MEDICAL SCIENCE MONITOR BASIC RESEARCH

LABORATORY RESEARCH

e-ISSN 2325-4416
© Med Sci Monit Basic Res, 2019; 25: 218-228
DOI: 10.12659/MSMBR.918134

Received: 2019.06.16
Accepted: 2019.09.13
Published: 2019.10.24

Exosome-Mediated MiR-155 Transfer Contributes to Hepatocellular Carcinoma Cell Proliferation by Targeting PTEN

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Source of support: This work was supported by the Special Funds of the National Natural Science Foundation of China (81872255), Key Medical Talents Fund of Jiangsu Province (2016KJQWZDRC-03), 333 High-level Talents Training Project of Jiangsu Province (BRA2016508), the Six Talents Peak Foundation of Jiangsu Province Project (WSW041)

Background: Most eukaryocytes release nano vesicles (30–120 nm), named exosomes, to various biological fluids such as blood, lymph, and milk. Hepatocellular carcinoma (HCC) is one of the tumors with the highest incidence rate in primary malignant carcinoma of the liver. However, the mechanism of HCC proliferation remains elusive. In this study, we aim to explore whether HCC cell-derived exosomes affect the proliferation of cancer cells.

Material/Methods: Exosomes were isolated from HCC cells by ultracentrifugation and were visualized the phenotype by transmission electron microscopy. Cell proliferation was detected by Cell Counting Kit-8 assays and EdU (5-ethynyl-2-deoxyuridine) incorporation assays. Dual-luciferase assays were performed to validate the paired correlation of miR-155 and 3'-UTR of *PTEN* (gene of phosphate and tension homology deleted on chromosome 10). A xenograft mice model was constructed to verify the effect of exosome-mediated miR-155 on cell proliferation *in vivo*.

Results: Our finding showed that miR-155 was enriched in exosomes released from HCC cells. The exosome-containing miR-155 transferred into new HCC targeted cells and lead to the elevation of HCC cells' proliferation. Besides, the exosomal miR-155 directly bound to 3'-UTR of PTEN leading to the reduction of relevant targets in recipient liver cells. The knockdown of PTEN attenuated the proliferation of HCC cells treated with the exosomal miR-155. Moreover, nude-mouse experiment results revealed a promotional effect of the exosomal miR-155 on HCC cell-acquired xenografts.

Conclusions: Our study indicated that exosomal-specific miR-155 transfers to adjacent and/or more distant cells and stimulates the proliferation of HCC cells.

MeSH Keywords: Carcinoma, Hepatocellular • Exosomes • MicroRNAs • Phosphatidylinositol 3-Kinases • PTEN Phosphohydrolase

Full-text PDF: https://www.basic.medscimonit.com/abstract/index/idArt/918134

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Background

Hepatocellular carcinoma (HCC) is the highest incidence type of primary hepatic malignancy cancer and occurs mainly in people with hepatitis B and C and cirrhosis. HCC is the 3rd most high cause of cancer deaths all over the world, leading to over 500 000 people affected, particular in developing countries due to late diagnosis and poor treatment options [1–4]. However, the pathogenesis of HCC is still far from clear. The discovery of a type of highly conserved small non-coding RNAs with pleiotropic regulatory functions, microRNA (miRNA), has a significant contribution to the deep molecular understanding of hepatocarcinogenesis [5,6]. Owing to the short sequence requirement for incomplete complementarity, an independent miRNA has the capacity to modulate hundreds of targeted genes. It is widely accepted that miRNAs potentially regulate many aspects of tumorigenesis, including proliferation and development, differentiation metabolism, and apoptosis [6–10]. For instance, miR-194 targets *Polycomb repressive complex 1 (PRC1)* and downregulates its expression, leading to the decrease of the migration and growth of primary liver cancer cells through blocking Wnt/β-catenin signaling pathway [11]. MiR-424-5p plays a suppressor role of inhibiting cell histiocytosis and invasion by directly targeting *TRIM29* [12].

Exosomes are nanometer-sized (30–120 nm) membrane vesicles that are secreted from almost all cells, including cancer cells. Ever-increasing studies confirm that proteins, mRNA, and non-coding RNA participate in all biological processes of malignant carcinoma [13–15]. Remarkably, cancer-released exosomes make up a network of the exchange between tumors and non-carcinoma tissues. The evolution of exosome-associated tumor depends on the external stroma of cancer, such as affecting metastasis and development [16–18]. Fu et al. demonstrated that attachment to HCC cell-released exosomes contain SMAD3 protein and stimulate the dispersion of HCC cells [19]. MiR-18a reduces the expression of estrogen receptor-α to promote HCC proliferation and development in women [20].

Exosomal RNAs are released into recipient cells and regulate their biological functions [21]. RNA sequencing (RNA-seq) has shown that miRNAs are abundant in exosomes [22]. In the current study, we discovered that miR-155 was preferentially enriched in exosomes that released from HCC cells. *In vitro* and *in vivo* assays revealed that the exosome-containing miR-155 was transported into HCC recipient cells and promoted HCC cells' proliferation by directly targeting 3'-UTR of *PTEN*. Our study provides evidence to demonstrate that exosomal-specific miRNAs carry proliferative content into recipient cells to stimulate HCC growth.

Material and Methods

Cell culture

We purchased HCC cell lines (MHCC97H, SMMC7721, Hep3B, and HepG2) and human immortalized normal hepatocyte cell line (LO2) from American Type Culture Collection. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, MA, USA) containing 10% fetal bovine serum (FBS), with 100 U/mL penicillin, 100 μg/mL streptomycin (Hyclone, USA) in a 5% CO_2 incubator at 37°C.

