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miR-1297 mediates PTEN expression and contributes to cell progression in LSCC

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression after transcription, and are involved in cancer development. Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignant neoplasms with increasing incidence in recent years. In this paper, we report the overexpression of miR-1297 in LSCC and Hep-2 cells. In addition, PTEN was identified to be directly regulated by miR-1297 through western blot and luciferase activity assay. Furthermore, downregulation of miR-1297 in Hep-2 cells was shown to inhibit cancer cell proliferation, migration, and tumor genesis. Our results document a new epigenetic mechanism for PTEN regulation in LSCC, which is crucial for the development of these tumors.

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is an aggressive malignancy with higher incidence in men than in women. Tobacco smoking and alcohol drinking are the two major determinants of LSCC risk [1,2]. Air pollution and unhealthy diet are also associated with LSCC risk [3]. Laryngeal cancer treatment procedures include radiotherapy, surgery, and chemotherapy. Despite the effect of multimodal therapies, the quality of life and survival rate remain poor [4]. Understanding the molecular mechanism of LSCC progression may aid in improving diagnosis and therapy.

MicroRNAs (miRNAs) are a class of nonconding small regulated RNAs. In the past decade, hundreds of miRNAs have been well studied, such as miR-21, the miR-let-7 family, and the miR-17–92 cluster. However, more new miRNAs have been found in recent studies, including miR-1297. miRNAs regulate gene expression after transcription by targeting the 3′-untranslated region (3′-UTR) sites. Furthermore, the role of miRNAs in cancer are mediated by their target genes, which are crucial for cancer progression in this manner [5]. Phosphatase and tensin homolog (PTEN) is a well-known tumor suppressor gene mutated in a large number of cancers at high frequency, containing a tensin-like domain and a catalytic domain. Generally, PTEN acts as tumor suppressor by negatively regulating the AKT/PKB signaling pathway. PTEN activity is lost via mutations or promoter methylation at high frequencies in many primary human cancers [6,7]. The deregulation of PTEN contributes to tumor genesis, metastasis, and proliferation [8–12].

In this study, we investigated the global miRNA expression by q-PCR array in LSCC to evaluate their involvement in the malignant progression of this tumor. We report that miR-1297 is significantly overexpressed in LSCC. Furthermore, downregulation of miR-1297 in Hep-2 cells inhibits cell proliferation in vitro and decreases tumor genesis in vivo. In addition, we found that miR-1297 directly targets PTEN and suppresses PTEN expression levels in Hep-2 cells and solid tumors.

2. Materials and methods

2.1. Cell lines and human tissues

Hep-2 cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Hep-2 cells were maintained in RPMI 1640 containing 10% fetal bovine serum at 37 °C in 5% CO_2. Human Laryngeal squamous cell carcinoma specimens ($n = 10$) and corresponding adjacent non-neoplastic tissues ($n = 10$) were obtained from patients of the Tianjin First Center Hospital with documented informed consent in each case.

2.2. microRNA qPCR and qPCR-array

RNA was isolated using the mirVana™ miRNA Isolation Kit (Ambion) according to the instructions. Then, miRNA-specific reverse transcription and PCR amplification were performed using the Taqman miRNA detected kit (ABI). The expression level was quantified using the $2^{-\Delta\Delta Ct}$ method. qPCR was performed in

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96-well plates using an ABI 7500 instrument. qPCR was performed in duplicate for each miRNA, and a non-template control was included on the same plate. All samples were analyzed on the same plate for a specific miRNA to control inter-assay variation. The qPCR reaction mixture consisted of 10 μl 2× all-in-one qPCR Mix, 2 μl all-in-one miRNA qPCR primer, 2 μl universal adaptor primer, 0.4 μl 50× ROX reference dye, and 5.6 μl cDNA. All required reagents were supplied in an all-in-one miRNA qPCR kit (Genecopoeia, Rockville, USA). Amplification was performed on an Applied Biosystems 7500 thermal cycler at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting analysis was carried out at the end of the amplification cycle to verify non-specific amplification.

