

## Research Article

# Human Bone Marrow Mesenchymal Stem Cell (hBMSCs)-Derived miR-29a-3p-Containing Exosomes Impede Laryngocarcinoma Cell Malignant Phenotypes by Inhibiting PTEN

Tingting Yu, Hong Yu, Dong Xiao, and Xiangyan Cui 

Department of Otolaryngology, Head and Neck Surgery, The First Hospital, Jilin University, Changchun, Jilin, China

Correspondence should be addressed to Xiangyan Cui; [xyxui@jlu.edu.cn](mailto:xyxui@jlu.edu.cn)

Received 30 January 2022; Revised 29 August 2022; Accepted 21 September 2022; Published 18 October 2022

Academic Editor: Stefan Arnhold

Copyright © 2022 Tingting Yu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Although microRNA-29a-3p was reported to inhibit laryngocarcinoma progression, the potential mechanisms have not been explored clearly. Laryngocarcinoma tissues were collected for analyzing the levels of miR-29a-3p and phosphatase and tensin homolog (PTEN). The miR mimics or inhibitor was transfected into laryngocarcinoma cell lines M4E and Hep2 for the investigation of the biological functions (proliferative, invasion, migratory rates, and apoptotic rates) of this miRNA. The exosomes (Exo) from human bone marrow mesenchymal stem cells (hBMSCs) after the transfection of miR mimics/inhibitor/si-PTEN were isolated and used to stimulate M4E and Hep2 cells. The *in vivo* mouse model was constructed to verify our findings. The miR-29a-3p level was decreased, and PTEN level was elevated in laryngocarcinoma tissues and the cancer cell lines. MiR mimics could inhibit proliferative, invasive migratory rates while promoting apoptotic rates of M4E and Hep2 cells. MiR-29a-3p was enriched in hBMSC-derived Exo, and the Exo from miR-29a-3p mimics transfected hBMSCs could inhibit laryngocarcinoma cell malignant phenotypes *in vitro* and prevent tumor progression *in vivo*. In addition, the direct binding relationship between miR-29a-3p and PTEN in this disease was determined. In conclusion, hBMSC-derived Exo with upregulated miR-29a-3p inhibited laryngocarcinoma progression via regulating PTEN, providing a potential diagnostic and therapeutic target in this disease.

## 1. Introduction

As a common malignant tumor in the upper respiratory tract, laryngocarcinoma accounts for approximately 25 to 30% of cancer cases worldwide [1, 2]. Although several efficient therapies for this disease including surgical operation, and the combination of radiotherapy and chemotherapy, have been developed in recent years, the prognosis of patients at advanced stage is still poor [3]. Therefore, the investigation of the underlying mechanisms during laryngocarcinoma progression may be helpful to find novel treatment strategies in this disease.

microRNAs (miRNAs) have been emerged as a class of novel noncoding RNA regulator, and their length are approximately 22 nucleotides in length [4]. Increasing evidences determined that the occurrence and development of laryngocarcinoma were involved in the dysregulation of a

large number of miRNAs. For example, miR-552 promotes cell proliferative rate of laryngocarcinoma cells by modulating p53 signaling [5]. MiR-125b participates in the oncogenic role of lncRNA MALAT1 in the phenotypes including cell proliferative and invasive rates in laryngocarcinoma [6]. MiR-29a-3p, a miRNA, was identified as a tumor-suppressing factor in various human cancers such as endometrial cancer [7], gastric cancer [8], and cervical cancer [9]. Recently, few studies revealed that miR-29a-3p could suppress the proliferative and invasive rates in laryngocarcinoma cells [10, 11]. Although the tumor-suppressive role of this miRNA in laryngocarcinoma is known, its potential mechanisms remain unclear.

Exosome (Exo) (30–200 nm) is a small single-membrane vesicle and exists in almost all mammalian cells [12, 13]. It has been reported that exosomes always act as the cargo carrier for miRNAs and is a promising therapeutic tool [14].

Previous studies indicated that exosomal miR-29a-3p derived from human bone mesenchymal stem cells (hBMSCs) could efficiently suppress cell migrative ability and vasculogenic mimicry in glioma [15]. Due to its role in laryngocarcinoma, we speculated that the exosomal miR-29a-3p derived from hBMSCs might also exert a potential impact in cancer progression.

Phosphatase and tensin homolog (PTEN) is a well-known tumor suppressor protein and has been identified to participate in various tumor progression including prostate cancer [16], osteosarcoma [17], non-small-cell lung cancer [18], and laryngeal carcinomas [19]. Previous studies revealed that inhibition of miR-744-3p could restore PTEN expression, and then suppressed laryngeal squamous cell carcinoma progression [20], suggesting that upregulation of PTEN could contribute to cancer development. More interestingly, PTEN was identified to be one of the downstream genes of miR-29a-3p and participated in the regulation of this miRNA during human diseases [21, 22]. This study aimed to investigate the role of exosomal miR-29a-3p in laryngocarcinoma, as well as the regulatory relationship between miR-29a-3p and PTEN.

In this study, we further verified the downregulation of miR-29a-3p in laryngocarcinoma. Notably, exosomal miR-29a-3p-derived hBMSCs could prevent against the development of this disease, and the impact was involved in the change of PTEN level. The binding relationship between miR-29a-3p and PTEN was also elucidated in our study. Our results provided that hBMSC-derived exosomes might be a potential treatment target for laryngocarcinoma.

## 2. Materials and Methods

**2.1. Clinical Samples.** A total of 30 laryngocarcinoma patients were recruited at the First Hospital of Jilin University, and the tumor tissues as well as the adjacent normal tissues were collected and snap-frozen in liquid nitrogen for the gene expression analysis. All patients have signed the informed consents.

**2.2. Cell Culture and Transient Transfection.** Human bone marrow mesenchymal stem cells (hBMSCs), laryngocarcinoma cell lines SUN-899, M4E, TU212, and the normal pharyngeal epithelial cell line NP69 were obtained from Bank of Chinese Academy of Sciences (Shanghai, China). The Hep-2 cell line was purchased from American type culture collection (ATCC; Manassas, VA, USA). All cell types were grown within Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) in a 37°C incubator.

To manipulate the expression levels of miRNA and PTEN in cells, the small interfering RNA for PTEN (si-PTEN) and nonspecific control (si-NC) were synthesized from Shanghai GenePharma Co., Ltd, and miR mimics/miR-NC and miR inhibitor/inhibitor NC were purchased from Guangzhou Ribobio Co., Ltd. The siRNAs/mimics/inhibitor were transiently transfected into Hep2 and M4E cells under the help of Lipofectamine 2000 (Invitrogen).

**2.3. Exosome Isolation and Identification.** The surface markers of hBMSCs (CD14, CD29, CD34, CD45, D73, CD90, and CD105) were analyzed by flow cytometry. HBMSCs on passage 4-6 were cultured with serum-free medium for 72 h, and the supernatant was centrifuged through differential centrifugation as previously described [23]. After that, the pellet was washed by phosphate buffered saline (PBS) for twice, and the final pellet was collected in PBS through 2 h of ultrahigh speed ( $100,000 \times g$ ), that is, hBMSC-derived exosomes (BMSC-Exo). The concentration of BMSC-Exo was determined by BCA kit (Beyotime, China).

The BMSC-derived Exo was visualized under a transmission electron microscopy (TEM; Hitachi-7500; Tokyo, Japan). Meanwhile, the exosomes were lysed by RIPA lysis buffer to extract the Exo protein, and the Exo-specific markers (CD9, CD63, and TSG101) were detected by western blot. To obtain the hBMSC-derived Exo with gene expression change of interest genes, hBMSCs were transfected with siRNA/mimics/inhibitor, followed by the isolation of Exo.

**2.4. Exosome Treatment.** To investigate the impact of hBMSC-derived Exo on biological functions of laryngocarcinoma cell lines, Exo derived from hBMSCs transfected with mimics/inhibitor/siRNAs and named by Exo miR-NC, Exo miR mimic, Exo si-PTEN, Exo si-NC, Exo miR inhibitor, Exo inhibitor NC, or Exo miR inhibitor + si-PTEN. Then, 200  $\mu$ g hBMSC-Exo (after transfection) or negative control PBS were added into Hep2 and M4E cells. Two days later, cells were harvested for the subsequent function analysis.

**2.5. Cell Proliferation.** Cell counting kit (CCK)-8 (Dojindo, Tokyo, Japan) was used to evaluate cell viabilities as previously reported [24]. Absorbance values at time point of 24, 48, 72, and 96 h were detected at 450 nm. For colony formation,  $1 \times 10^3$  cells were plated into 6-well plates and cultured for two weeks. Then cells were fixed with methanol for 10 min and stained by 0.1% crystal violet for 15 min. The images were photographed with a light microscope, and colony numbers were counted by naked eyes.

**2.6. Flow Cytometry.** Cells were seeded into 6-well plates with  $1 \times 10^6$  cells/well and cultured overnight, and the supernatant was removed by centrifugation. By using the Annexin V-FITC cell apoptosis detection kit (Biovision, USA), cell pellets were incubated with 500  $\mu$ L loading buffer, 5  $\mu$ L Annexin V-FITC, and 10  $\mu$ L propidium iodide (PI) solution for 15–20 min. Apoptosis rate was measured using the flow cytometry (BD Biosciences).