Exosome isolation

The cells were cultured in a medium supplemented with 2% exosome depleted FBS (SBI, USA). Centrifuge cultural supernatants for 10 minutes at 1500 g to remove dead cells. After discarding the pellet, the supernatant was transferred to a new collection tube. Then the supernatant was ultra-centrifuged at 12 000 g for 30 minutes (Beckman Coulter Optima MAX-XP), and exosomes were pelleted by ultracentrifugation at 100 000 g for 70 minutes. After discarding the supernatant, the exosome pellet was re-suspended in phosphate-buffered saline (PBS) and centrifuged at 100 000 g for 70 hours.

Transmission electron microscopy

An equal volume of 4% paraformaldehyde was added to the exosome sample and incubated for 2 hours. Then 3 μL aliquots of exosomes were dropped onto the grids which were then incubated in 2% paraformaldehyde for 20 minutes. The grids were transferred to a wax strip and washed with 100 mL PBS. The grids were incubated in 50 mM glycine/PBS for 5 minutes and blocked in 5% bovine serum albumin (BSA)/PBS for 10 minutes and washed with PBS followed by incubation in 2.5% glutaraldehyde for 5 minutes. Following washes of 2 minutes with H_2O, the grids were incubated for 5 minutes in uranyl oxalate and in 1% methyl cellulose. Excess liquid was removed with a filter paper, and the grids were air-dried for 30 minutes. Exosomes were visualized under a Hitachi 7000 transmission electron microscope (Hitachi, Tokyo, Japan) operated at 120 kV.

Cell Counting Kit-8 (CCK-8) assay

We measured cell proliferation using the Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Japan). Dispense 100 μL of cell suspension (5000 cells/well) in a 96-well plate. The plates were pre-incubated for 24 hours in a humidified incubator (37°C, 5% CO_2). Then 10 μL of different concentrations of the test substance were added to the plate. Incubate the plate for an appropriate time in the incubator. Add 10 μL of CCK-8 solution to each well of the plate. The plates were incubated for

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1 hour in an incubator. Measure the absorbance at 450 nm using a microplate reader (Bio-Tek, Elx800, USA).

Generation of miR-155 knockout cells line

The lentiviral packaging plasmid pMD2.G (Addgene 12259), psPAX2 (Addgene 12260), and lenti-CRISPRv2 (Addgene 52961) were obtained from Addgene. The gRNA targeting miR-155 genomic sequence was sub-cloned into the lenti-CRISPRv2 vector. The gRNA sequence (5'-GTTAATGCTAATCGTGATAG-3') was designed online (*http://www.genome-engineering.org/crispr/*). The lentiCRISPRv2 and packaging plasmids were transfected into HEK-293T cells to package virus. Then 5×10^5 Hep3B cells were infected with 100 µL lentivirus for 48 hours with 5 ug/mL polybrene. After limiting dilution, single clones were selected in the presence of puromycin (2 µg/mL) for 3 weeks and then assayed for the expression measurement by polymerase chain reaction (PCR) or western blot.

EdU incorporation assay

The 5-ethynyl-2-deoxyuridine (EdU) incorporation assay was measured as previously described using the Click-iT®EdU [23].

Nanoparticle tracking analysis

Exosomes particles, using nanoparticle tracking analysis (NTA), were measured as previously described [24].

Western blotting

Western blotting was used to analyze protein expression according to standard procedures. After various treatments, exosomes fraction or HCC cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz, LA, USA).The isolated total protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Blocked with 5% non-fat dried milk, the membranes were subsequently incubated with appropriate primary and secondary antibodies and washed. The membranes were imagined using Li-COR Odyssey Infrared Imager (Li-COR Bioscience). The primary antibodies used were CD63 (Santa Cruz), TSG101 (Proteintech), PTEN, Akt, p-Akt, and GAPDH (all from Abcam).

RNA preparation and quantitative real-time PCR

Total RNA was isolated from HCC cells after treatment by using TRIzol reagent (TaKaRa) and operated strictly according to the manufacturer's instructions. Reverse transcription was performed on 2.0 µg of total RNA from each sample using the First Strand cDNA Synthesis Kit (TaKaRa). We performed quantitative real-time (RT)-PCR reaction by using SYBR Green Master Mix (Life Technologies). Quantitative PCR was performed with the ABI Step OnePlus™ system (Applied Biosystems). We purchased RT and PCR primers for miR-155 and U6 from RiBoBio (Guangzhou, China). All miRNA quantification data were normalized U6 snRNA. The Data were analyzed using $2^{-\Delta\Delta Ct}$ method.

Luciferase assays

We used the dual-luciferase reporter assay system (Promega, USA) to detect firefly and Renilla luciferase activity. The *PTEN* 3'-UTR report was obtained from HepG2 cDNA and cloned into the downstream of luciferase in the pLUC-report. The pLUC mutant construct containing seed sequences of miR-155 was produced by mutagenesis PCR. HepG2 cells seeded in 48-well plates to approximately 70% to 80% confluence and transfected with wild type or mutant 3'-UTR of PTEN report. After 6 hours, the transfected cells were incubated with a different type of exosomes. Luciferase activities were measured through firefly luciferase activity normalized to Renilla luciferase activity. All experiments were performed 6 times, and the results were expressed as firefly luciferase activity normalized to Renilla luciferase activity.

In vivo tumorigenesis

Animal experimental protocols were approved by the Animal Experimentation Ethics Committee of Nanjing Medical University. As regard tumor growth analysis in a xenograft model, 5-week-old male nude mice (BALB/c nu/nu) were from the Model Animal Research Center of Nanjing University (Nanjing, China) under specific pathogen-free conditions. We injected HepG2 cells (2×10^6) subcutaneously into the posterior side of each mouse. Once the tumors formed after 15 days, 100 ug exosomes were injected into each tumor. After 15 days, mice were sacrificed, and tumors weight was measured.

