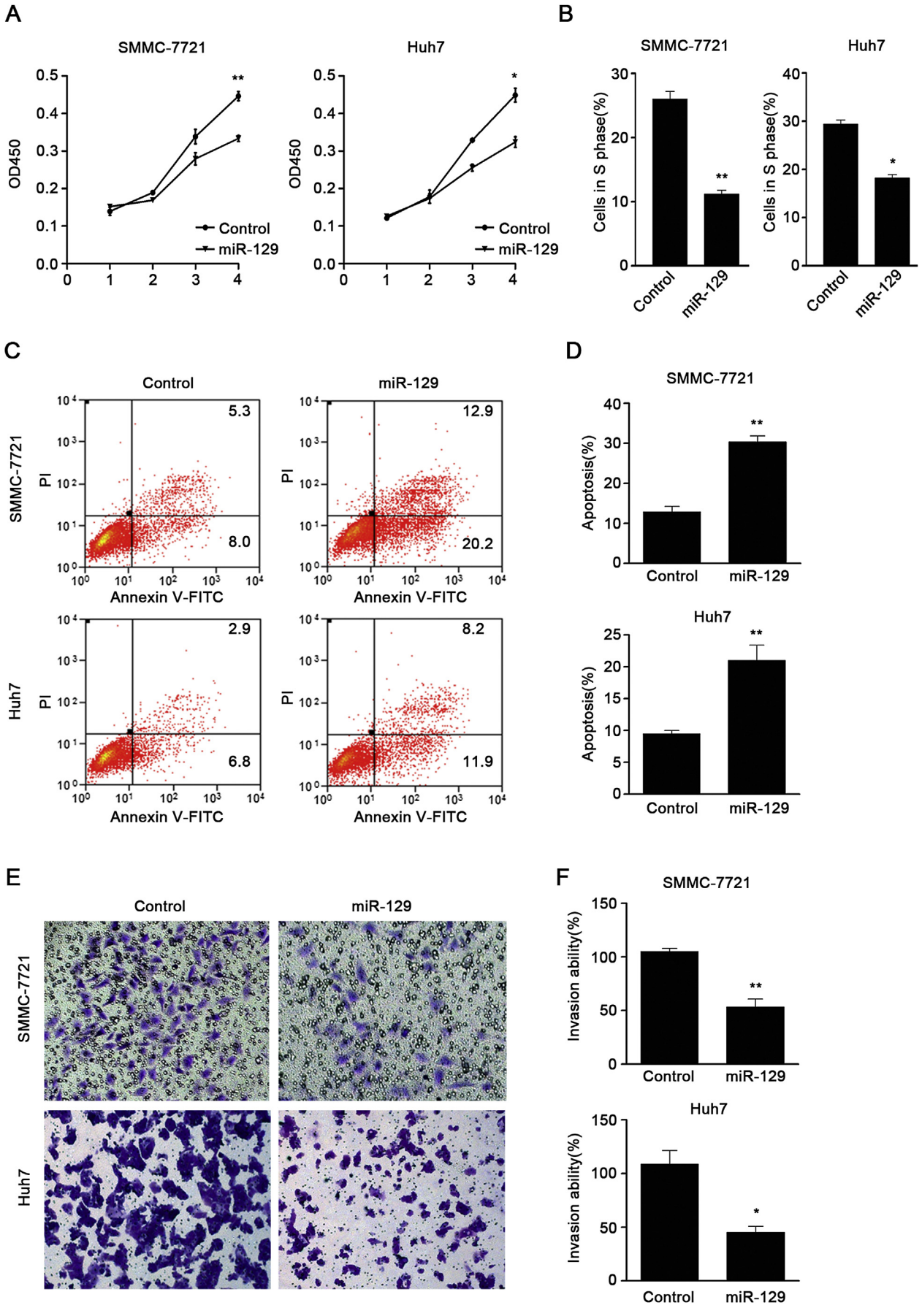


Fig. 1. Level of miR-129 is downregulated in human HCC tissues and its expression correlated with HCC malignant phenotype. A. Relative mRNA levels of miR-129 in 76 paired samples of HCC tissues and adjacent nontumorous tissues were evaluated by qRT-PCR. B. The downregulation of miR-129 was found in 74% of HCC tissues compared with corresponding nontumorous tissues, the fold change of relative miR-129 expression (N/T)>2or < 1/2 was defined as significant. C. miR-129 expression in different clinical stages of HCC according to the 7th Edition of the AJCC Cancer's TNM Classification. D. Downregulation of miR-129 was associated with vascular invasion.