**2.7. Transwell Assay.** An 8  $\mu$ m well size Transwell chamber (Corning, N.Y., USA) was used for Transwell assay. For cell invasion, 100  $\mu$ L of 50 mg/L Matrigel (1:40) was coated into the upper surface of chamber bottom membrane. 100  $\mu$ L of cell suspension with  $2 \times 10^5$  cells was added to the upper chamber, and the lower chamber was filled with 600  $\mu$ L 20% FBS contained DMEM medium. After 24 h, cells were fixed by methanol for 10 min and stained with 0.1% crystal violet for imaging and counting under five random fields. Cell migration assay was performed as same to invasion assay without coating the Matrigel.

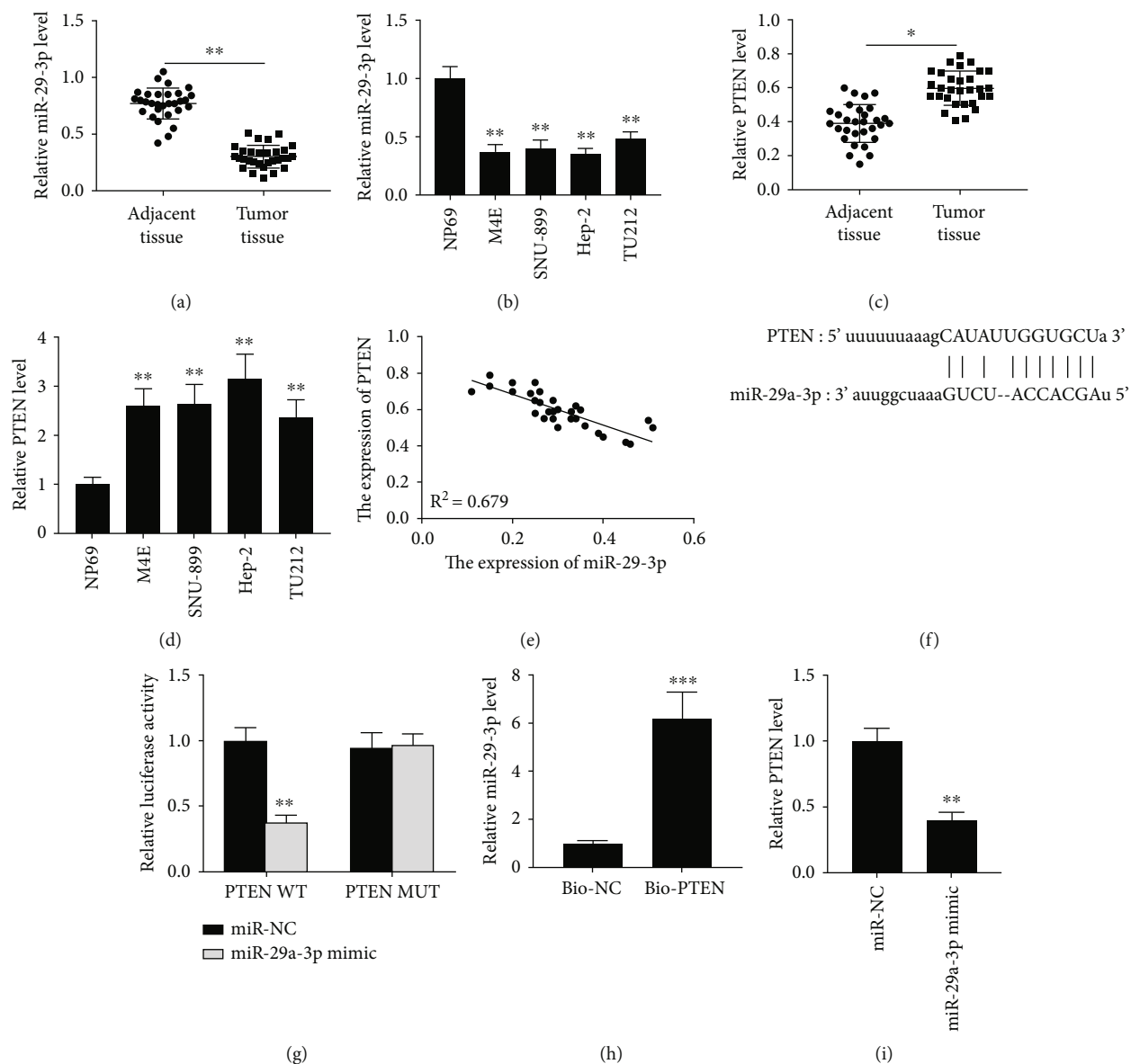


FIGURE 1: The relationship between miR-29a-3p and PTEN in laryngocarcinoma. (a–b) QRT-PCR analysis of miR-29a-3p in laryngocarcinoma tissues (a) and cancer cell lines (b). (c–d) QRT-PCR analysis of PTEN in laryngocarcinoma tissues (c) and cancer cell lines (d). (e) Correlation analysis between miR-29a-3p and PTEN level in laryngocarcinoma tissues. (f) Base sequence of miR-29a-3p and the wild-type as well as the mutant PTEN 3'UTR by Targetscan. (g) Luciferase activity detection after transfection in M4E cells. (h) The enrichment analysis of miR-29a-3p in M4E cells using biotin labelled PTEN probe. (i) QRT-PCR analysis of PTEN in M4E cells after transfected with miR mimics. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

**2.8. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).** Total RNA was extracted by TRIzol reagent (Invitrogen), and the cDNAs were synthesized using specific Reverse Transcription Kit (Applied Biosystems). The subsequent PCR reactions were conducted with SYBR green Super-mix (Thermo Fisher) on ABI 7500 PCR fast detection system. The gene expression analysis was evaluated based on the  $2^{-\Delta\Delta CT}$  method. GAPDH/U6 was used as the normalization control. The sequences of primers in this study are as follows: miR-29a-3p, F: 5' AACAGGTGACTGGTTAGACAA 3', R: 5' GTGCAGGGTCCGAGGT 3'; U6, F: 5' TCGCTTCGGCAGCACATATACT 3', R: 5'

ACGCTTCACGAATTTGCGTGT 3'; GAPDH, F: 5' GGCCAGAAATGCAGTTCGCCTT 3', R: 5' AATGGCACCCTGCTCAGCA 3'; PTEN, F: 5' CTTACAGTTGGCCCTGTACCATCC 3', R: 5' TTTGATGCTGCCGGTAACTCCA 3'.

**2.9. Western Blot.** Total protein samples were extracted by RIPA buffer. A total of 50  $\mu$ g protein sample/lane was separated by 10% SDS-PAGE. After transferring into PVDF membranes, the membrane was incubated with the appropriate primary antibodies CD9 (1:1000), CD63 (1:1000), Tsg101 (1:1000), Calnexin (1:1000), and PTEN (1:1000)

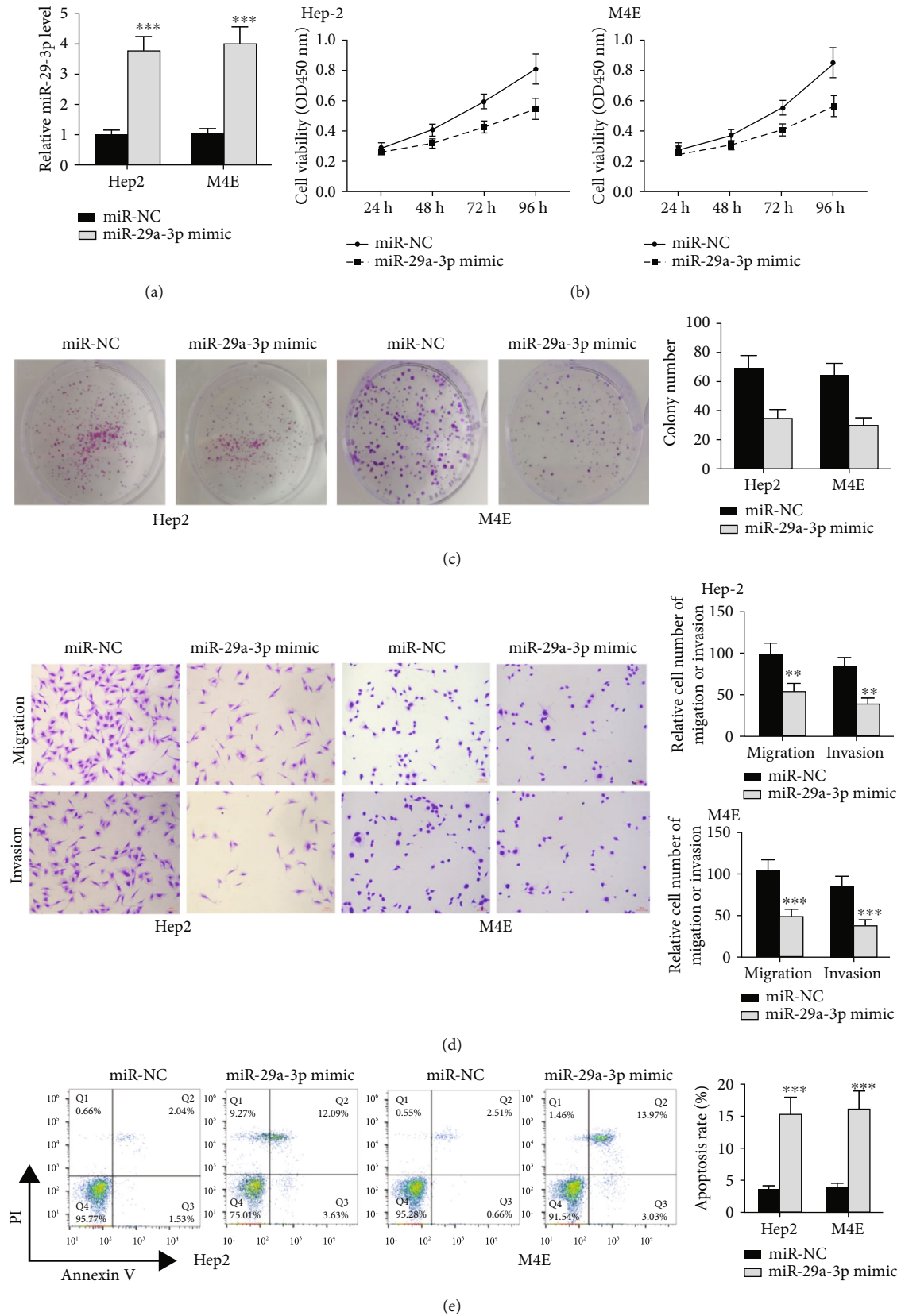


FIGURE 2: MiR-29a-3p upregulation reduced laryngocarcinoma cell malignant progression. M4E and Hep2 cells were transfected with miR-29a-3p mimics or miR-NC. (a) QRT-PCR analysis of miR-29a-3p. (b) CCK-8 assay. (c) Colony formation assay. (d) Transwell assay for detecting cell invasive and migratory rates. (e) Flow cytometry analysis of apoptotic rate. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