Immunohistochemistry

The slides were heated for 15 minutes and subjected to antigen retrieval with citrate buffer (pH 6.0) after deparaffinization and rehydration. After blocking, sections were incubated overnight at 4°C with the PTEN antibodies (Abcam). Two-step technique (SuperPicture™ 3rd Gen IHC Detection kit; Invitrogen, CA, USA) was used for visualization, with diaminobenzidine as a chromogen. In the end, the sections were counterstained with hematoxylin and mounted. We considered immunostaining was apparent in the nucleus as positive PTEN expression.

Statistical analysis

Data from at least 3 independent experiments was analyzed using the GraphPad Prism program (version 7.00 for Windows, San Diego, CA, USA). All data were expressed as mean±standard

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error of mean (SEM) and analyzed with Student's *t*-test or one-way ANOVA followed by Bonferroni's test. *P* values of less than 0.05 were regarded as statistically significant.

Results

Profiles of exosomal miRNAs that are upregulated in HCC via measuring the series of RNA

We utilized the sequencing of RNA to explore the enrichment level of microRNA (miRNA) in exosomes which release from HCC cells. To compare the exosomal RNA profiles that derived from HCC cells or normal hepatic cells, exosomes were isolated from the cultural clear liquid on the surface of MHCC97H (an HCC cell line), as well as LO2 (the people's immortalized hepatocyte line) as a control. Using transmission electron microscope (TEM) the sizes of the extracellular vesicles were predominantly in almost 100 nm in the radius of the isolated exosomes fraction (Figure 1A). This data was consistent with the nanoparticle tracking analysis (NTA); NTA showed the peak size of 100 nm (Figure 1B). Moreover, the features of isolated exosomes were identified by western blotting through detecting special exosomal protein CD63 and TSG101, which was consistent with commercial exosome-standard samples (Exo standard) (Figure 1C). The data demonstrated that the isolated exosomes were in line with the size and morphology reported as before. Compared with LO2 cells, 36 miRNAs were significantly gathered in exosomes released from MHCC97H cells (Figure 1D). For miRNAs in exosomes, we were concerned about miR-155 presenting the highest expression in exosomes (Figure 1D). Quantitative detection by RT-PCR was used to determine RNA sequencing result and found the enrichment level of miR-155 in exosomes of other HCC cells lines. The expression of miR-155 in Hep3B, HepG2, and SMMC7721 HCC cell lines derived exosomes was higher than that in LO2 cells (Figure 1E). These results showed that miR-155 was enriched in exosomes from HCC cells.

The exosomal miR-155 promoted HCC cell proliferation

To explore whether exosomal miR-155 has functional effects on HCC cell proliferation, we constructed a miR-155 gene-knockout Hep3B cell line (Hep3B-miR-155-KO) through the Clustered Regulatory Interspaced Short Palindromic Repeat-cas9 (CRISPR-CAS9) method. MiR-155 was deleted in Hep3B cells (Figure 2A). We collected the exosomes from the cell culture of Hep3B cells (Exo-WT) as well as Hep3B-miR-155-KO cell (Exo-miR-155-KO) to incubate HepG2 cells for 24 hours (Figure 2B). The proliferation ability of HepG2 cells was significantly weaker in Exo-miR-155-KO treatment group than that in Exo-WT incubated group by CCK-8 assay (Figure 2C). Because the most accurate method for testing cell growth is the direct measurement of DNA synthesis, we chose the 5-ethynyl-2'-deoxyuridine (EdU) assay to confirm the proliferation ability of HCC cells. The proliferation ability of HepG2 cells showed a significantly elevation when incubated with Exo-miR-155-KO reference to the treatment group with Exo-WT (Figure 2D). This result indicated that miR-155 has a potential ability of affecting HCC proliferation. At the same time, we co-cultured the Hep3B or Hep3B-miR-155-KO cells with HepG2 cells for 24 hours in a Transwell system. A higher OD450 value of HepG2 cells co-cultured with Hep3B-HepG2 than that of miR-155-KO-HepG2 co-culture system (Figure 2E), suggesting that the soluble factors from Hep3B cells facilitated the proliferation of HepG2 cells. Together, these observations indicated that exosomal miR-155 is transported to a new HCC cells to promote the proliferation.

Exosomal miR-155 directly targets *PTEN*

To explore the molecular mechanism underlying the biological effects of exosomal miR-155 to promote HCC cell proliferation, we analyzed the potential miR-155 target genes by the miRNA online target databases (miRDB, TargetScan, multiMiR, and miRNA.org). Then we analyzed the RNA structure and calculated the scores of each feature to predict the most effectively targeted mRNAs. Among these predicted target genes, we focus on *PTEN* gene, which was predicted by all above miRNA database. PTEN is known to play as a tumor suppressor which dephosphorylates PIP3 produced by phosphatidylinositol-3-kinase (PI3K) to regulate cell biological processes including proliferation [25–28]. Moreover, PTEN was downregulated in rat HCC model and HCC tissue specimens as previous report [29]. To explore whether that miR-155-containing exosome carried into other cells caused a decrease of the predicted target gene, the PTEN 3'-UTRs were linked into luciferase-reporter plasmids (Figure 3A). HepG2 cells were transfected with PTEN 3'-UTRs luciferase-reporter plasmids and then incubated with Exo-WT or Exo-miR-155-KO. The reporter assays indicated that Exo-WT significantly decreased the relative luciferase ability of the reporter construct containing wild-type PTEN 3'-UTR in HepG-2 cells, while the luciferase ratio of the mutational PTEN 3'-UTR reporter vector had no change (Figure 3B). These results indicated the predicted miR-155 targeted sites in the PTEN 3'UTRs were essential for exosomal miR-155-mediated suppression.

