MiR-375 and Doxorubicin Co-delivered by Liposomes for Combination Therapy of Hepatocellular Carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies, and it threatens the human health severely. Although various surgical operations and chemotherapeutic interventions have been endorsed, the prognosis of HCC patients is still poor due to metastasis and the high post-surgical recurrence rate. Doxorubicin (DOX) is one of the most frequently used anticancer drugs and the front line option for hepatocellular carcinoma (HCC) treatment. However, the clinical applications of DOX are restricted largely due to its toxicity and chemoresistance. Here, we report that miR-375 and DOX were co-delivered by liposomes (named L-miR-375/DOX-NPs) for combination therapy of HCC and drug resistance reversion of DOX. In vitro, L-miR-375/DOX-NPs could deliver DOX and miR-375 efficiently and simultaneously into HCC cells and ensure the successful release of mature miR-375 and DOX. Then, the released miR-375 suppressed the malignant hallmarks of HCC by significantly decreasing the expression of AEG-1, YAP1, and ATG7, while the released DOX evidently accelerated cell apoptosis and blocked cycle at a G2/M stage by activating the P53/Bax/Bcl-2, caspase-3, and P-JNK, P-P38 pathway. Furthermore, miR-375 dramatically inhibited drug resistance of DOX by reducing the expression of multidrug resistance gene 1 (MDR1). In vivo, L-miR-375/DOX-NPs exhibited enhanced anti-tumor efficiency in xenograft HCC mouse models with mild adverse effects compared with doxorubicin or miR-375 alone. In conclusion, our research demonstrated that L-miR-375/DOX-NPs had significant synergetic anti-tumor effects and added values in overcoming drug resistance, which may represent a promising approach for the therapy of HCC.

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In vitro, L-miR-375/DOX-NPs could deliver DOX and miR-375 efficiently and simultaneously into HCC cells and ensure the successful release of mature miR-375 and DOX. Then, the released miR-375 suppressed the malignant hallmarks of HCC by significantly decreasing the expression of AEG-1, YAP1, and ATG7, while the released DOX evidently accelerated cell apoptosis and blocked cycle at a G2/M stage by activating the P53/Bax/Bcl-2, caspase-3, and P-JNK, P-P38 pathway. Furthermore, miR-375 dramatically inhibited drug resistance of DOX by reducing the expression of multidrug resistance gene 1 (MDR1). In vivo, L-miR-375/DOX-NPs exhibited enhanced anti-tumor efficiency in xenograft HCC mouse models with mild adverse effects compared with doxorubicin or miR-375 alone. In conclusion, our research demonstrated that L-miR-375/DOX-NPs had significant synergetic anti-tumor effects and added values in overcoming drug resistance, which may represent a promising approach for the therapy of HCC.

Micro (mi)RNA modulates cellular behavior via specifically regulating gene expression by nearly perfect base pairing at the post-transcriptional and translational level. Accordingly, it has been identified as a potential novel class of therapeutic agent due to its ability to specifically silence the expression of cancer-related genes or regulate various biological pathways that underlie liver tumor formation. Previously, we and others have found that miR-375 was downregulated significantly in HCC tissues and could inhibit the core hallmarks of HCC by targeting several important oncopgenes like AEG-1, YAP1, and ATG7. Meanwhile, it has been confirmed that miR-375 played a strong tumor suppressive role in the AKT/Ras driven hepatocarcinogenesis. It has been widely acknowledged that
multidrug resistance gene 1 (MDR1) encoding broad-spectrum efflux pump P-glycoprotein (P-gp) contributes significantly to drug resistance. In addition, it has been reported that the miR-375 targeting gene AEG-1 could increase the expression of MDR1, when we upregulate the expression of miR-375, it could decrease the expression of MDR1 by targeting gene AEG-1, which results in increased accumulation of doxorubicin, thus inhibiting doxorubicin resistance.39 Therefore, how to successfully co-deliver miR-375 and doxorubicin into HCC cells and exert the synergistic anti-cancer role has become an urgent issue to be solved.