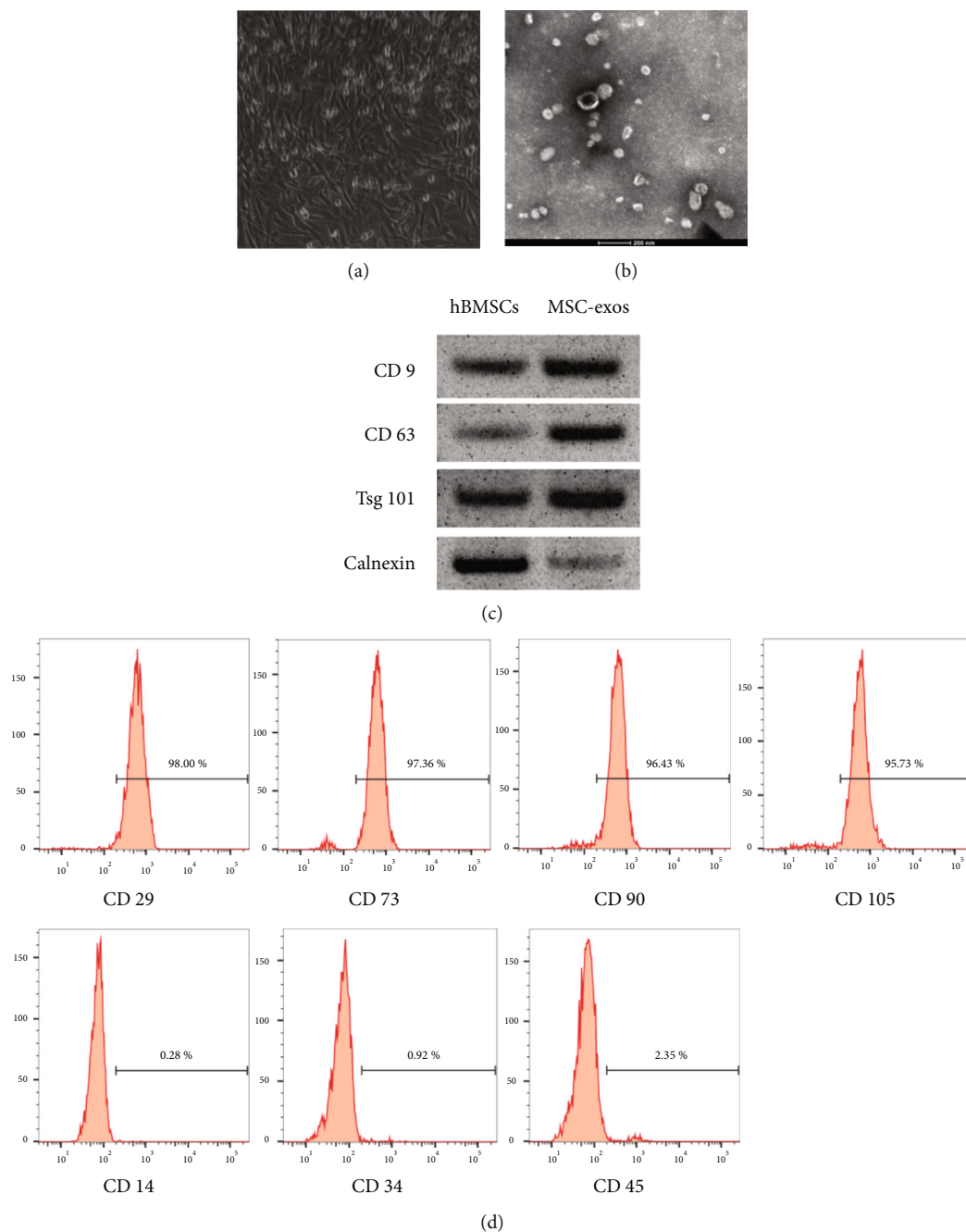


FIGURE 3: Identification of hBMSC-derived exosomes. (a) The morphology of hBMSCs by inverted microscope. Magnification  $\times 400$ . (b) The ultrastructure of hBMSC-derived Exo by a TEM (scale bar = 200 nm). (c) Western blot analysis of Exo surface markers. (d) Flow cytometry analysis of hBMSCs' surface markers.

overnight at  $4^{\circ}\text{C}$ , followed by the incubation with HRP-conjugated secondary antibody (1:10000) for 1 h. All antibodies were purchased from Abcam. The images of protein bands were developed using chemiluminescence reagent, and Image J software was used to gray value analysis.

**2.10. Luciferase Reporter Assay.** The full length of PTEN 3' UTR covering wild-type or mutant miR-29a-3p binding sites were subcloned into pmirGLO dual-luciferase reporter vectors (Promega). After that, the recombinant vectors (PTEN WT and PTEN MUT (the sequence of 3'UTR of PTEN that

predicted to interact with miR-29a-3p was mutated into base complementary sequences)) were individually cotransfected into M4E cells with miR mimics or miR-NC. Two days later, the luciferase activity was tested by a dual-luciferase reporter assay system.

**2.11. RNA Pull-down Assay.** PTEN biotin and PTEN non-biotin labelled probes (Bio-PTEN and Bio-NC) were constructed by Shanghai GenePharma Co., Ltd. and treated with M-280 streptavidin-magnetic beads (Invitrogen) for 2 h at  $4^{\circ}\text{C}$ . Then M4E cells were lysed, and the whole lysates

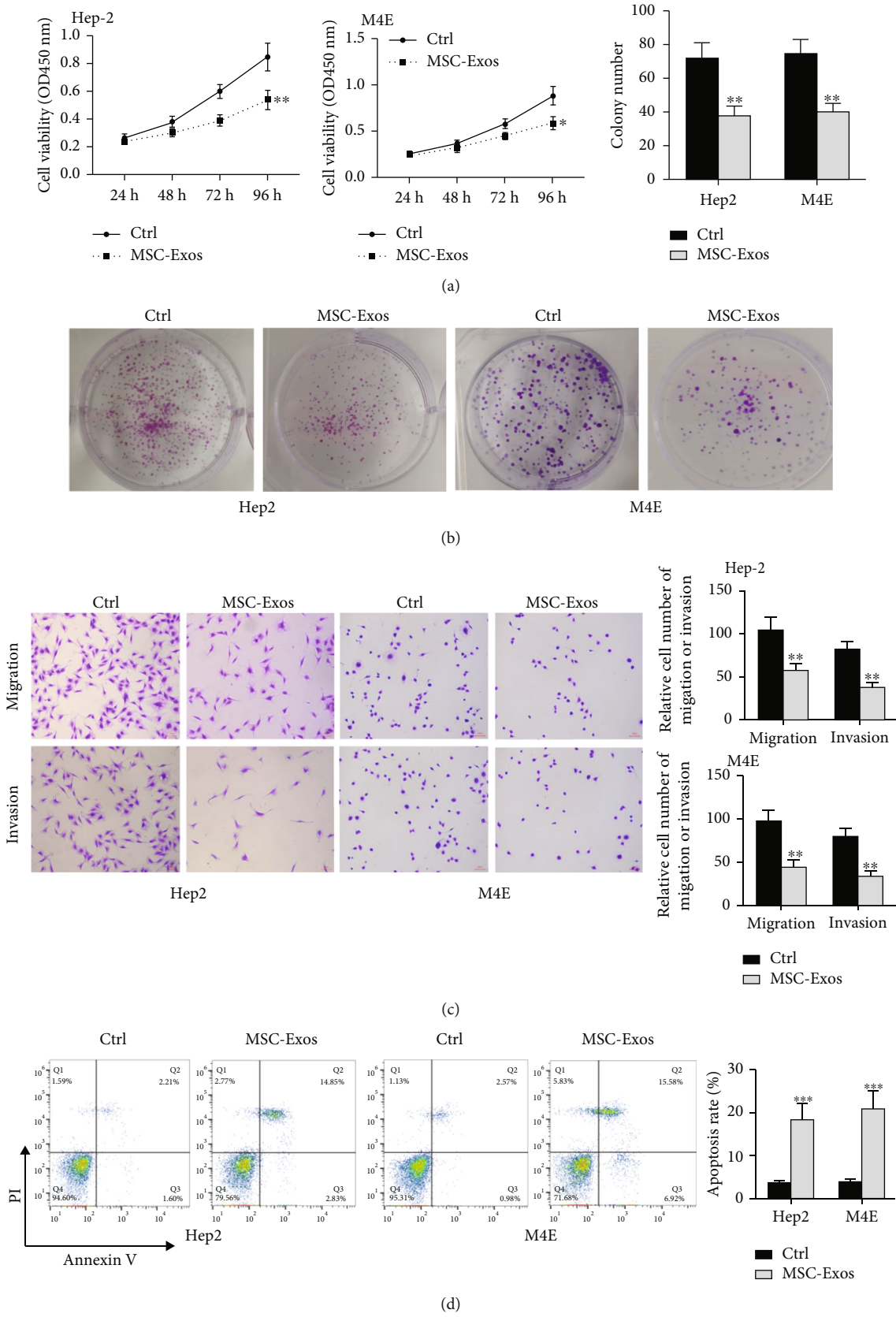


FIGURE 4: HBMSC-derived Exo reduced laryngocarcinoma cell malignant progression. M4E and Hep2 cells were treated with hBMSC-derived Exo for 48 h. (a) CCK-8 assay. (b) Colony formation assay. (c) Transwell assay for detecting cell invasive and migratory rates. (d) Flow cytometry analysis of apoptotic rate. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