Meaningfully, the Exo-miR-155-KO treatment had no effect on the relative luciferase ability to wild-type receptor or mutational PTEN 3'-UTR. Furthermore, the transfer of Exo-WT decreased the expression of target PTEN proteins in recipient HepG2 cells using western blotting, while Exo-miR-155-KO treatment showed no significant changes in PTEN expression (Figure 3C). Because PTEN plays a major negative role on PI3K-Akt pathway [30], we explored the function of the exosome-laden miR-155 on PI3K-Akt pathway. We found the

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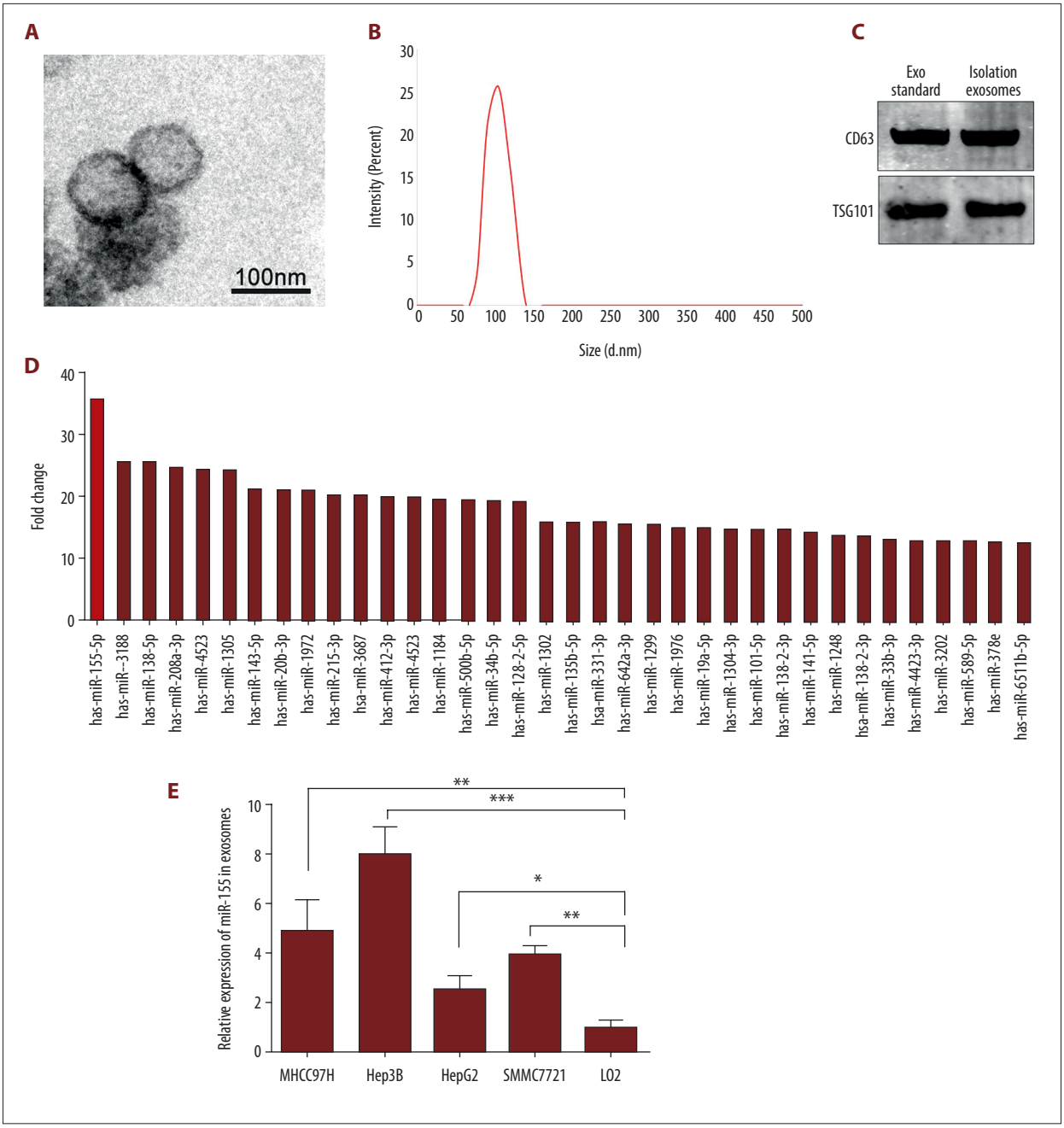

Figure 1. Elevated exosomal miRNAs in hepatocellular carcinoma (HCC) cells were detected via deep sequencing of RNA. (**A**) Electron microscope photographs of exosomes displayed in Hep3B cells. The sizes of the extracellular vesicles were predominantly in almost 100 nm. (**B, C**) Histogram revealing the exosomes distribution of hepatocellular carcinoma as analyzed via nano-tracking analysis (**B**) and western blotting (**C**). (**D**) Constitution of microRNAs in Hep3B acquire exosomes. Data show fold-change in Hep3B versus LO-2 cells. (**E**) Expression of miR-155 in exosomes secreted from HCC cell lines, and the normal hepatic cell line LO2 derived exosomes as the control.

phosphorylated-Akt (p-Akt) were dramatically decreased in Exo-WT treatment group compared with that in NC or Exo-miR-155-KO treatment group (Figure 3C). Our results concluded that exosomal miR-155 contributed to directly inhibit PTEN expression and activated the PI3K-Akt pathway.