2.3. MTT assay

Hep-2 Cells were seeded in a 96-well plate at a concentration of 10^3 cells/well. The cells were then maintained at 37 °C for 24, 48, and 72 h after transfection. The cells were treated with 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL) for 4 h at 37 °C. The medium was removed and the precipitate was dissolved in 100 μl DMSO. The absorbance at 570 nm was determined with a microplate reader.

2.4. Flow cytometry analysis

Forty eight hour after transfection, Hep-2 cells were fixed with 75% ethanol after treatment. An indirect labeling method was used to incubate PI; its fluorescence used the FL3 channel. C6 flow cytometry (Accuri) was used for determination.

2.5. Colony formation assay

Twenty-four hours after transfection, Hep-2 cells were seeded in a six-well plate at a density of 1000 cells/well. After two weeks, clones were fixed with methanol and stained with 2% Giemsa solution (Merck, Darmstadt, GERMAN) for 10 min, and then photographed.

2.6. Cell invasion assay

Transfected Hep-2 cells were plated in Transwell cell culture inserts (Invitrogen) at a concentration of 10^5 cells/well. Then, the cells were maintained and allowed to migrate for 24 h after transfection. The passed cells were stained with crystal violet solution and photographed under the microscope.

2.7. DNA construction

For the construction of miR-1297 inhibitor, A DNA fragment with an antisense sequence of miR-1297 was cloned into pmiRZIP (System Biosciences). An empty vector was used as the control. PTEN shRNA (small hairpin RNA) was synthesized in and inserted into pSilencer2.1-U6 vector by BamHI and HindIII. The target sequence for PTEN was 5′-GTATAGAGCGTGCAGATAA-3′. For the luciferase reporter assay, the fragment of PTEN 3′UTR (PTEN UTR) with miR-1297 binding sites or a mutated PTEN 3′UTR (PTEN-UTR-M) was inserted into the pmirGLO dual-luciferase reporter vector using PmeI and XbaI. The primer sequences were as follows: PTEN-UTR-Top, 5′-AAACATTTTTTTTTATCAAGAGGGATAAAACACCATGAAAATAAACTTGAATAAACTGAAAT-3′; PTEN-UTR-Bot, 5′-CTAGATTTCAGTTTATTCAAGTTTATTTTCATGGTGTTTTATCCCTCTTGATAAAAAAAAAATGTTT-3′; PTEN-UTR-M-Top, 5′-AAACATTTTTTTTTATCAAGAGGGATAAAACACCATGAAAATAATGAACTTTAAACTGAAAT-3′; and PEN-UTR-M-Bot, 5′-CTAGATTTCAGTTTAAAGTTCATTATTTTCATGGTGTTTTATCCCTCTTGATAAAAAAAAATGTTT-3′.

2.8. Luciferase reporter assay

The luciferase reporter plasmids (PTEN-UTR or PTEN-UTR-M) were transfected separately or cotransfected with miR-1297 inhibitor or its control vector using Lipofectamine 2000 (Invitrogen) into Hep-2 cells cultured in 48-well plates. Approximately 48 h after transfection, the luciferase activity was tested using the Dual-Glo® Luciferase Assay System (Promega) and an FL-800 Microplate Fluorescence Reader (Bio-Tek).

2.9. Western blot

For Western blot analysis, the amounts of loaded lysates were normalized before SDS gel electrophoresis and the transfer of proteins to PVDF membranes (Millipore). Membranes were blocked and incubated with the PTEN primary antibody (Abcam) and were subsequently incubated with an HRP-labeled secondary antibody (Santa Cruz). GAPDH was used as the loading control. The expression of protein was assessed via enhanced chemiluminescence.

2.10. Murine xenograft model

Stable cell lines with low expressions of miR-1297 were established by transfecting Hep-2 cells with a miR-1297 inhibitor, followed by selection for 30 days to obtain stable cell lines. Up to 1×10^7 cells were mixed with matrigel (BD) and injected into athymic nude mice (six weeks old). The mice were monitored and the tumor size was measured daily, and tumor volumes were calculated as width (mm) × width (mm) × length (mm) × 0.5. The mice used in the experiments were handled in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. After 27 days, the mice were killed.