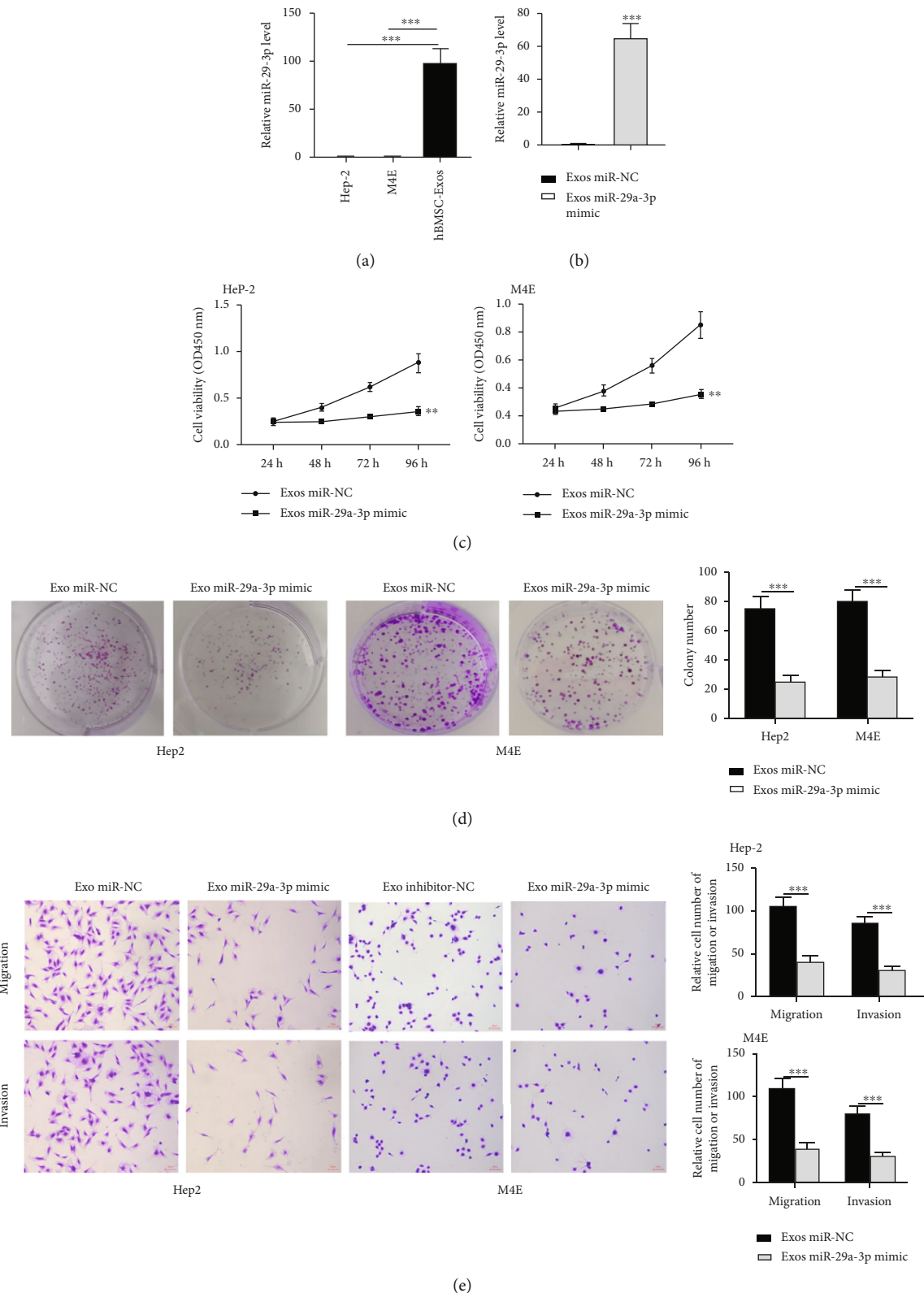


FIGURE 5: Continued.

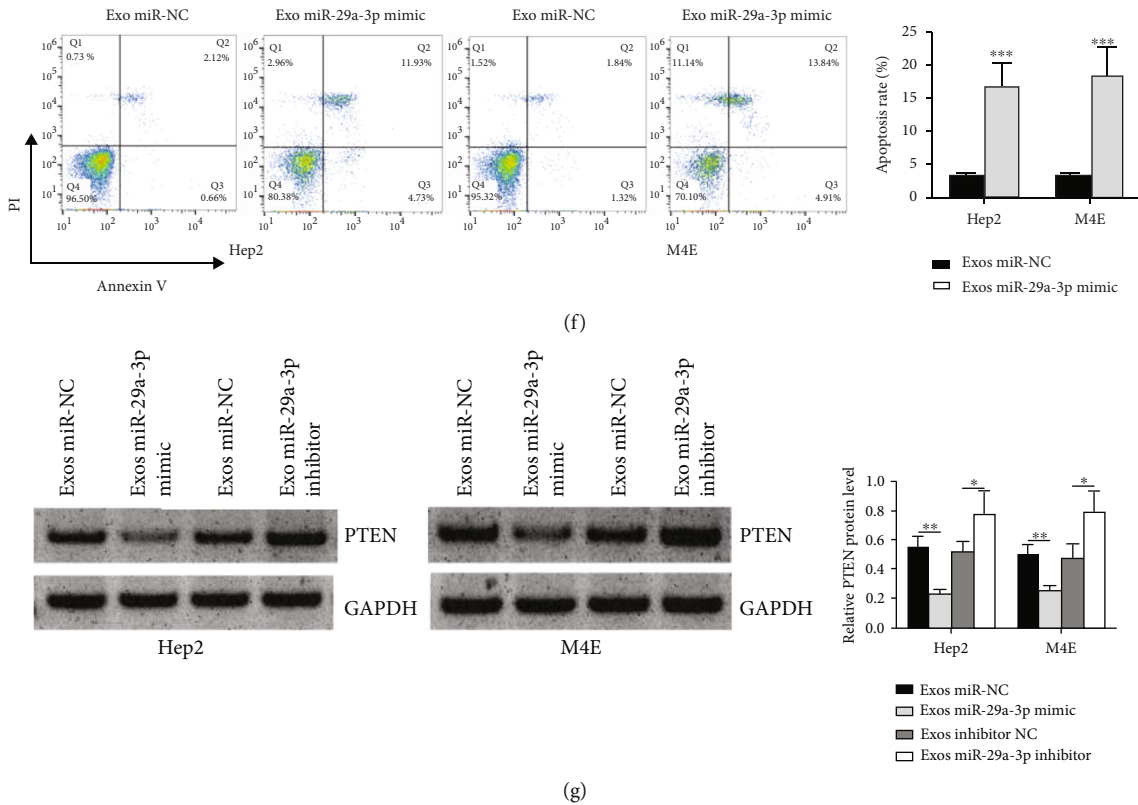


FIGURE 5: HBMSC-derived Exo with upregulated miR-29a-3p inhibited laryngocarcinoma cell malignant progression. (a) QRT-PCR analysis of miR-29a-3p in hBMSC-derived Exo, M4E, and Hep2 cells. (b) QRT-PCR analysis of miR-29a-3p in hBMSC-derived Exo after the transfection of miR mimics and miR-NC. (c–f) The Exo derived from miR mimics or miR-NC-transfected hBMSCs were used to stimulate M4E and Hep2 cells for 48 h. (c) CCK-8 assay. (d) Colony formation assay. (e) Transwell assay for detecting cell invasive and migratory rates. (f) Flow cytometry analysis of apoptotic rate. (g) Western blot analysis of PTEN in two cell types. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

were incubated with probe-coated beads overnight at 4°C. Finally, the RNA was extracted and subjected to qRT-PCR analysis for miR-29a-3p enrichment.

**2.12. In Vivo Model.** A total of 20 BALB/c nude mice (4–6 weeks) were used to construct the animal model. Approximately  $1 \times 10^6$  M4E cells were injected into flank regions of mice via subcutaneous injection as previously described [25]. After the mean tumor volume reaching  $100 \text{ mm}^3$ , all mice were randomly divided into four groups: Exo miR-NC, Exo miR-29a-3p mimics, Exo miR-29a-3p inhibitor, and Exo miR-29a-3p mimics/inhibitor. Five mice were in each group. A dose of  $200 \mu\text{g}/\text{mouse}$  hBMSC-derived Exo was intravenously injected into mice every two days for a total of ten times. Four weeks later, mice were killed by asphyxiation in a  $\text{CO}_2$  chamber; tumors were collected and weighted. The tumor volume was evaluated by  $(\text{length} \times \text{width}^2)/2$ . Meanwhile, the immunohistochemistry (IHC) assay was performed to assess Ki67 positive cells in tumor tissues using anti-Ki67 primary antibody (1:200, Abcam) as previously reported [26]. Slides were observed under a light microscope (Olympus, Tokyo, Japan).