3.2. miR-129 suppresses proliferation and invasion of HCC cells

To confirm the impact of miR-129 in HCC cells, we used miR-129 mimics and antagonist to perform the gain and loss function analysis. The cell proliferation assays revealed that overexpression of miR-129 in SMMC-7721 and Huh7 cells could significantly suppress cell proliferation (Fig. 2A). Furthermore, the cell cycle analysis also showed that the percentage of cells in S phase were declined indicating that DNA synthesis was retarded (Fig. 2B). While decreased expression of miR-129 could notably promote cell proliferation, which was accompanied by the increased S phase cells (Supplementary Fig. S1A and S1B). Meanwhile, we hypothesized that miR-129 affected cell apoptosis as well. We transfected HCC cells with miR-129 mimics and assessed apoptosis by FACS and found there had slight difference when cells were cultured in normal conditions (data not shown). So we changed to the low FBS (1%) medium and our results showed that miR-129 increased apoptosis significantly in HCC cell lines, as assessed by the proportion of cells that are Annexin V positive (Fig. 2C). In the subsequent experiments, we verified the anti-apoptosis effects caused by the anti-miR-129 as we expected (Supplementary Fig. S1C). Collectively, both cell proliferation and survival assays indicated that miR-129 inhibited cell proliferation and promoted apoptosis. Given that miR-129 expression was highly associated with the metastasis property of HCC, we inferred that miR-129 had impacts in cell invasion ability. So we carried out transwell assays with Metrigel and demonstrated that overexpression of miR-129 could also significantly suppressed invasion of SMMC-7721 and Huh7 cells when compared with control groups (Fig. 2D). Inhibited expression of miR-129 could significantly promote invasion abilities of both cells (Supplementary Fig. S1D). In conclusion, we


A
SMMC-7721
Huh7
OD450
Control
miR-129
B
SMMC-7721
Huh7
Cells in S phase(%)
Control
miR-129
C
Control
miR-129
SMMC-7721
Huh7
PI
Annexin V-FITC
5.3
8.0
12.9
20.2
2.9
6.8
8.2
11.9
D
SMMC-7721
Huh7
Apoptosis(%)
E
Control
miR-129
SMMC-7721
Huh7
F
SMMC-7721
Huh7
Invasion ability(%)