In this study, we successfully combined the miR-375 and doxorubicin by liposome nanoparticles and demonstrated that it could not only inhibit the malignant phenotypes of HCC cells with a high efficiency by simultaneously playing the tumor suppressor role of doxorubicin and miR-375 in vitro and vivo, but also partially reverse the doxorubicin resistance by downregulating MDR1 expression through targeting AEG-1. The schematic illustration and mechanism of L-miR-375/DOX-NPs synergistic anti-tumor action in HCC are shown in Figure 1.

RESULTS
L-miR-375/DOX-NPs Were Successfully Synthesized and Characterized

Empty liposomes (L), L-DOX-NPs, and L-miR-375/DOX-NPs were synthesized (Figure S1) as previously described.20 The average particle sizes of L, L-DOX-NPs, and L-miR-375/DOX-NPs detected by dynamic light scattering (DLS) were 117.7 ± 0.5, 122.4 ± 0.3, and 162.5 ± 0.7 nm, respectively, which indicated that L-miR-375/DOX-NPs could accumulate in liver easily. The zeta potentials of empty L, L-DOX-NPs, and L-miR-375/DOX-NPs were 52.4 ± 2.05, 47.8 ± 3.14, and 15.3 ± 5.37 mV, respectively. The reduced zeta potential of L-miR-375/DOX-NPs was due to the incorporation of the miR-375 which carries a negative potential. The encapsulation of L-DOX-NPs and L-miR-375/DOX-NPs were 85.9% and 84.7% (Table S1). To sum up, these data demonstrated that L-miR-375/DOX-NPs were successfully synthesized as expected.

L-miR-375/DOX-NPs Were Uptaked by HepG2 Cells and SMMC-7721 Cells with a High Efficiency

Firstly, we checked the co-delivery and localization of miR-375 and DOX co-delivered by L-miR-375/DOX-NPs with fluorescence (Figure 2A). L-miR-375/DOX-NPs could deliver both the miR-375 (FAM labeled, green) and DOX (red) to SMMC-7721 cells with high efficiency as the same as free DOX, L-DOX-NPs, and L-miR-375-NPs. Meanwhile, we found that doxorubicin and miR-375 located in the nucleus and cytoplasm, respectively. Flow cytometry analysis also proved that the combination of miR-375 and DOX were simultaneously absorbed by HepG2 and SMMC-7721 cells efficiently. The uptake ratio was 97.20% in HepG2 cells and 99.54% in SMMC-7721 after incubated with L-miR-375/DOX-NPs for 1 hr (Figure 2B). Bulge-Loop miRNA qRT-PCR showed that L-miR-375/DOX-NPs enormously upregulated the expression of miR-375 after treated for 48 hr (Figure 3A). These data illustrated that L-miR-375/DOX-NPs could deliver both DOX and miR-375 efficiently and simultaneously into HCC cells and ensure the successful release of mature miR-375 and DOX.

L-miR-375/DOX-NPs Inhibited the Malignant Tumor Characteristics and Induced G2/M Arrest and Apoptosis In Vitro