**2.13. Statistical Analysis.** All data were shown as mean  $\pm$  standard deviation (SD), and each experiment was repeated

for three independent times. The differences between groups were analyzed by using the paired Student's  $t$ -test followed by Tukey's post hoc tests when only two groups were compared, or by one-way analysis of variance (ANOVAs) followed by Dunnett's post hoc tests when more than two groups were compared. Statistical significance was set at  $p < 0.05$ .

### 3. Results

**3.1. MiR-29a-3p and PTEN Might Play Crucial Roles in Laryngocarcinoma.** Firstly, qRT-PCR analysis confirmed that miR-29a-3p level was dramatically decreased in laryngocarcinoma tissues from patients and cancer cell lines (Figures 1(a) and 1(b), all  $p < 0.01$ ). On the contrary, PTEN expression was significantly elevated in tumor tissues and cancer cell lines (Figures 1(c) and 1(d), all  $p < 0.01$ ). The levels of miR-29a-3p and PTEN in tumor tissues were negatively correlated (Figure 1(e),  $R^2 = 0.679$ ). Although previous studies have reported the binding sites between miR-29a-3p and PTEN 3'UTR sequence (Figure 1(f)), we reverified their interaction. After transfected with miR mimics in M4E cells, the luciferase activity of PTEN WT was significantly reduced, while the PTEN MUT remained unchanged (Figure 1(g),  $p < 0.01$ ). RNA pull-down assay further



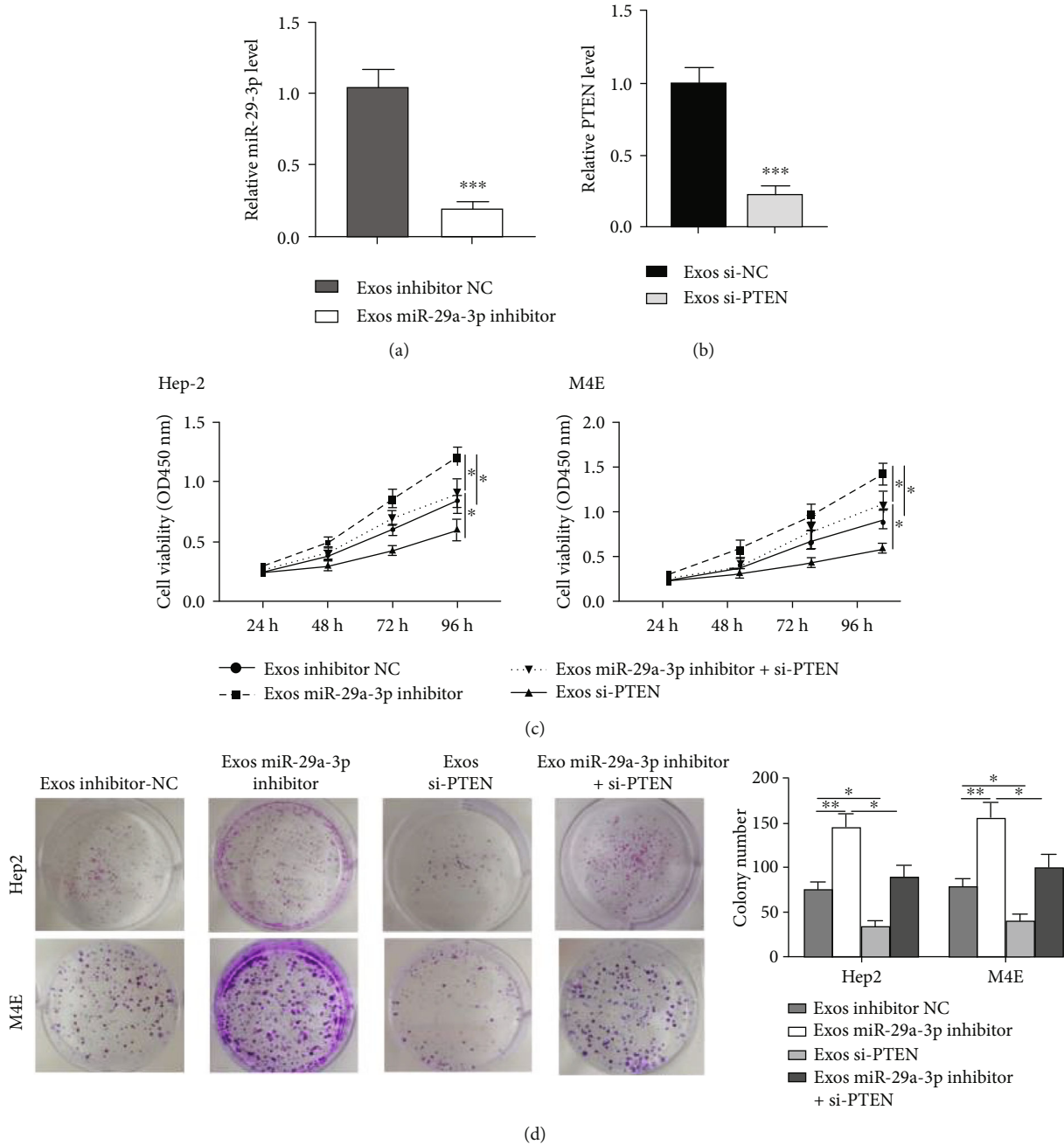


FIGURE 6: Continued.

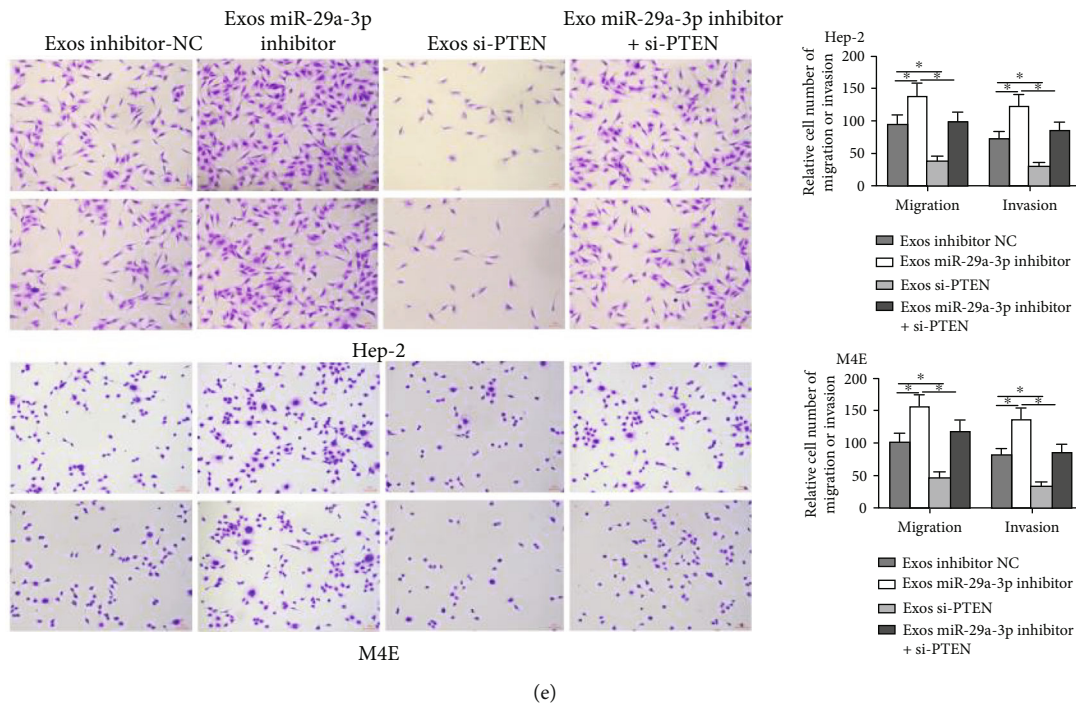


FIGURE 6: The impacts of Exo miR-29a-3p inhibitor and Exo si-PTEN in laryngocarcinoma cell progression. (a–b) MiR-29a-3p inhibitor and si-PTEN were transfected into hBMSCs, and the Exo was isolated. QRT-PCR analysis of the levels of miR-29a-3p (a) and PTEN (b). (c–f) The Exo derived from inhibitor NC, miR inhibitor, si-PTEN, or miR inhibitor + si-PTEN transfected hBMSCs were used to stimulate M4E and Hep2 cells for 48 h. (c) CCK-8 assay. (d) Colony formation assay. (e) Transwell assay for detecting cell invasive and migratory rates. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

determined their direct binding M4E cells (Figure 1(h),  $p < 0.001$ ). In addition, after transfected with miR mimic M4E cells, PTEN level was notably reduced compared to miR-NC (Figure 1(i),  $p < 0.01$ ). It postulates that this axis may play potential roles in laryngocarcinoma.