Exosomal miR-155 promotes the proliferation of HCC cells through PTEN

To validate the function of PTEN on the growth of HCC, we investigated the miRNA-155 effects on differentiation of HCC

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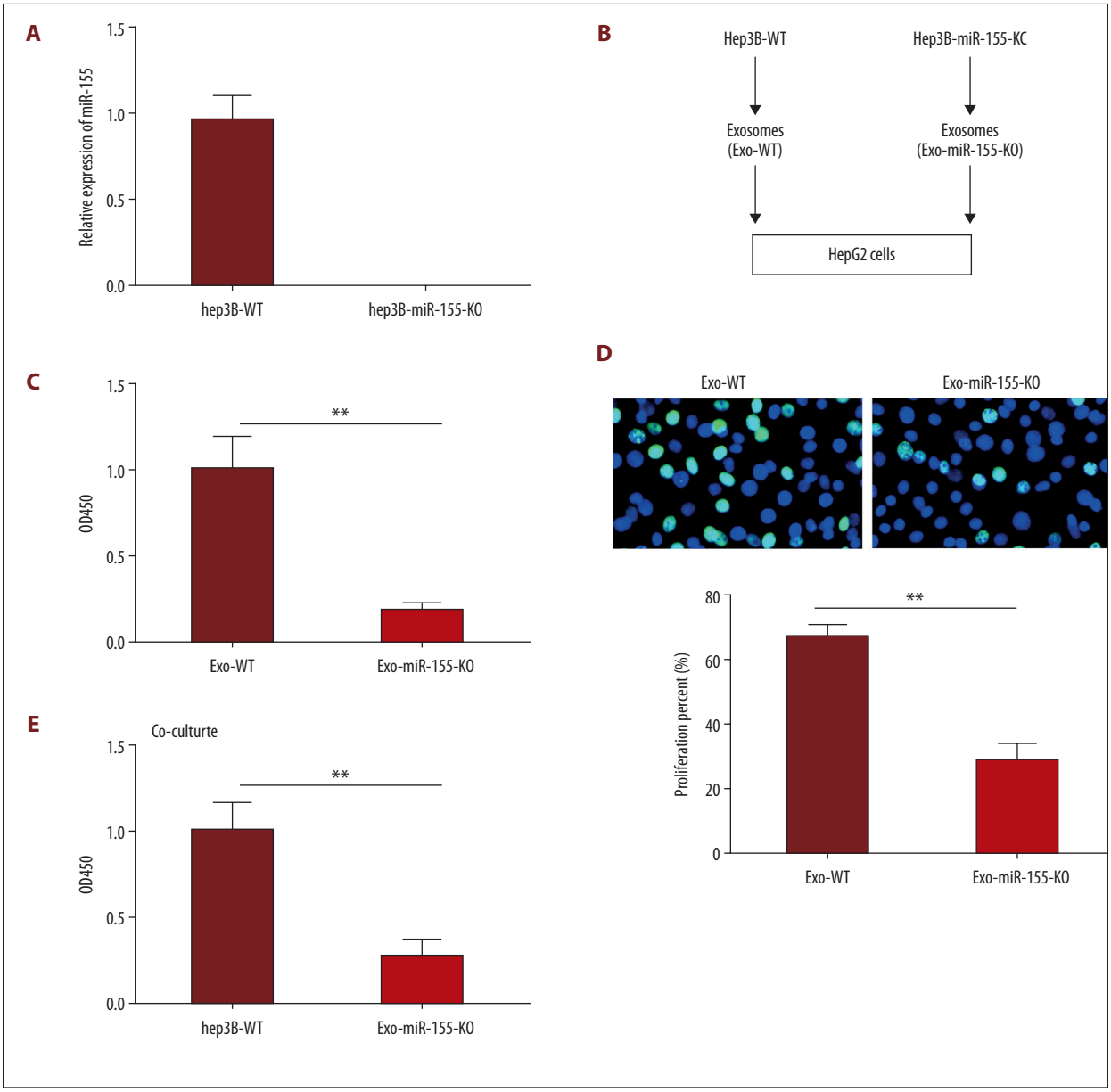

Figure 2. The exosomal miR-155 promotes hepatocellular carcinoma (HCC) cell proliferation. (**A**) Hep3B lines with miR-155 genome elimination was established via CRISPR-CAS9 technology. RT-PCR showed that miR-155 lost miR-155-KO cells and WT cells were not lost. (**B**) Schematic demonstration of the exosome migration. (**C**) The growth of HepG2 cells detected via the CCK-8 assay. (**D**) Cells were marked via EdU and stained with azide-conjugated Alexa-488 (green) and Hoechst 33342 (blue) after incubated exosomes from miR-155-KO or WT cells. 200×. (**E**) The Transwell co-culture with miR-155-KO Hep3B or Hep3B WT cells on the top well and HepG2 in the ground well. A porous (0.4 μm) membrane allows transfer of exosomes but precludes direct cell-cell contact. The growth of HepG2 cells detected via the CCK-8 assay. * $P<0.05$. ** $P<0.01$. *** $P<0.001$. RT-PCR – real-time polymerase chain reaction; CCK-8 – Cell Counting Kit-8; EdU – 5-ethynyl-2-deoxyuridine.

cells according to PTEN expression knockdown using lentivirus shRNA-PTEN vectors. Western blotting demonstrated that PETN was significantly knockdown in HepG2 cells, suggesting the stable PTEN-KD HepG2 cells was successfully constructed (Figure 4A). Then, we treated HepG2 PTEN-KD cells with an equal quality of Exo-WT as well as Exo-miR-155-KO for 24 hours. The proliferation of HepG2 PTEN-KD cells had no significant difference between Exo-WT and Exo-miR-155-KO treatment using CCK-8 assay and EdU staining (Figure 4B, 4C). These results demonstrated that PTEN functions as a crucial function in the differentiation of HCC cells induced via exosome-laden miRNA-155.