To assess whether L-miR-375/DOX-NPs could suppress malignant behaviors of HCC cells, we employed the Cell Counting Kit-8 (CCK-8), Transwell assay, and wound healing assay. First, we carried out the CCK-8 experiment to select the optimal concentration of doxorubicin. As shown in Figure S2A, doxorubicin shows an inhibitory effect at the concentration of 0.25 μg/mL, since the cell toxicity of doxorubicin was delayed, we chose 0.25 μg/mL as the suitable dosage, while the concentration of miR-375 we adopted was 100 nM, as the specification recommended. SMMC-7721 and HepG2 cells transfected with L-miR-375/DOX-NPs markedly suppressed proliferation and presented a time-dependent character (Figure 3B). A colony formation assay also confirmed that L-miR-375/DOX-NPs decreased the cell growth remarkably compared to the controls (Figure S3). A Transwell assay indicated that L-miR-375/DOX-NPs groups obviously inhibited the SMMC-7721 cells invasion and migration ability compared with free DOX, L-DOX-NPs, and L-miR-375-NPs groups (Figure 3C). We also performed a wound-healing assay to estimate the cell motility, and the result was consistent with the Transwell assay (Figure 3D). Morphological changes of apoptosis by Hoechst 33342 staining revealed that the apoptosis rate of
L-miR-375/DOX-NPs was higher than the L-DOX-NPs, free DOX, and L-miR-375-NPs groups (Figure 3E). Finally, we analyzed the effect of L-miR-375/DOX-NPs on cell cycle progression in SMMC-7721 cells by fluorescence-activated cell sorting (FACS), after being treated with L-miR-375/DOX-NPs for 72 hr, SMMC-7721 cells were arrested in G2/M phase, suggesting that the cells were blocked in G2 phase and unable to progress to M phase (Figure 3F). In general, the L-miR-375/DOX-NPs displayed a synergistic anti-tumor efficiency due to the combination of doxorubicin and miR-375.

L-miR-375/DOX-NPs Played an Anti-tumor Role by Simultaneously Targeting Downstream Target Genes of Doxorubicin and miR-375

To confirm the specific anti-cancer mechanism of L-miR-375/DOX-NPs, we screened the downstream target genes from previous investigation and verified its expression. As shown in Figure 4, both the SMMC-7721 and HepG2 cell lines treated with L-miR-375/DOX-NPs groups not only showed a dramatic downregulation of AEG-1, YAP1, and ATG7, as similar as the L-miR-375-NPs groups (Figures 4A and 4B), but also activated the P53/Bax/Bcl-2, caspase-3, and P-JNK, P-P38 pathway just as free doxorubicin and L-DOX-NPs treating groups (Figures 4C and 4D). In a word, these results demonstrated that L-miR-375/DOX-NPs promoted the tumor killing efficiency by giving full play of the miR-375 and doxorubicin.

L-miR-375/DOX-NPs Inhibited Tumor Growth In Vivo

Potentially therapeutic use of L-miR-375/DOX-NPs was also explored in a subcutaneous tumor model in mice. The tumor models were randomly divided into five groups after the tumor volume reached 100 mm³, and each group was injected with PBS, free doxorubicin, L-DOX-NPs, L-miR-375-NPs, and L-miR-375/DOX-NPs, respectively, for four times per 3 days. Compared with the PBS group, the other groups showed obvious suppressing effects of tumors growth, which could be presented by gross morphology (Figure 5A). Notably, the L-miR-375/DOX-NPs displayed the most visible tumor-suppressor role. As shown in Figure 5C, the tumor growth curves indicated that the L-miR-375/DOX-NPs treated groups maintained persistent tumor suppression and had the smallest volume (79.3 mm³), which demonstrated a superior therapeutical effect than free DOX (293 mm³), L-DOX-NPs (247.11 mm³), or L-miR-375-NPs (762.7 mm³). Furthermore, the body weight of L-miR-375/DOX-NPs groups showed no distinct decline among these groups (Figure 5B). HE staining indicated that the free DOX groups showed apparent inflammation of heart and liver, while L-miR-375/DOX-NPs groups appeared to have mild inflammation (Figure 5F). Similarly, liver function tests showed the variation trends of alanine transaminase (ALT) and aspartate transaminase (AST) were consistent with histological results (Figure 5D), while the renal function reflected by blood urea nitrogen (BUN) and Creatinine (Cr) levels changed slightly (Figure 5E). In order to verify the apoptosis effect of L-miR-375/DOX-NPs on tumor tissue, the expression of several relative genes was measured by immunohistochemistry. As shown in Figure S4B, the expressions of Bax and Caspase-3 were significantly increased, while the expression Bcl-2 was downregulated, evidently. As a marker of proliferation, the expression of Ki-67 was detected and found to decrease remarkably. All of these data fully demonstrated the therapeutic efficacy of L-miR-375/DOX-NPs, which was significantly higher than sole treatment in vivo.