**3.2. MiR-29a-3p Upregulation Reduced Laryngocarcinoma Cell Malignant Progression.** To further confirm its function, we transfected miR mimics into M4E and Hep2 cell lines, and the level of miR-29a-3p was determined by qRT-PCR analysis (Figure 2(a), both  $p < 0.001$ ). As shown in Figures 2(b)–2(e), we observed that the transfection of miR mimics notably reduced cell proliferative, invasive, and migratory rates while enhanced cell apoptotic rate of two cell lines compared to miR-NC (cell viability, both  $p < 0.05$ ; colony number, both  $p < 0.01$ ; cell invasion and cell migration, all  $p < 0.01$ ; cell apoptosis, both  $p < 0.001$ ). It suggests that miR-29a-3p upregulation can inhibit laryngocarcinoma cell malignant progression *in vitro*.

**3.3. Identification of hBMSC-Derived Exosomes.** To isolate the exosomes from hBMSCs, we observed the morphology of hBMSCs (Figure 3(a)) and hBMSC-derived Exo (Figure 3(b)). Meanwhile, western blot analysis revealed that Exo surface makers CD9, CD63, and Tsg101 exhibited a relative high level, while Calnexin level showed a low level in BMSC-Exo than that in hBMSCs (Figure 3(c)). In addition, flow cytometry analysis indicated that hBMSCs' surface makers CD29 (98%), CD73 (97.36%), CD90 (96.43%), and

CD105 (95.73%) were highly expressed, while CD14 (0.28%), CD34 (0.92%), and CD45 (2.35%) were barely expressed (Figure 3(d)). The data suggest that hBMSC-derived Exo was successfully isolated and can be used for the subsequent studies.

**3.4. HBMSC-Derived Exo Reduced Laryngocarcinoma Cell Malignant Progression.** Subsequently, we isolated hBMSC-derived Exo, then stimulated M4E and Hep2 cells with PBS as the control. As shown in Figures 4(a)–4(d), the proliferative, invasive, and migratory rates of two cell lines were significantly suppressed, while cell apoptotic rate was oppositely enhanced by hBMSC-derived Exo compared to control group (cell viability: Hep-2,  $p < 0.01$ , M4E,  $p < 0.05$ ; colony number, both  $p < 0.01$ ; cell invasion and migration, all  $p < 0.01$ ; cell apoptosis, both  $p < 0.001$ ). Based on these results, we think that hBMSC-derived Exo can attenuate laryngocarcinoma cell progression.

**3.5. HBMSC-Derived Exo with Upregulated miR-29a-3p Inhibited Laryngocarcinoma Cell Malignant Progression.** Interestingly, we observed that miR-29a-3p level in hBMSC-derived Exo was notably higher than that in M4E and Hep2 cells (Figure 5(a), both  $p < 0.001$ ). Then we introduced miR-29a-3p mimics into hBMSCs and isolated miR mimics transfected hBMSC-derived Exo to stimulate M4E and Hep2 cells. MiR-29a-3p level in miR mimics transfected hBMSC-derived Exo was significantly higher than that in miR-NC transfected hBMSC-derived Exo (Figure 5(b),

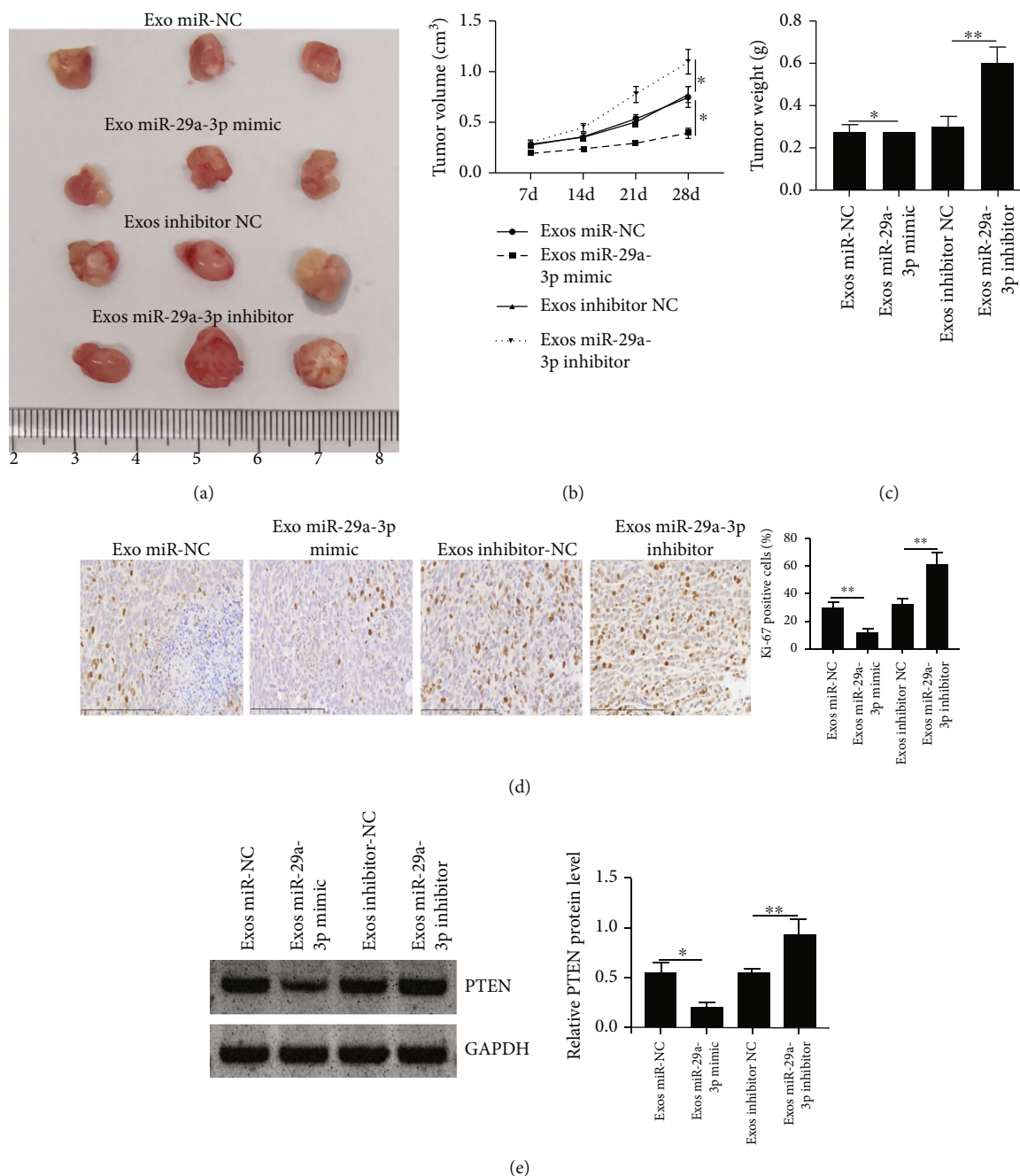


FIGURE 7: The impacts of Exo miR mimics and Exo inhibitor in tumor progression *in vivo*. (a) Representative images of tumors. (b) Tumor volume. (c) Tumor weight. (d) IHC assay using anti-Ki67 antibody in tumor tissues. Scale bar = 100  $\mu$ m. (e) Western blot analysis of PTEN in tumor tissues. \* $p < 0.05$  and \*\* $p < 0.01$ .

$p < 0.001$ ). As shown in Figures 5(c)–5(f), we found that Exo miR-29a-3p mimics notably reduced the proliferative, invasive, and migratory rates of two cell lines, while it enhanced the apoptotic rate of two cell types compared to Exo miR-NC (cell viability, both  $p < 0.01$ ; colony number, both  $p < 0.001$ ; cell invasion and migration, all  $p < 0.001$ ; cell apoptosis, both  $p < 0.001$ ). Exo miR-29a-3p mimics reduced PTEN expression compared with Exo miR-NC, and Exo miR-29a-3p inhibitor enhanced PTEN expression in comparison to Exo inhibitor NC (Figure 5(g), mimic, both  $p < 0.01$ ; inhibi-

tor, both  $p < 0.05$ ). These data indicate that the inhibition of hBMSC-derived Exo on laryngocarcinoma cell malignant progression is associated with the upregulation of miR-29a-3p.

**3.6. HBMSC-Derived Exo with Downregulated miR-29a-3p Enhanced, and Downregulated PTEN Inhibited Laryngocarcinoma Cell Progression.** To confirm our findings, we cointroduced miR inhibitor and si-PTEN into hBMSCs and isolated hBMSC-derived Exo. The levels of miR-29a-

3p and PTEN in Exo miR-29a-3p inhibitor and Exo si-PTEN were both reduced compared to that in Exo inhibitor NC and Exo si-NC, respectively (Figures 6(a) and 6(b), both  $p < 0.001$ ). As shown in Figures 6(c)–6(e), Exo miR-29a-3p inhibitor enhanced the proliferative, invasive, and migratory rates of two cell lines (cell viability, both  $p < 0.05$ ; colony number, both  $p < 0.01$ ; cell invasion and migration, all  $p < 0.05$ ); Exo si-PTEN reduced the proliferative, invasive, and migratory rates of two cell lines (cell viability, both  $p < 0.05$ ; colony number, both  $p < 0.05$ ; cell invasion and migration, all  $p < 0.05$ ), while cotreatment of Exo miR-29a-3p inhibitor and Exo si-PTEN notably attenuated the impacts of Exo miR-29a-3p inhibitor on these cell malignant phenotypes (cell viability, both  $p < 0.05$ ; colony number, both  $p < 0.05$ ; cell invasion and migration, all  $p < 0.05$ ). These data suggest that exosomal miR-29a-3p from hBMSCs inhibits the laryngocarcinoma progression via PTEN.