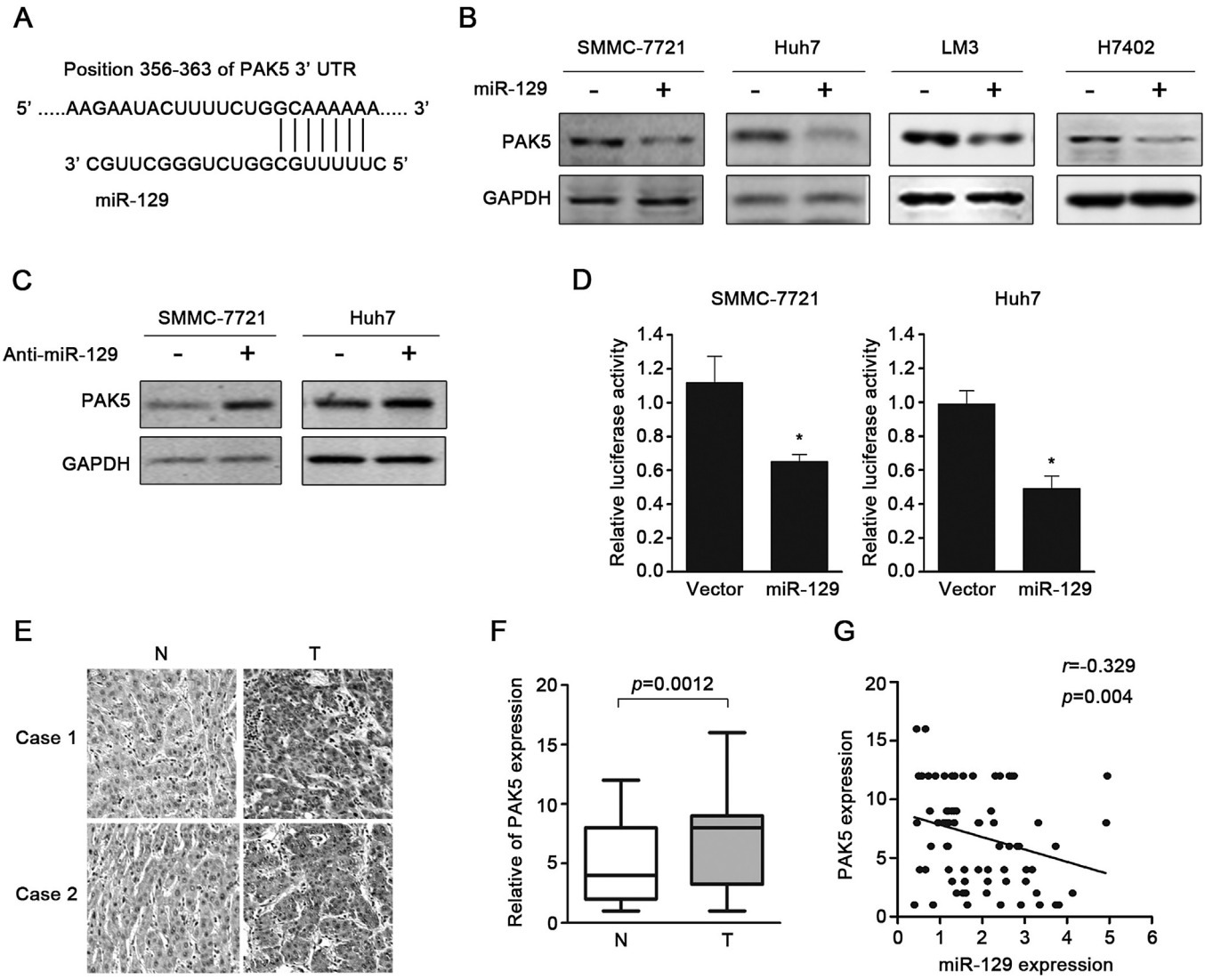


Fig. 3. miR-129 negatively regulates PAK5 by binding to the 3′ UTR of PAK5. A. Putative miR-129–binding site in PAK5 3′ UTR and the corresponding binding site were shown. B. Western blot analysis was performed to detect the expression of endogenous PAK5 in four HCC cells (SMMC-7721, Huh7, LM3, H7402) after transfected with miR-129 mimics or control. GAPDH served as an internal control. C. Western blot analysis was used to detect the expression of PAK5 in SMMC-7721 and Huh7 cells after transfection with NC or miR-129 antagonist. GAPDH served as an internal control. D. Relative luciferase activity assays of luciferase reporters with PAK5 3′ UTR were performed after cotransfection with miR-129 mimics or control. The Renilla luciferase vector was co-transfected as an internal control. E. Representative images of immunohistochemical staining of PAK5 is shown in HCC (T) and adjacent nontumorous tissue (N) (magnification × 100). F. Relative score of PAK5 immunochemistry staining for HCC tissues and adjacent nontumor tissues. G. The relevance of miR-129 and PAK5 and the statistical significance were performed using spearman analysis. (*$P < 0.05$).

determined that miR-129 was a negative regulator for HCC progression.

3.3. *miR-129 post-transcriptionally down-regulates PAK5 expression by directly targeting its 3′ UTR*

To explore the mechanism by which miR-129 affects HCC progression, we employed bioinformatics analysis using TargetScan and attempted to find potential genes which might be involved in miR-129 mediated tumor-suppression functions. Previous studies have reported that CDK6 and BCL2 were two target genes of miR-129 [17,18]. However, the miR-129 regulation network was still incomplete. In our study, we identified PAK5 as the direct targets of miR-129 as well (Fig. 3A). Western blot analysis in four HCC cell lines with overexpressed miR-129 mimics showed a silencing effect on PAK5 expression (Fig. 3B). Furthermore, after transfection with anti-miR-129 in SMMC-7721 and Huh7 cells, the expression of PAK5 was obviously increased (Fig. 3C). To confirm the direct binding between miR-129 and PAK5 3′ UTR, we constructed 3′ UTR sequence of PAK5 and inserted into luciferase reporter vector. The

Fig. 2. miR-129 suppresses HCC cells proliferation and invasion *in vitro*. A. Proliferation effects of overexpression of miR-129 in SMMC-7721 and Huh7 cells by miR-129 mimics were assessed by CCK8 assay. Data were representative of three independent experiments. B. The cell cycle analysis of SMMC-7721 and Huh7 transfected with miR-129 mimics was performed by FACS and the S phase were showed. Data were representative as the mean ± SEM of three independent experiments. C. The cell apoptosis rates were analyzed by FACS. D. Data were representative as the mean ± SEM of three independent experiments. E. Representative results of transwell assays on invasion abilities of SMMC-7721 and Huh7 cells transfected with miR-129 mimics (magnification × 100). F. Results of invasive cells were described as the average number of invasion cells from five randomly selected fields. Data were representative as the mean ± SEM of triplicated independent experiments. (*$P < 0.05$, **$P < 0.01$).

relative luciferase activity was remarkably reduced by miR-129 transfection (Fig. 3D). Taken together, our findings indicated that miR-129 played a critical role in regulating PAK5 expression.