L-miR-375/DOX-NPs Increased Chemosensitivity in SMMC-7721/ADM Cells

First, we identified the drug resistance of SMMC-7721/ADM cells, the gene level of MDR-1 and the expression of P-gp both were upregulated, obviously (Figures 6A and 6B). Second, we used Bulge-Loop miRNA qRT-PCR to detect the release of mature miR-375 in SMMC-7721/ADM cells and found that the miR-375 expression level of SMMC-7721/ADM cells treated with L-miR-375/DOX-NPs increased about 100,000 times (Figure 6D). We also employed flow
cytometry techniques to quantify the fluorescence intensity and discovered that the L-miR-375/DOX-NPs groups displayed a stronger fluorescence intensity than free DOX and L-DOX-NPs treated groups after being treated for 20 hr, which implied the miR-375 could promote the accumulation of DOX in SMMC-7721/ADM cells (Figure 6C). Finally, we performed a CCK-8 assay to test the proliferation inhibitory effect. After being treated with L-miR-375/DOX-NPs, L-DOX-NPs, and free DOX for 24 hr, the SMMC-7721/ADM cells were apparently suppressed by the L-miR-375/DOX-NPs and showed a dose-dependent manner. More importantly, L-miR-375/DOX-NPs greatly reduced the IC50 compared with the free DOX treated group (Figure 6F). Western blot explained the specific molecular mechanism for the decrease in drug resistance of L-miR-375/DOX-NPs because it could not only inhibit the expression of P-gP, but also promote apoptosis (Figure 6E). A Transwell assay indicated that L-miR-375/DOX-NPs groups obviously inhibited the SMMC-7721/ADM cells invasion ability compared with free DOX, L-DOX-NPs, and L-miR-375 treated groups (Figure 6G). In conclusion, L-miR-375/DOX-NPs possess a great deal of potential in reversing resistance.

**DISCUSSION**

Clear evidences have indicated that the combination delivery of siRNA/miRNA and drugs using nanoparticles are indeed beneficial in suppressing the tumor growth and significantly enhancing chemosensitivity compared to naked counterparts. We have demonstrated that miR-375 was a downregulated tumor suppressing miRNA in HCC, and overexpression of miR-375 inhibits liver cancer cell growth in vitro and vivo via directly targeting AEG-1. Interestingly, it has been confirmed that AEG-1 obviously increased the expression of MDR-1, thus, the overexpression of miR-375 could decrease the expression of MDR1 and may seem a superior target for liver cancer therapy. It has been widely acknowledged that doxorubicin has shown great treatment potential and remains an indispensable cornerstone of cancer chemotherapy. As far as we know, although the combination therapy has been recommended for the treatment of cancer due to its additive or synergistic anti-cancer efficiency, the combination of miR-375 and doxorubicin has been rarely reported. Liposomes are biocompatible and can be modified to render the chemotherapy agents long circulatory lifetime and specific delivery to a tumor site, which functioned as an outstanding nano-carrier.

In this article, L-miR-375/DOX-NPs were successfully synthesized and provided an excellent platform to effectively, safely, and simultaneously deliver miR-375 and DOX into HCC cells. The uptake assay have revealed the excellent uptake efficiency as the same as miR-375 and DOX alone. PCR confirmed that mature miR-375 was released enormously after L-miR-375/DOX-NPs were taken up by HCC cells.
Meanwhile, L-miR-375/DOX-NPs present synergistic anti-tumor efficiency, as we expected. On the one hand, L-miR-375/DOX-NPs exert predominant inhibitory effects on cell proliferation, migration, and invasion ability, also an enhanced apoptosis promoting effect and G2/M cell cycle arrest than miR-375 and DOX applied individually. On the other hand, in a mouse subcutaneous tumor model, L-miR-375/DOX-NPs treated groups could obviously inhibit tumor growth and significantly alleviate the toxicity of liver and heart and seems to have no obvious toxicity on the lung, spleen, and kidney. What’s more, L-miR-375/DOX-NPs treated groups could decrease the expression of P-gp, thus causing an increased intracellular accumulation than free DOX and triggering the apoptosis in SMMC-7721/ADM cells, both abnormal expression of P-gp efflux pumps and the activation of an anti-apoptotic pathway are the key mechanisms by which cancer cell attains chemoresistance.24,25 Taken together, these results demonstrated that L-miR-375/DOX-NPs enjoyed an unexhausted repertoire of activities in both HCC cells and HCC cells insensitive to doxorubicin.