2.11. Statistical analysis

All data in the study were evaluated using SPSS 11.5 (SPSS Inc., USA). Student's *t* test or one-way ANOVA test were performed to analyze the significance of differences between sample means ob-

Table 1
Top 10 significant expressions of miRNA in LSCC. After RNA extraction, miRNA expression in laryngeal squamous cell carcinoma specimens ($n = 10$) and corresponding adjacent non-neoplastic tissues were analyzed by qPCR array. Data show that miR-1297 was the most significant miRNA in LSCC.

miRNA	Signal ratio (Log2)	*p*-Value
Top 10 up-regulated miRNA in microarray analysis		
hsa-miR-1297	8.41	4.34×10^{-04}
hsa-miR-21	6.10	1.12×10^{-05}
hsa-miR-1308	4.45	2.42×10^{-03}
hsa-let-7b	3.87	1.33×10^{-03}
hsa-miR-20a	3.17	1.49×10^{-03}
hsa-miR-923	2.14	1.44×10^{-03}
hsa-miR-27a	2.13	1.62×10^{-03}
hsa-miR-214	2.10	1.44×10^{-03}
hsa-miR-23b	1.72	1.76×10^{-03}
hsa-miR-24	1.43	3.22×10^{-04}
Top 10 down-regulated miRNA in microarray analysis		
hsa-miR-34a	−6.08	2.13×10^{-03}
hsa-miR-663	−5.21	3.02×10^{-03}
hsa-miR-22	−4.97	2.65×10^{-03}
hsa-miR-205	−4.64	1.57×10^{-03}
hsa-miR-18a*	−3.11	2.92×10^{-04}
hsa-miR-193a	−3.03	3.41×10^{-03}
hsa-miR-26a	−2.95	4.03×10^{-03}
hsa-let-185–3p	−2.57	2.19×10^{-04}
hsa-miR-16	−1.85	1.97×10^{-04}
hsa-miR-449a	−1.48	2.02×10^{-03}

* Mature sequence hsa-miR-18a-3p.

tained from three independent experiments. Differences were considered significant at values of $p < 0.05$.

3. Results

3.1. Aberrant expression pattern of miRNAs in LSCC

The results of comparison between human LSCC specimens ($n = 10$) and corresponding adjacent non-neoplastic tissues ($n = 10$) via qPCR array revealed that various miRNAs exhibited differentiation between the two groups. The top 10 different miRNA samples are shown in Table 1. Among these samples, miR-1297 was the most significant in LSCC.

3.2. miR-1297 is highly expressed and inversely correlated with PTEN expression in LSCC tissues

To analyze the expression of miR-1297 and PTEN in normal tissues and those in LSCC, real-time qRT-PCR was employed to quantify the level of miR-1297 in ten pairs of matched human LSCC tissues/surrounding tissues and Hep-2 cells. As shown in Fig. 1A, the expression of miR-1297 is significantly higher in LSCC specimens and Hep-2 cells than that in normal tissues. Furthermore, we investigated the protein expression of PTEN in these tissues and Hep-2 cells by western blot analysis. As shown in Fig 1B, the protein expression of PTEN is significantly lower, whereas the expression of miR-1297 is higher in LSCC specimens and cell lines than that in normal tissues. Moreover, we found the expression of miR-1297 was inversely correlated with protein expression of PTEN in LSCC tissues (Fig. 1C).

3.3. Downregulation of miR-1297 inhibits LSCC cell progression

The aberrant expression of miR-1297 suggested its positive role in LSCC progression. Therefore, MTT, colony formation, Transwell, and flow assay were performed in Hep-2 cells. Compared with the control group, Hep-2 cells exhibited a more significant reduction in cellular proliferation after miR-1297 downregulated (Fig. 2).