3.4. Upregulation of PAK5 inversely correlated with miR-129 expression in HCC

To further elucidate the relationship between miR-129 and PAK5 in HCC, we detected the PAK5 expression in the same 76 paired HCC and adjacent nontumorous tissues using IHC. We found that the 53 cases of tumors showed enhanced PAK5 expression when compared with adjacent nontumorous tissues (Fig. 3E, F). More importantly, the expression levels of PAK5 in tumor tissues inversely correlated with the miR-129 levels (Spearman $r = -0.329$, $p = 0.004$; Fig. 3G). These data suggested that miR-129 negatively regulated PAK5 expression and their inversely correlation could be determined in clinical samples.

3.5. PAK5 is involved in miR-129 mediated suppression of HCC cells proliferation and invasion

Since PAK5 is frequently up-regulated in HCC and correlated with decreased miR-129 expression, we speculated that down-regulation of PAK5 directly participated in miR-129 mediated suppression effects. To further address this issue, we used siRNA to knockdown the expression of PAK5 after anti-miR-129 transfection and the western blot analysis confirmed these results (Fig. 4A). Importantly, cell viability assays and transwell assays indicated that the repression of PAK5 significantly reduced the HCC cells proliferation and invasion that could be augmented by anti-miR-129 (Fig. 4B, C). These results provided evidences that miR-129 suppressed cell proliferation and metastasis through targeting PAK5.

4. Discussion

There are many reports that demonstrate miRNA aberrant expression in various tumors. Whereas miRNAs that modulate the HCC carcinogenesis and progression have not been precisely explored. miR-129 is reported dysregulation in lung cancer, colorectal cancer, esophageal carcinoma and gastric cancer which impacts cell proliferation, death and drug resistance [19]. However, its roles in HCC remains inadequately understood. Here, we identified a novel regulatory mechanism of miR-129 in HCC which was to mediate the expression of PAK5. miR-129 is commonly down-regulated in HCC and can suppress HCC cells proliferation and invasion. We found that HCC patients with lower expression of miR-129 tended to have more advance stages. And there is also significant difference between the miR-129 expression in metastatic tumors and non-metastatic tumors. The target gene, PAK5, is frequently up-regulated and could promotes cell viability and invasion. Taken together, these results indicate that miR-129 plays a fundamental role in HCC and have important clinical implications.

The tumor suppressor function of miR-129 has been determined in the present works, mostly functioned as pro-apoptotic miRNA. It's found that epigenetic repression might lead to altered expression of miR-129 and frequent DNA methylation of miR-129 was found in hematological malignancies, gastric cancer and liver cancer [15,20]. As mentioned before, some molecules were also found to be directly targets of miR-129. It was reported that miR-129 gain of function resulted in G1 phase arrest and repressed proliferation rate, causing cell apoptosis. These effects were partially due to downregulation of CDK6. Similarly, miR-129 could sensitized colorectal carcinoma cells to 5-FU treatment, triggering apoptosis. The mechanisms underlying these effects is related to the decreased expression of BCL2. Recently, wang et al. found the migration