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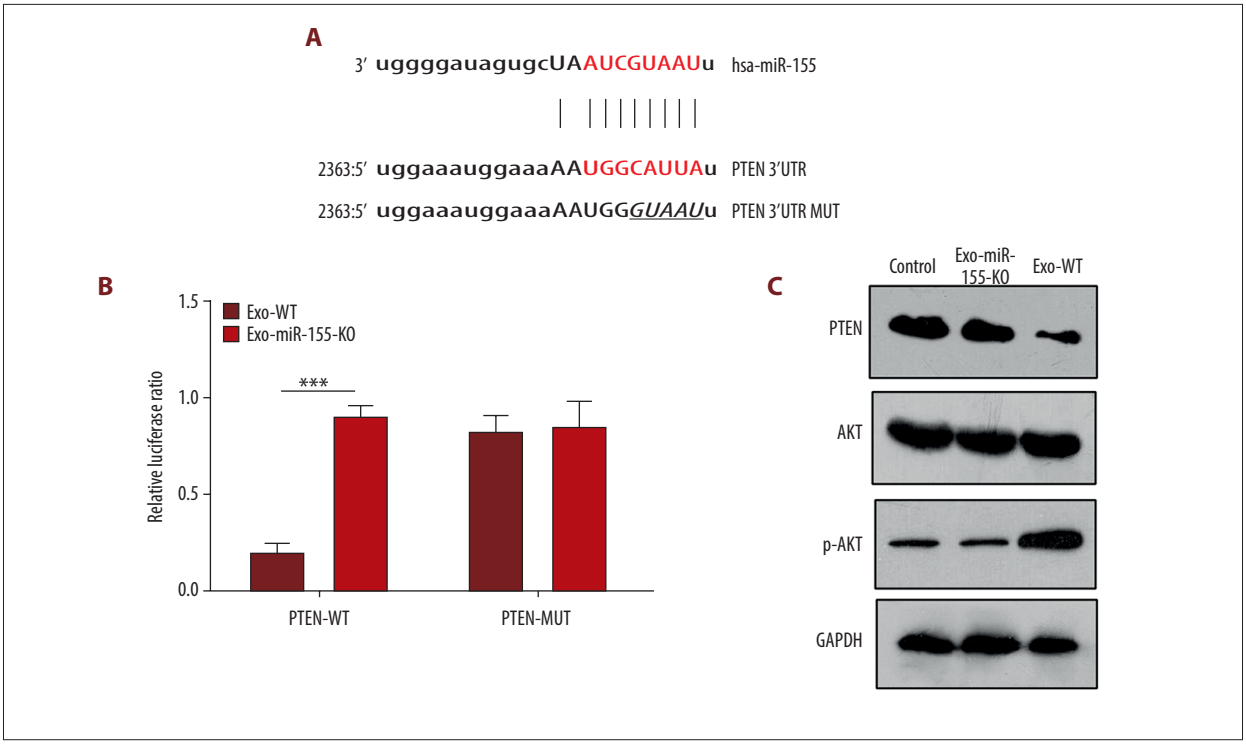

Figure 3. MiR-155 binds to the target site of PTEN. (**A**)The assumed binding position of miR-155 at site of 2363 in *PTEN* 3′-UTR are checked by TargetScan (red). (**B**) 3′UTR luciferase results from transfection of HepG2 with PTEN 3′-UTR vector and/or mutant vector were conducted 6 hours with exosomes of Hep3B WT and exosomes of miR-155-KO cells. (**C**) Protein levels of PTEN, Akt, and p-Akt were determined in HepG2 cells via western blotting after incubated exosomes that from Hep3B WT (Exo-WT) or miR-155-KO (Exo-miR-155-KO) Hep3B cells. *** $P<0.001$.

The exosomal miR-155 promotes HCC proliferation *in vivo*

To confirm the proliferation ability of miR-155 in HCC tumor formation, we injected HepG2 cells subcutaneously into nude mice to form tumor masses. After 15 days, the mouse with the tumor mass was injected with an equal protein quality Exo-WT or Exo-miR-155-KO to assess whether exosome-laden miR-155 had effect on HCC cell tumor formation *in vivo* (Figure 5A). As shown in Figure 5B, the volume of tumors grew obviously faster when injected with Exo-WT from 7 days with 490 mm^3 than Exo-miR-155-KO-injected group with 445 mm^3. The tumor weight from Exo-WT injection were significantly higher than Exo-miR-155-KO-injected group, with average of 1.654 g in Exo-WT group and 0.8926 g in Exo-miR-155-KO-group (Figure 5C). Moreover, the PTEN antigen staining density in Exo-WT-injected tumor tissue was decreased compared to the Exo-miR-155-KO-injected tumor by immunohistochemistry analysis (Figure 5D). Thus, we provide evidence that exosomal miR-155 promotes HCC growth through targeting PTEN in animal experiment.

Discussion

In most countries, the high mortality rate of HCC is almost equal to the incidence of HCC, indicating the lack of effective therapies for HCC [2,31]. Many studies have shown that DNAs, exosome proteins and RNA, make contributions to the development of HCC [3,17,18]. MiRNAs package in exosomes should be shift to neighboring recipient and then exert the regulation of target genes [16,21,32]. Therefore, miRNAs in cancer-secreted exosomes play a critical role in controlling internal environment around the tumor and lead to the uncontrollable growth of tumor. However, the function and mechanism of HCC-derived exosomes have not been well established. In our study, we aimed to validate whether exosomes released from HCC cells regulate the proliferation of HCC cells. Our finding showed that miR-155 was expressed in exosomes that are released from HCC lines. Then, we found that the exosome-containing miR-155 was taken up by targeted cell, which promoted the cell proliferation of liver carcinoma. Further study demonstrated that the exosome-laden miR-155 directly targeted the *PTEN* gene and then suppressed its expression in recipient cells.