3.4. miR-1297 directly targets PTEN

miRNAs are regulator small RNAs, so finding miR-1297 targeting genes, which are critical for LSCC, is important. After prediction via the Targetscan database, we determined PTEN as a candidate target gene of miR-1297 (Fig. 3A). Luciferase reporter assay and Western blot were performed to validate the relationship between

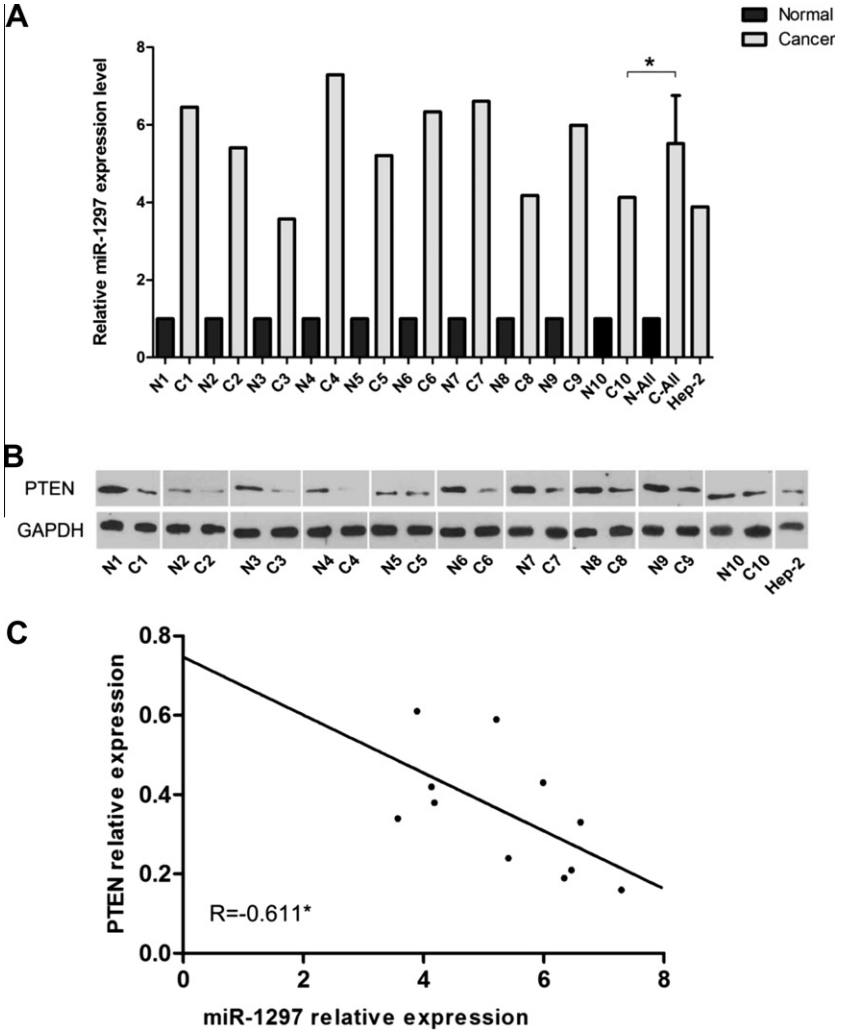

Fig. 1. Identification the overexpression of MiR-1297 and inversely expression pattern of PTEN in LSCC tissues and Hep-2 cells. The expression level of miR-1297 (A) and PTEN protein level (B) were measured in ten pairs of LSCC tissues/surrounding tissues and Hep-2 cells by real-time qRT-PCR and Western blot, U6 snRNA and GAPDH as the control. The expression of miR-1297 was determined by triplicate measurements for each sample and were quantified using the $2^{-\Delta\Delta Ct}$ method. The expression level in normal tissues is set to 1. (C) The correlation of PTEN protein and miR-1297 levels ($r = -0.611$) are shown. $^{*}p < 0.05$.