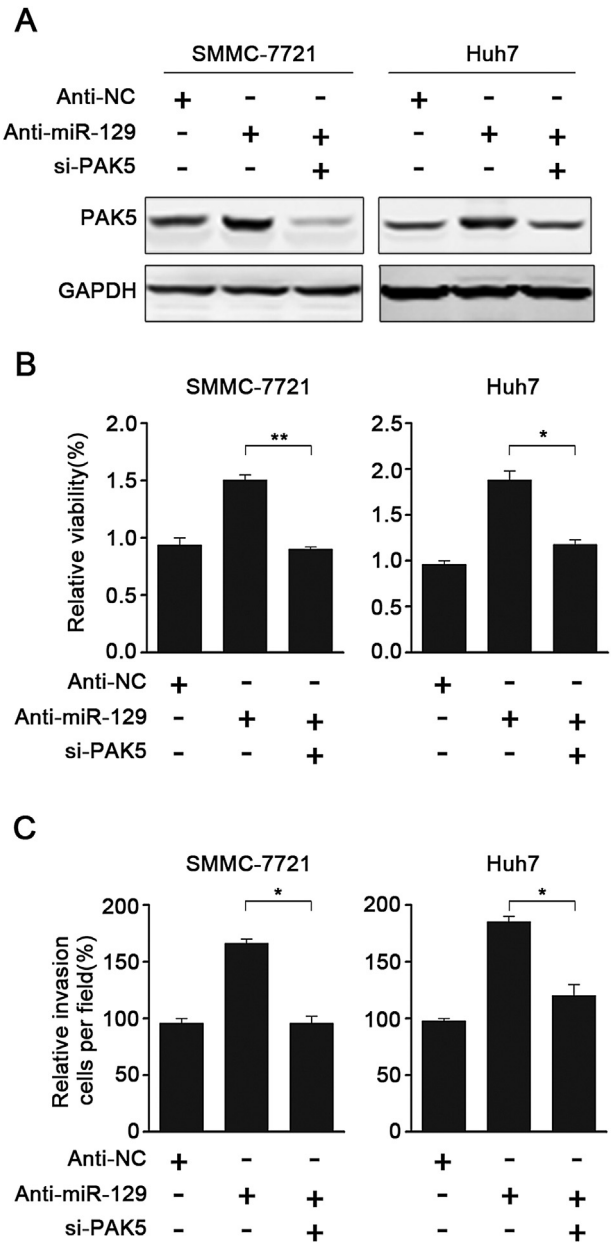


Fig. 4. PAK5 involved in miR-129 mediated suppression of HCC cells proliferation and invasion. A. Western blot analysis was used to detect the PAK5 expression in SMMC-7721 and Huh7 cells after transfection with miR-129 antagonist, si-PAK5, or NC. GAPDH served as an internal control. B. The proliferation abilities of SMMC-7721 and Huh7 cells after transfection with miR-129 inhibitor, si-PAK5, or NC were analyzed by CCK8. C. Representative results of transwell assays on invasion abilities of cells. Results of invasive cells were described as the average number of invasion cells from five randomly selected fields. Data were representative as the mean ± SEM of triplicated independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$).

inhibitory of miR-129 in gastric cancer by targeting BDKRB2 [21]. In our study, we identified that overexpression of miR-129 in HCC cells suppressed cell proliferation through inducing cell cycle arrest and promoting apoptosis. Also, we found that miR-129 inhibited cell migration and invasion as well. These results partially couldn't explained by the current investigations. So with these in mind, we focused on some new targets of miR-129 which might also attribute to the suppression of cell viability and invasion ability.

PAK5, also known as PAK7, has been implicated in several kinds of tumors such as breast cancer, colon cancer and liver cancer [22].

The previous studies reported that PAK5 harbored diverse roles in regulating cell signal pathways. Han et al. found that down-regulation of PAK5 inhibited glioma cell migration and invasion potentially through Egr1-MMP2 pathway, which was intriguing, that miR-129 was also reported to target Egr1 [23]. So these implicates the complicated and precise regulation networks. PAK5 was also found to promote paclitaxel-chemoresistance of epithelial ovarian cancer [12]. However, chemical therapeutic suffers great changes in HCC, so our study on miR-129-PAK5 axis offers great potential to provide new strategy for these situation. More specifically, in the reverse experiments, we used siRNA to diminish the expression of PAK5 which was up-regulated by anti-miR-129 and the results supported the regulatory concern.

We attempt to analyze the relationship of miR-129 and PAK5 expression in clinical samples. As Fang et al. reported, PAK5 expression was elevated in HCC tissues [14]. Consistent with this result, our study found that the expression of PAK5 in 76 paired HCC samples was also enhanced in 53 cases. Subsequently, we evaluated the relationship of miR-129 and PAK5. We determined that PAK5 expression was inversely correlated with miR-129 expression. So the elevated expression of PAK5 might be prevented or ameliorated by reinforcement of miR-129 expression.

In conclusion, this study reveals that miR-129 is frequently decreased in HCC and involved in regulating HCC development and progression. Further experiments demonstrates that PAK5 is considered to be a target of miR-129 and downregulation of PAK5 is at least partially responsible for miR-129 mediated tumor suppression functions. Thus, restoration of miR-129 and inhibition of PAK5 could be further investigated which allow for more effective therapeutic strategy, as well as additional targets for the clinical drugs development.

Conflict of interest

No potential conflicts of interest were disclosed.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.108.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.108.

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