The miR-155 is located in 21q21.3 of human chromosome and is originally defined as an insertion site of the Poultry

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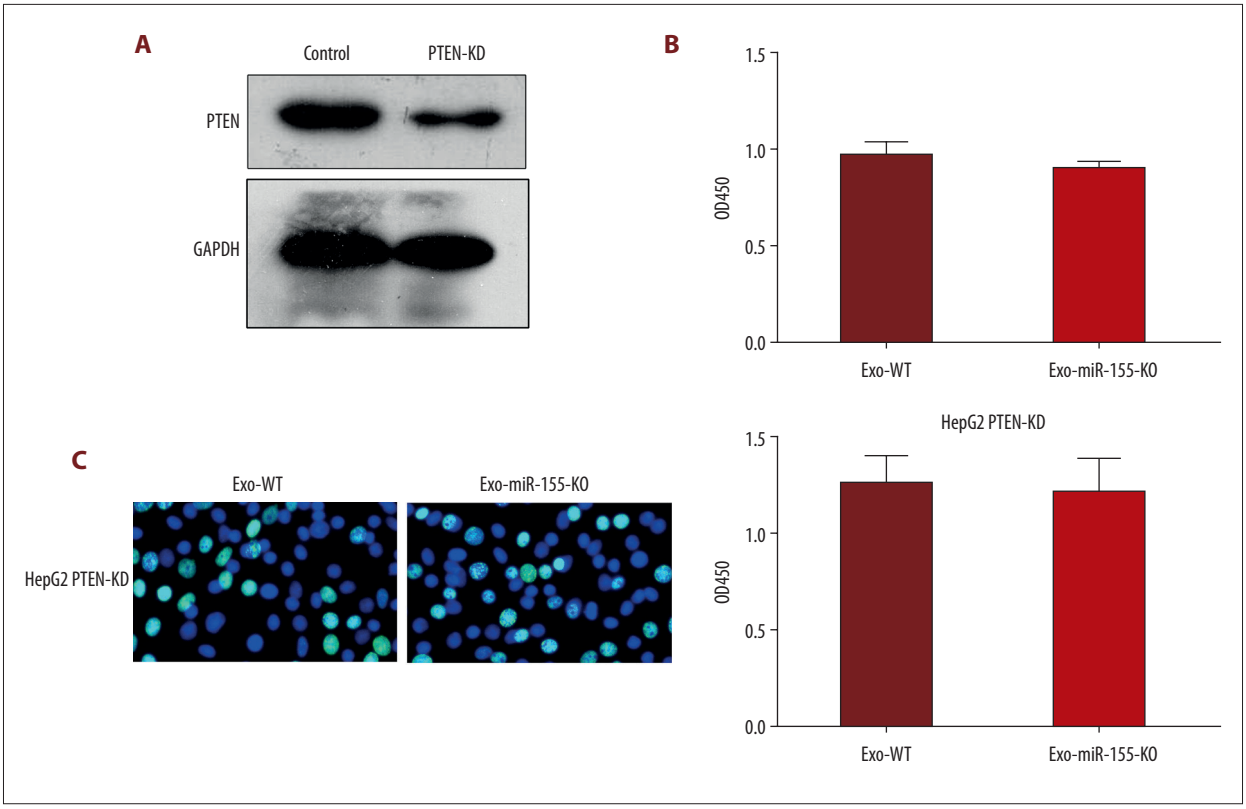

Figure 4. The combination PTEN and miR-155 promotes the proliferation of hepatocellular carcinoma cells. (**A**) PTEN-knockdown HepG2 cells was generated using lentiviral shRNA vectors and confirmed by western blotting. (**B, C**) The proliferation of PTEN-knockdown HepG2 reaction to exosomes of Hep3B or miR-155-KO cells by CCK-8 kit (**B**) and EdU assay (**C**). CCK-8 – Cell Counting Kit-8, EdU – 5-ethynyl-2-deoxyuridine.

leukocyte virus [33–35]. The previous study demonstrated that miR-155 was increased in primary liver cancer specimens and in the early stages of hepatocarcinogenesis [36]. MiR-155 is considered to be predictive as a poor survival marker in liver transplantation [37]. Several studies have indicated that miR-155 plays an importance on positive neoplastic epithelial adhesion molecules of HCC [38]. Nowadays, miR-155 has been confirmed to participate in immune system response, cardiovascular diseases, and tumorigenesis [33,34,37]. However, the mechanisms of exosomal miR-155 of HCC development has not been explored. In our study, we showed that miR-155 was preferentially enriched in exosomes that released from HCC lines.

Because the exosomal miRNAs can be carried into neighboring cells or distant cells and lead to the modulation of recipient cells, we constructed a miR-155-KO Hep3B cell line to confirm whether exosome-laden miR-155 has functional effects on HCC cell proliferation. Exosomes from wild-type Hep3B were enriched with miR-155 according to the RNA-Seq data and RT-PCR analysis. The exosomes release from miR-155-KO Hep3B cells were lacking miR-155. Using the KO cells model, we found that the exosome-containing miR-155 from wild-type Hep3B cells was transferred into HepG2 cells and promoted the cell proliferation.

Generally, the miRNA regulates the expression of hepatocellular carcinoma genes via the 3'-UTR region of targeted mRNAs at the post-transcriptional level to inhibit translation and/or induce mRNAs degradation [9]. To explore the biological mechanisms of miR-155 on cell proliferation of HCC, we utilized the miRNA prediction databases to analyze the potential target genes. PTEN is a dominant cancer-suppressor gene blocking PI3K-Akt pathway via PIP3. Emerging evidence revealed PTEN deficiency, mutation or inexpression has been discovered in a lot of malignant tumors, such as the liver, breast, lung, brain, and prostate cancers [25,39–44]. Our findings showed that the exosome-laden miR-155 directly targeted *PTEN* and lead to the decrease of respective target protein in recipient cells, and then activated the PI3K-Akt pathway. The PI3K-Akt pathway is verified to play a key role in controlling cancer cell growth and progression, which could be activated by different kinds of cytokines and integrin signaling [27,30]. It has been displayed that knockdown of PTEN expression triggers the activation of the PI3K/Akt pathway and sequentially facilitates tumor growth and ultimately contributes to the biological progression of the tumor. Finally, a model of xenograft cancer was applied to certify exosomal miR-155-mediated tumorigenesis by targeting PTEN *in vivo*.