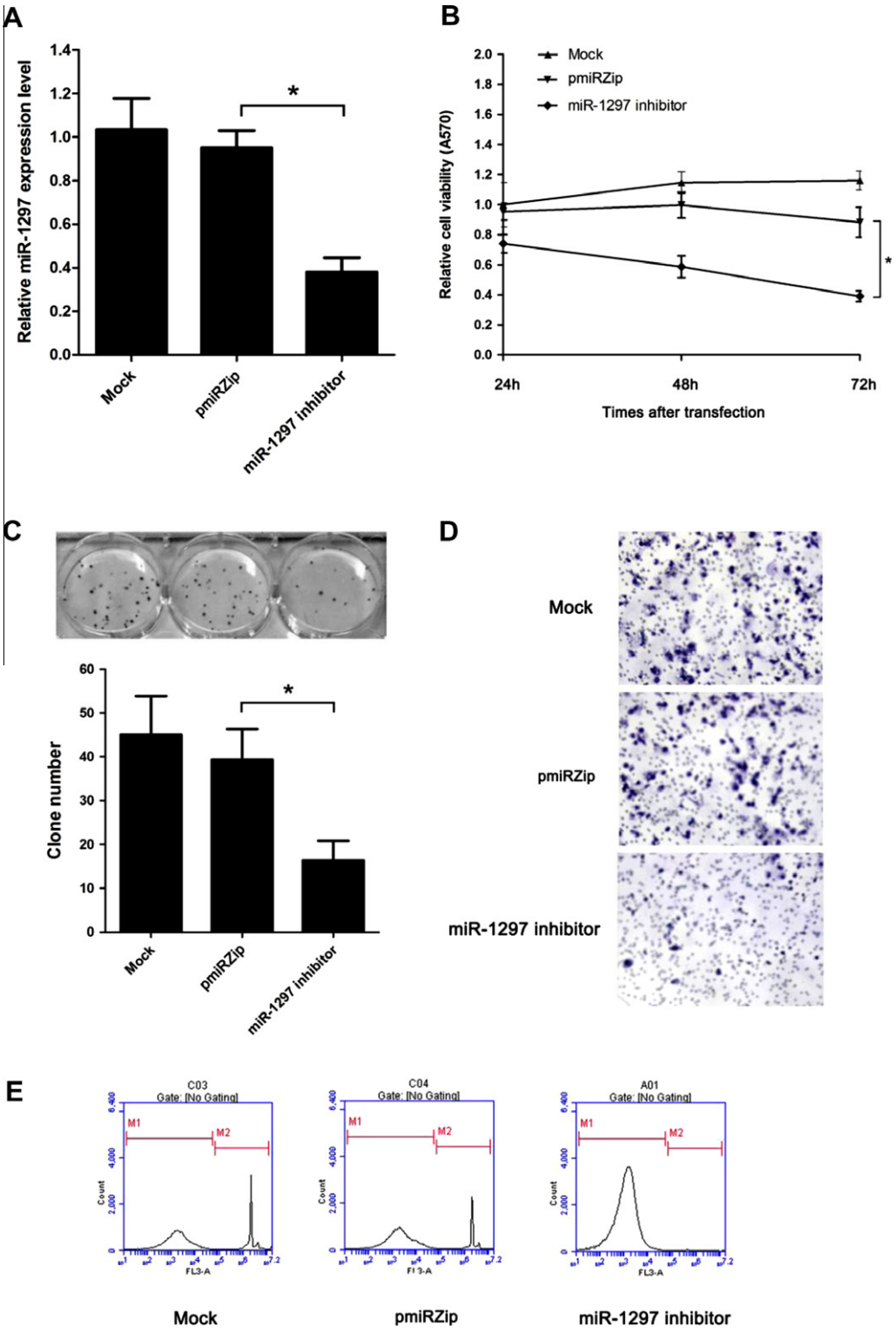

Fig. 2. Downregulation of miR-1297 inhibits the proliferation of the Hep-2 cell line. Hep-2 cells were transfected with an miR-1297 inhibitor and an empty vector as the control. (A) The transfection of miR-1297 inhibitor decreased the expression of miR-1297 in Hep-2 cell lines. MTT assay (B), colony formation assay (C), invasion assay (D), and flow cytometry analysis (E) revealed that the downregulation of miR-1297 in Hep-2 cells inhibited cell proliferation more significantly than that in the control group. $^{*}p < 0.05$.

miR-1297 and PTEN. In the Western blot analysis, the downregulation of miR-1297 could significantly upregulate PTEN expression compared with the control group (Fig. 3B). As for the luciferase reporter assay, miR-1297 decreased luciferase activity with PTEN 3′UTR, but had no effect on luciferase reporter with mutated miR-1297 binding elements (Fig. 3C). We have proofed that miR-1297 is overexpressed, whereas PTEN is downregulated in Hep-2 cells, and miR-1297 can inhibit the expression of PTEN directly. So we speculate that miR-1297 regulate Hep-2 cell progression through PTEN. To support this hypothesis, we constructed PTEN shRNA plasmid to reverse the effect on PTEN caused by miR-1297 downregulation in Hep-2 cells. As shown in Fig 3D,