**3.7. hBMSC-Derived Exo with Upregulated miR-29a-3p Inhibited Tumor Growth, and That with Downregulated miR-29a-3p Enhanced Tumor Growth In Vivo.** Finally, the *in vivo* animal model was constructed to verify our findings. As shown in Figures 7(a)–7(c), we found that Exo miR-29a-3p mimics inhibited tumor growth, tumor volume, and weight (all  $p < 0.05$ ), while Exo miR-29a-3p inhibitor enhanced tumor progression (all  $p < 0.01$ ). From Ki67 staining results, the number of Ki67 positive cells in tumor tissues was significantly reduced by Exo miR-29a-3p mimics and elevated by Exo miR-29a-3p inhibitor (Figure 7(d), all  $p < 0.01$ ). In addition, western blot analysis showed that Exo miR-29a-3p mimics decreased PTEN level ( $p < 0.01$ ), and Exo miR-29a-3p inhibitor increased PTEN level in tumor tissues ( $p < 0.05$ ) (Figure 7(e)). It indicates that hBMSC-derived Exo can inhibit laryngocarcinoma progression via exosomal miR-29a-3p mediated suppression of PTEN.

#### 4. Discussion

In the past decades, stimulating literatures have pointed out the potential of stem cell-derived exosomes in the treatment for different human cancers [27, 28]. Despite of these important significances, more molecular mechanisms involved in exosome treatment have not been well studied. In this study, we revealed that hBMSC-derived exosomes could efficiently inhibit laryngocarcinoma cell malignant phenotypes *in vitro* and tumor growth *in vivo*. Moreover, our data demonstrated that miR-29a-3p and PTEN regulation might account for the suppressive role of hBMSC-derived exosomes in laryngocarcinoma. This study contributed us to understand the potential action mechanism of stem cell-derived exosomes in cancer progression and also develop exosome therapeutic strategy. How to deliver exosomes with changed miRNA level to body and how to make it work without no side effect is becoming a big challenge in laryngocarcinoma.

MiR-29a-3p was observed to be downregulated in many human cancers and thought as a potential tumor-suppressive factor [29]. MiR-29a-3p inhibited the proliferative, migratory, and invasive rates of endometrial cancer cells [7]. Downregulated miR-29a-3p might account for the tumor promotion of

the lncRNA KCNQ1OT1 in hepatocellular carcinoma [30]. In addition, miR-29a-3p was also shown to inhibit the proliferative ability of prolactinoma cells via modulating the  $\beta$ -catenin signaling pathway [31]. Although the oncogenic role of this miRNA in laryngocarcinoma has been reported, its deliver from hBMSCs and the complex mechanisms remains unclear. Here, we found that miR-29a-3p was significantly enriched in hBMSC-derived exosomes. hBMSC-derived exosomes and hBMSC-derived exosomes with upregulated miR-29a-3p could both inhibit laryngocarcinoma cell malignant phenotypes. The results *in vivo* animal model were keeping with that *in vitro* cell experiments. The hBMSC-derived exosomes carrying miRNA mimics might be a novel therapeutic strategy for laryngocarcinoma and other miR-29a-3p downregulated human cancers.

PTEN is a tumor-related gene and was identified to be associated with the process of multiple malignant tumors including prostate cancer [32], acute myeloid leukemia [33], papillary thyroid cancer [34], and also laryngocarcinoma [35]. A series of previous studies indicated that PTEN was a downstream mRNA target of miR-29a-3p and participated in the regulation of this miRNA in tumor progression. For example, miR-29a-3p regulated the development and progression of abdominal aortic aneurysm via direct interaction with PTEN [21]. MiR-29-3p also regulated the progression of hepatocellular carcinoma through targeting PTEN followed by the NF- $\kappa$ B signaling pathway [36]. However, there were few related reports about the regulation of miR-29a-3p and PTEN in the development of laryngocarcinoma. Our study revealed the binding relationship between miR-29a-3p and PTEN, and further confirmed that this axis closely impacted the progression of laryngocarcinoma by performing the rescue experiments. As we known, one miRNA might target one or multiple downstream mRNAs simultaneously in eukaryotic cells. Except for PTEN, whether there were other potential target mRNAs that participated in regulation of miR-29a-3p in laryngocarcinoma, which should be well investigated in the subsequent experiments. Interestingly, several potential targets of miR-29a-3p have been reported in previous studies including STX17 [37], COL4A1 [38], CCNT2 [39], HDAC4 [40], and so on. We will explore these potential mechanisms in the future.

The PTEN/PI3K/AKT axis is reported to implicate in diverse physiological and pathological conditions and plays an important role in the regulation of cell growth and apoptosis in diverse human cancer including prostate cancer, hepatocellular carcinoma, and pancreatic cancer [41–44]. As the key target of PTEN, PTEN is identified to negatively regulate PI3K/AKT signaling [45, 46]. Based on the regulation relationship between PTEN and PI3K/AKT signaling, we speculated that the effect of miR-29a-3p/PTEN regulation axis in laryngocarcinoma might be also mediated by PI3K/AKT signaling. This needed to be further investigated in the subsequent experiments.

#### 5. Conclusion

In summary, our findings thought that hBMSC-derived exosomes could inhibit laryngocarcinoma progression via exosomal miR-29a-3p-mediated suppression of PTEN, suggesting that exosome therapy might be a promising choice for this disease.

## Abbreviations

Exo:	Exosomes
hBMSCs:	Human bone marrow mesenchymal stem cells
miRNAs:	microRNAs
PTEN:	Phosphatase and tensin homolog
PI:	Propidium iodide
SD:	Standard deviation.

## Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Ethical Approval

The present study was approved by the Ethics Committee of the First Hospital of Jilin University. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. The study was conducted in strict accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

## Consent

All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

## Conflicts of Interest

The authors declare that they have no competing interest.

## Authors' Contributions

TY and XC designed and carried out the study. TY and HY participated in experiments and statistical analysis. TY and XC wrote the manuscript. DX help interpreting the data. All authors revised this manuscript. All authors read and approved the final manuscript.

## Acknowledgments

This work was supported by the Natural Science Foundation of Science and Technology Department of Jilin Province grant (20190201223JC).

## References

- [1] C. E. Steuer, M. El-Deiry, J. R. Parks, K. A. Higgins, and N. F. Saba, "An update on larynx cancer," *CA: a Cancer Journal for Clinicians*, vol. 67, no. 1, pp. 31–50, 2017.
- [2] S. McGuire, "Geneva, Switzerland: World Health Organization, International Agency for Research on Cancer, WHO press, 2015," *Advances in Nutrition*, vol. 7, no. 2, pp. 418–419, 2016.
- [3] S. Y. Zhang, Z. M. Lu, X. N. Luo et al., "Retrospective analysis of prognostic factors in 205 patients with laryngeal squamous cell carcinoma who underwent surgical treatment," *PLoS One*, vol. 8, no. 4, article e60157, 2013.
- [4] S. Vienberg, J. Geiger, S. Madsen, and L. T. Dalgaard, "MicroRNAs in metabolism," *Acta physiologica*, vol. 219, no. 2, pp. 346–361, 2017.
- [5] J. Gu, T. Han, L. Sun, A. H. Yan, and X. J. Jiang, "miR-552 promotes laryngocarcinoma cells proliferation and metastasis by targeting p53 pathway," *Cell Cycle*, vol. 19, no. 9, pp. 1012–1021, 2020.
- [6] G. Zong, X. Feng, X. Sun, J. Du, G. Wang, and T. Song, "LncRNA promote cell proliferation and invasion by sponging miR-125b to modulate HMGA1 expression in laryngocarcinoma," *Iranian Journal of Public Health*, vol. 50, no. 5, pp. 959–969, 2021.
- [7] A. Geng, L. Luo, F. Ren, L. Zhang, H. Zhou, and X. Gao, "miR-29a-3p inhibits endometrial cancer cell proliferation, migration and invasion by targeting VEGFA/CD C42/PAK1," *BMC Cancer*, vol. 21, no. 1, p. 843, 2021.
- [8] Z. Zhao, L. Wang, W. Song et al., "Reduced miR-29a-3p expression is linked to the cell proliferation and cell migration in gastric cancer," *World Journal of Surgical Oncology*, vol. 13, no. 1, p. 101, 2015.
- [9] Y. Chen, W. Zhang, L. Yan, P. Zheng, and J. Li, "miR-29a-3p directly targets Smad nuclear interacting protein 1 and inhibits the migration and proliferation of cervical cancer HeLa cells," *PeerJ*, vol. 8, article e10148, 2020.
- [10] T. Liu, W. Wang, Y. C. Xu, Z. W. Li, and J. Zhou, "Long non-coding RNA NEAT1 functions as an oncogene in human laryngocarcinoma by targeting miR-29a-3p," *European Review for Medical and Pharmacological Sciences*, vol. 23, no. 14, pp. 6234–6241, 2019.
- [11] J. Su, E. Lu, L. Lu, and C. Zhang, "MiR-29a-3p suppresses cell proliferation in laryngocarcinoma by targeting prominin 1," *FEBS Open Bio*, vol. 7, no. 5, pp. 645–651, 2017.
- [12] Z. Yao, J. Li, H. Xiong et al., "MicroRNA engineered umbilical cord stem cell-derived exosomes direct tendon regeneration by mTOR signaling," *Journal of Nanobiotechnology*, vol. 19, no. 1, p. 169, 2021.
- [13] D. M. Pegtel and S. J. Gould, "Exosomes," *Annual Review of Biochemistry*, vol. 88, no. 1, pp. 487–514, 2019.
- [14] S. P. Li, Z. X. Lin, X. Y. Jiang, and X. Y. Yu, "Exosomal cargo-loading and synthetic exosome-mimics as potential therapeutic tools," *Acta Pharmaceutologica Sinica*, vol. 39, no. 4, pp. 542–551, 2018.
- [15] Z. Zhang, X. Guo, X. Guo et al., "MicroRNA-29a-3p delivery via exosomes derived from engineered human mesenchymal stem cells exerts tumour suppressive effects by inhibiting migration and vasculogenic mimicry in glioma," *Aging*, vol. 13, no. 4, pp. 5055–5068, 2021.
- [16] H. M. Wise, M. A. Hermida, and N. R. Leslie, "Prostate cancer, PI3K, PTEN and prognosis," *Clinical Science*, vol. 131, no. 3, pp. 197–210, 2017.
- [17] C. Zheng, F. Tang, L. Min, F. Hornicek, Z. Duan, and C. Tu, "PTEN in osteosarcoma: recent advances and the therapeutic potential," *Biochimica Et Biophysica Acta. Reviews on Cancer*, vol. 1874, no. 2, article 188405, 2020.
- [18] C. Pérez-Ramírez, M. Cañadas-Garre, M. Molina, M. J. Faus-Dáder, and M. Calleja-Hernández, "PTEN and PI3K/AKT in non-small-cell lung cancer," *Pharmacogenomics*, vol. 16, no. 16, pp. 1843–1862, 2015.
- [19] V. Ragos, P. P. Fotiadis, E. Tsiambas, and D. Peschos, "PTEN in laryngeal carcinomas," *Journal of BUON*, vol. 21, no. 4, pp. 1024–1025, 2016.