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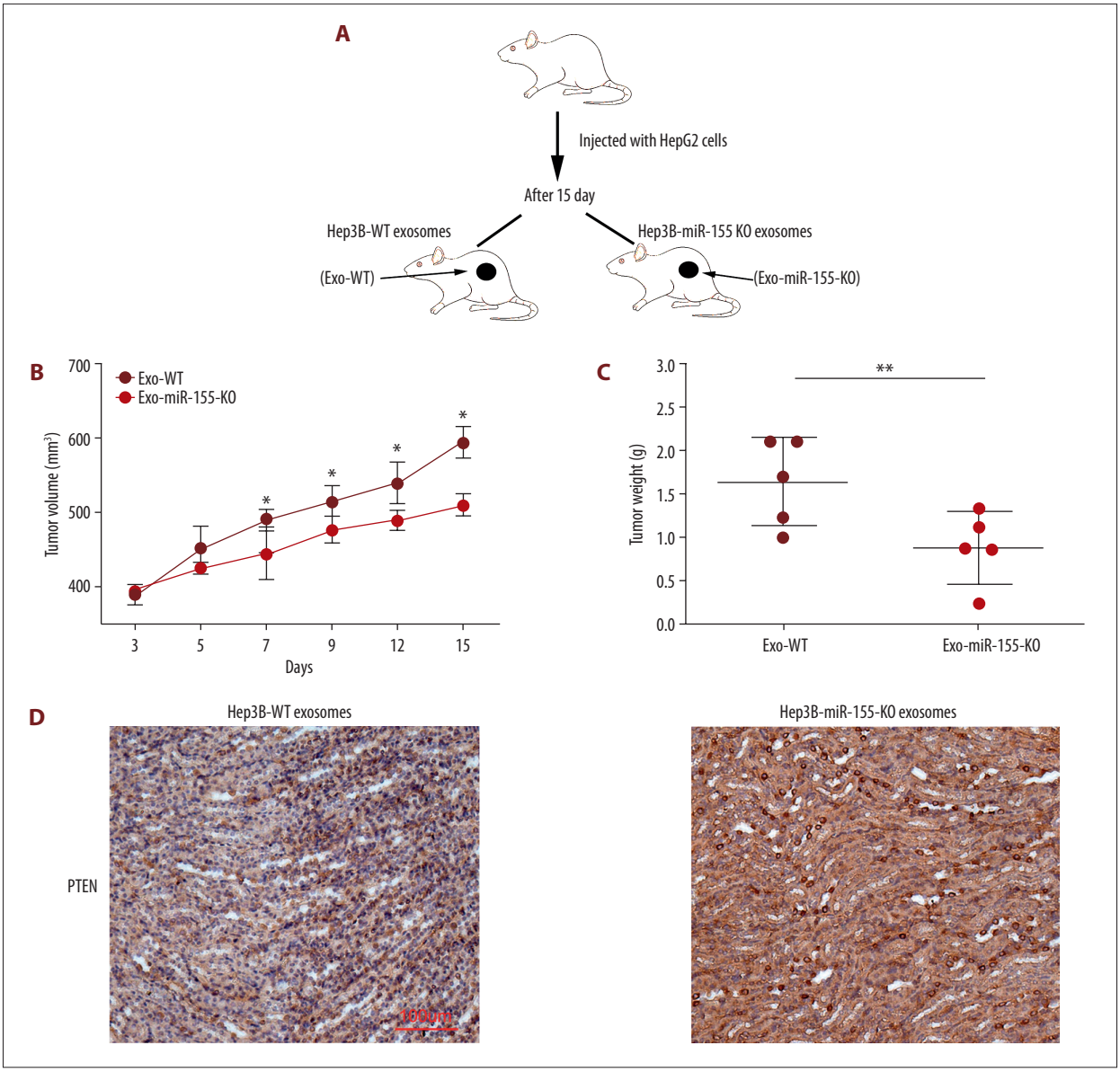

Figure 5. The exosomal miR-155 promotes hepatocellular carcinoma cell growth *in vivo*. (**A**) Schematic demonstration of miR-155 in cancer proliferation trials. The equal number of HepG2 was be applied to *in vivo* tumor induction. The induced tumors were then treated with 100 ug Exo-WT or Exo-miR-155-KO. The tumors were removed and tested after 15 days. (**B, C**) The tumor weight and growth curves were tested three days after detection of induced tumor, and the size of the cancer was tested every 3 days. (**D**) Expression of PTEN in both groups of induced tumors were tested via immunohistochemistry. Bar: 100 um. * $P<0.05$, ** $P<0.01$.

More and more evidence has demonstrated that the exosome-associated miRNAs are defined as novel biological biomarkers in HCC. Previous studies have shown that miR-122, miR-1246, and miR-148a in serum exosomes in HCC were significantly higher than hepatitis C and healthy liver samples [45]. Fornari et al. showed that HCC-released exosomes, which contained miR-21, miR-221, miR-519d, and miR-1228 were correlated with circulating and tissue levels [46]. Lin et al. examined the miR-210 released from HCC stimulates cancer angiogenesis by blocking the entry of SMAD4 and STAT6 into vascular endothelial [47]. Corresponding to the results of our study, Matsuura et al. confirmed that HCC-related miR-155 was upregulated in hypoxic environment, resulting in the tube formation of HUVECs [48]. Our studies suggested that exosomal miR-155 could promote HCC cell proliferation and trigger the PI3K-Akt pathway activity by targeting PTEN *in vitro* and *in vivo*. However, we did not reveal the expression level of exosomal miR-155 in different HCC stages; further studies are needed using clinical samples.

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Conclusions

Our investigations suggest that exosomal miR-155 promotes HCC cell proliferation and triggers the PI3K-Akt pathway activity by suppressing PTEN *in vitro* and *in vivo*. All of our research data demonstrated that the enrichment of miR-155 in HCC-associated exosomes mediates HCC development and that it might be a novel biomarker for the treatment and/or diagnosis of HCC.

Conflicts of interest

None.

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