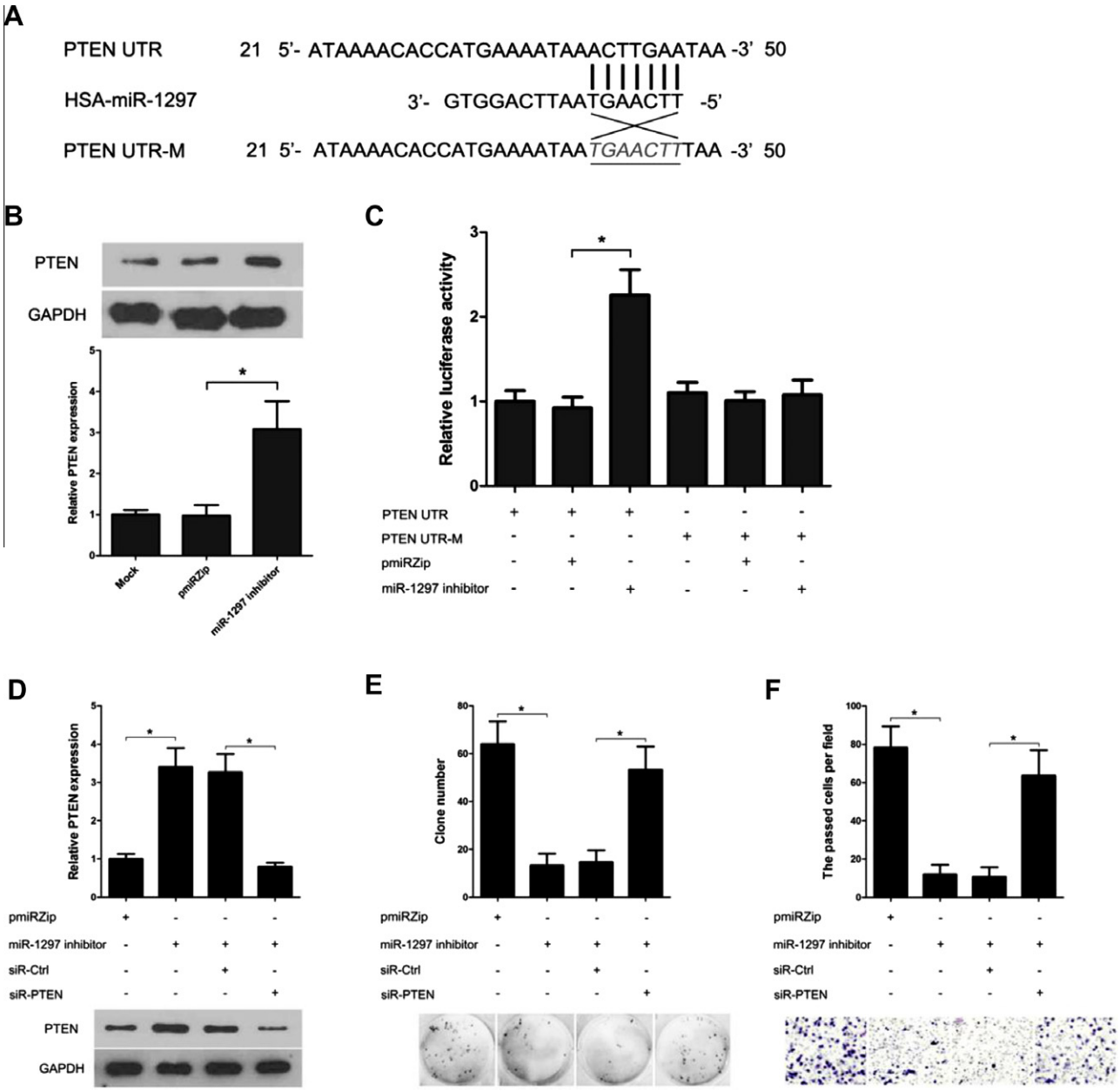

Fig. 3. MiR-1297 regulates the expression of PTEN. (A) PTEN 3′UTR with natured or mutated miR-1297 binding sites based on the Targetscan database. (B) Results of the Western blot analysis of PTEN expression after treatment with the miR-1297 inhibitor. The expression of PTEN in Hep-2 cells was lower in the miR-1297 downregulation group compared with the control group. (C) miR-1297 decreased luciferase activity by binding PTEN. PTEN UTR or PTEN UTR-M was transfected alone or cotransfected with miR-1297 inhibitor or control vectors into Hep-2 cells. 48 h later, cells were lyzed and analysed. The intensity of firefly luciferase in cells cotransfected with PTEN UTR and miR-1297 inhibitor was increased after 48 h ($p < 0.05$). However, miR-1297 inhibitor had no effect on the intensity of firefly luciferase when cells were transfected with the PTEN UTR-M. (D) PTEN expression level was upregulated after the Downregulation of miR-1297, but the following trasfection of PTEN shRNA restored the expression of PTEN. (E, F) The effects of PTEN shRNA on cell proliferation and invasion were evaluated by colony formation and invasion assays. Transfection of PTEN shRNA could significantly reverse the effect of miR-1297 downregulation on Hep-2 cells compared with control group. $^{*}p < 0.05$.

transfection of PTEN shRNA could significantly suppress the expression of PTEN. Furthermore, we evaluated the effects of PTEN shRNA on cell proliferation and invasion ability by colony formation and transwell assays. The results showed that PTEN shRNA can rescue cell colony formation and invasion ability after miR-1297 downregulation in Hep-2 cells. (Fig. 3E and F).

3.5. miR-1297 contributes to tumor genesis in vivo

Stable cell lines with downregulated miR-1297, as well as a control group, were established to generate tumor xenografts. Up to 1×10^7 cells with matrigel were injected into the mice. Tumors were measured daily in two dimensions with calipers. After 27 days, the mice were killed, and the tumors were removed. As shown in Fig. 4 compared with the control group, the tumor volume and PTEN expression level decreased after the miR-1297 downregulated.

4. Discussion

Uncontrolled cell growth and proliferation indicate that malignant tumors remain difficult to treat. Although cancer treatments, including radiotherapy, surgery, and chemotherapy are performed, the quality of life and extended survival rate remain poor.