- [20] J. Z. Li, W. Gao, W. B. Lei et al., "MicroRNA 744-3p promotes MMP-9-mediated metastasis by simultaneously suppressing PDCD4 and PTEN in laryngeal squamous cell carcinoma," *Oncotarget*, vol. 7, no. 36, pp. 58218–58233, 2016.
- [21] Y. Zhou, M. Wang, J. Zhang, P. Xu, and H. Wang, "MicroRNA-29a-3p regulates abdominal aortic aneurysm development and progression via direct interaction with PTEN," *Journal of Cellular Physiology*, vol. 235, no. 12, pp. 9414–9423, 2020.
- [22] S. He, Z. Wang, Y. Li et al., "MicroRNA-92a-3p enhances functional recovery and suppresses apoptosis after spinal cord injury via targeting phosphatase and tensin homolog," *Bioscience Reports*, vol. 40, no. 5, 2020.
- [23] C. Théry, S. Amigorena, G. Raposo, and A. Clayton, "Isolation and characterization of exosomes from cell culture supernatants and biological fluids," *Current Protocols in Cell Biology*, vol. 30, no. 1, pp. 3–22, 2006.
- [24] L. Z. Zhou, Y. X. Cui, W. Z. Wang, J. Wu, Z. Sun, and S. Y. Ma, "FFAR4 promotes cell proliferation and migration and serves as a potential biomarker for clinicopathological characteristics and prognosis in laryngocarcinoma," *European Review for Medical and Pharmacological Sciences*, vol. 23, no. 17, pp. 7438–7444, 2019.
- [25] J. Dong, J. Wang, C. Shan, H. Zhang, and O. Xu, "MicroRNA-892a regulates laryngocarcinoma cell proliferation via Dicer," *Experimental Biology and Medicine*, vol. 245, no. 14, pp. 1222–1232, 2020.
- [26] H. Y. Zhu, Y. J. Gao, Y. Wang, C. Liang, Z. X. Zhang, and Y. Chen, "LncRNA CRNDE promotes the progression and angiogenesis of pancreatic cancer via miR-451a/CDKN2D axis," *Translational Oncology*, vol. 14, no. 7, article 101088, 2021.
- [27] B. Bondhopadhyay, S. Sisodiya, F. A. Alzahrani et al., "Exosomes: a forthcoming era of breast cancer therapeutics," *Cancers*, vol. 13, no. 18, p. 4672, 2021.
- [28] N. Y. Kazemi, B. Gendrot, and E. Berishvili, "The role and clinical interest of extracellular vesicles in pregnancy and ovarian cancer," *Biomedicine*, vol. 9, p. 1257, 2021.
- [29] Y. Hozaka, N. Seki, T. Tanaka et al., "Molecular pathogenesis and regulation of the miR-29-3p-family: involvement of ITGA6 and ITGB1 in intra-hepatic cholangiocarcinoma," *Cancers*, vol. 13, no. 11, p. 2804, 2021.
- [30] W. Zhou, H. Li, S. Shang, and F. Liu, "LncRNA KCNQ1OT1 reverses the effect of sevoflurane on hepatocellular carcinoma progression via regulating the miR-29a-3p/CBX3 axis," *Brazilian Journal of Medical and Biological Research*, vol. 54, no. 7, article e10213, 2021.
- [31] J. Xia, S. Li, D. Ma, W. Guo, H. Long, and W. Yin, "MicroRNA-29-3p regulates the  $\beta$ -catenin pathway by targeting IGF1 to inhibit the proliferation of prolactinoma cells," *Molecular Medicine Reports*, vol. 23, no. 6, pp. 1–9, 2021.
- [32] T. Vlajnic, P. Brunner, S. Eppenberger-Castori, C. A. Rentsch, T. Zellweger, and L. Bubendorf, "High inter- and intratumoral variability of Ki67 labeling index in newly diagnosed prostate cancer with high Gleason scores," *Pathobiology*, vol. 1–7, 2021.
- [33] S. Esmaeili, S. Salari, V. Kaveh, S. H. Ghaffari, and D. Bashash, "Alteration of PPAR-GAMMA (PPARG; PPAR $\gamma$ ) and PTEN gene expression in acute myeloid leukemia patients and the promising anticancer effects of PPAR $\gamma$  stimulation using pioglitazone on AML cells," *Molecular Genetics & Genomic Medicine*, vol. 9, no. 11, article e1818, 2021.
- [34] K. Guo, J. Wang, L. Shu, and G. Zhou, "MiR-200c promotes papillary thyroid cancer cell proliferation, migration, and invasion by downregulating PTEN," *Tissue & Cell*, vol. 73, article 101647, 2021.
- [35] Q. Guo and Y. Li, "Research on the expression of Survivin and PTEN in laryngeal squamous cell carcinoma transplanted on the back sides of nude mice treated by gold throat tablets," *Lin Chuang Er Bi Yan Hou Tou Jing Wai Ke Za Zhi*, vol. 26, no. 1134–1137, p. 1143, 2012.
- [36] J. H. Ma, X. Bu, J. J. Wang, and Y. X. Xie, "MicroRNA-29-3p regulates hepatocellular carcinoma progression through NF- $\kappa$ B pathway," *Clinical Laboratory*, vol. 65, no. 5, 2019.
- [37] M. Zhou, X. Liu, E. Qiukai et al., "Long non-coding RNA *Xist* regulates oocyte loss via suppressing *miR-23b-3p* / *miR-29a-3p* maturation and upregulating STX17 in perinatal mouse ovaries," *Cell death & disease*, vol. 12, no. 6, p. 540, 2021.
- [38] H. Zhang, Y. Wang, and H. Ding, "COL4A1, negatively regulated by XPD and miR-29a-3p, promotes cell proliferation, migration, invasion and epithelial-mesenchymal transition in liver cancer cells," *Clinical & Translational Oncology*, vol. 23, no. 10, pp. 2078–2089, 2021.
- [39] R. Tian, X. Guan, H. Qian et al., "Restoration of NRF2 attenuates myocardial ischemia reperfusion injury through mediating microRNA-29a-3p/CCNT2 axis," *BioFactors*, vol. 47, no. 3, pp. 414–426, 2021.
- [40] W. Huang, F. Xiao, W. Huang, Q. Wei, and X. Li, "RETRACTED: MicroRNA-29a-3p strengthens the effect of dexmedetomidine on improving neurologic damage in newborn rats with hypoxic-ischemic brain damage by inhibiting HDAC4," *Brain Research Bulletin*, vol. 167, pp. 71–79, 2021.
- [41] A. Papa and P. P. Pandolfi, "The PTEN/PI3K axis in cancer," *Biomolecules*, vol. 9, no. 4, p. 153, 2019.
- [42] Q. Wang, S. Wu, Y. Gu et al., "RASAL2 regulates the cell cycle and cyclin D1 expression through PI3K/AKT signalling in prostate tumorigenesis," *Cell Death Discovery*, vol. 8, no. 1, p. 275, 2022.
- [43] Y. Zhou, J. Qiu, S. Liu et al., "CFDP1 promotes hepatocellular carcinoma progression through activating NEDD4/PTEN/PI3K/AKT signaling pathway," *Cancer Medicine*, 2022.
- [44] J. Yang, Y. Liu, S. Lu et al., "Coix seed oil regulates mitochondrial functional damage to induce apoptosis of human pancreatic cancer cells via the PTEN/PI3K/AKT signaling pathway," *Molecular Biology Reports*, vol. 49, pp. 5897–5909, 2022.
- [45] L. Shi, W. Zhu, Y. Huang et al., "Cancer-associated fibroblast-derived exosomal microRNA-20a suppresses the PTEN/PI3K-AKT pathway to promote the progression and chemoresistance of non-small cell lung cancer," *Clinical and translational medicine*, vol. 12, article e989, 2022.
- [46] X. Liu and X. Li, "Umbilical cord mesenchymal stem cell-derived small extracellular vesicles deliver miR-21 to promote corneal epithelial wound healing through PTEN/PI3K/Akt pathway," vol. 2022, Article ID 1252557, 15 pages, 2022.