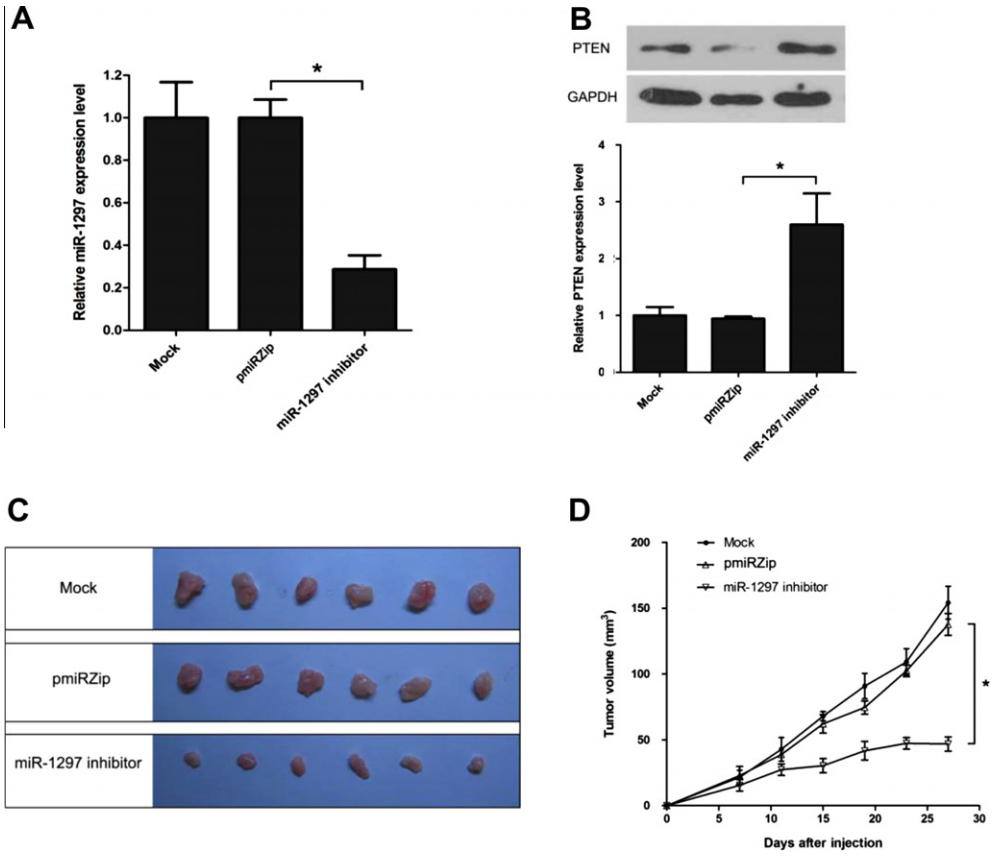

Fig. 4. Downregulation of miR-1297 inhibits tumor growth in vivo. Stable cell lines were injected into mice. Twenty-seven days after injection, the mice were killed. (A, B) miR-1297 expression and the protein level of PTEN in solid tumors were analysed. (A) Realtime PCR result suggested that miR-1297 was downregulated in solid tumors of miR-1297 inhibitor Hep-2 cells compared to control group. (B) PTEN expression level was more upregulated in this group than in the control group. (C, D) The downregulation of miR-1297 significantly decreased the volume of tumors compared with the control group. $^{*}p < 0.05$.

Furthermore, a number of types of tumors exhibit resistance to the universal application of chemotherapy drugs. As such, more efficient treatment tools, such as gene therapy, are important for cancer therapy. MiRNAs are nonconding RNAs, but they could regulate tumor progression through target genes, which are critical for cancer. The downregulation of miR-206 contributes to LSCC proliferation and invasion via VEGF [13]. The exogenous overexpression of miR-375 and the knockdown of the miR-106b-25 cluster inhibit the proliferation of HNSCC (head and neck squamous cell carcinomas) [14]. MiR-1297 is one poorly understood miRNA. In the recent study, miR-1297 was shown to be downregulated in colorectal cancer [15]. Here we found that miR-1297 was significantly upregulated in LSCC compared with normal tissue. Furthermore, the downregulation of miR-1297 has been proven to contribute to LSCC cell proliferation both in vitro and in vivo.

PTEN is a dual lipid and a protein phosphatase, as well as a well-known tumor suppressor gene downregulated in a variety of cancers [12,16]. The loss of PTEN function results in the accumulation of PIP3, triggering the activation of its downstream effectors, namely, PDK1, AKT/PKB, and Rac1/cdc42 [17]. The deregulation of AKT promotes cancer proliferation, invasion, and tumorigenicity [18–21]. PDK1 was found to regulate cancer cell motility via the inhibition of ROCK1 by RhoE, and has been found to be overexpressed in breast cancer, gastric carcinomas, and HNSCC [22–25]. The functions of PTEN as a tumor suppressor have been well documented in early studies. The PTEN gene is frequently deleted or mutated not only in human glioblastoma and canine osteosarcoma, but also in a wide range of advanced human malignancies, such as gastrointestinal stromal tumor, hepatoma, breast cancer, lung cancer, thyroid cancer, head and neck cancers, malignant melanoma, and lymphoma [26–33]. In this paper, we demonstrated that PTEN is downregulated in Hep-2 cells and is directly targeted by miR-1297, suggesting that the positive role of miR-1297 in LSCC may be represented by PTEN.

In summary, our study revealed the overexpression of miR-1297 in LSCC and the downregulation of inhibited LSCC proliferation both in vitro and in vivo. Moreover, we found a novel target gene of miR-1297: PTEN, a crucial tumor suppressor. Further research will focus on the mechanism of miR-1297/PTEN in LSCC. This newly identified miR-1297/PTEN link may be useful in understanding LSCC further, and provides a new, potential therapeutic target for treating LSCC.

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