In conclusion, our findings demonstrated that L-miR-375/DOX-NPs have significant synergetic anti-tumor effects and added values in overcoming drug resistance, which may represent a potential therapeutic candidate for HCC and deserves further investigation.

MATERIALS AND METHODS

Preparation of L-miR-375/DOX-NPs

FAM-labeled or non-labeled miR-375 and negative control miR-NC were synthesized by Guangzhou RiboBio. The Liposome nanoparticles (L) and L-DOX-NPs were synthesized by the Pharmacy School of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China20 (purity >95%). Characterization of liposomes and formation of L-miR-375/DOX-NPs are described in Figure S2. The L-miR-375-NPs and L-miR-375/DOX-NPs were mixed at v/v (miR-375 volume/liposome volume) ratio of 1:5. The samples were vortexed 1–2 min and then incubated at room temperature for 10–15 min to ensure formation of L-miR-375/DOX-NPs and L-miR-375-NPs.

Cell Culture

The human HCC cell lines HepG2 and SMMC-7721 stored by the Liver Disease Institute (Tongji Hospital, Wuhan, China) were resuscitated and cultured in DMEM containing 10% fetal calf serum (Invitrogen Gibco) and incubated in a 5% CO2 incubator at 37°C. The SMMC-7721/ADM was bought from the Saiqi Biotechnology Company and cultured in DMEM containing 10% fetal calf serum (Invitrogen Gibco).

Uptake of L-miR-375/DOX-NPs

The uptake ratio of doxorubicin and miR-375 were detected under fluorescence and analyzed by the flow cytometry method. SMMC-7721/HepG2 cells in logarithmic phase were seeded on a 12-well plate at 5 × 10⁴ cells per well and cultured for 24 hr, then treated with free DOX, L-DOX-NPs, L-miR-375-NPs, and L-miR-375/DOX-NPs and incubated at 37°C for 1 hr. After incubation, the cells were washed three times with cold PBS to remove unbound liposomes, and then fixed in 4% PFA for 15 min. Next, the nucleus was counterstained with DAPI for 10 min and washed with PBS for three times. Finally, the cells were imaged under a fluorescence microscope. For the flow cytometry method, after incubation for 1 hr, cells were harvested by trypsinization, washed, and suspended with PBS, then the cell suspension could be analyzed by FACS. MiR-375 was labeled with FAM; DOX can emit red fluorescence by itself. The concentration of miR-375 and DOX was (100 nM, 1 μg/mL).

CCK-8 Assay

The cytotoxic effects of L-miR-375/DOX-NPs on HepG2, SMMC-7721, and SMMC-7721/ADM cells were measured using a CCK-8 (Promoter). The cells were seeded into 96-well plates at a density of...
1 × 10^4 cells/well. After 24 hr, cells were treated with free DOX, L-DOX-NPs, L-miR-375-NPs, and L-miR-375/DOX-NPs, respectively. After incubation for an additional 24 hr, 48 hr, and 72 hr, the portions of viable cells was measured using CCK-8 kits according to the user’s manual. The cell viability of each group was measured by a percentage of the viability compared to untreated cells.

Cell Cycle Analysis
For cell cycles analyzed by FACS, SMMC-7721 cells were seeded in DMEM with 10% FBS on 6-well plates and allowed to attach overnight. Then cells were treated with free DOX, L-DOX-NPs, L-miR-375-NPs, or L-miR-375/DOX-NPs, respectively. The concentration of miR-375 and DOX was (100 nM, 0.1 µg/mL). After 72 hr, cells were harvested by trypsinization, collected, and fixed in 70% ethanol at 4°C overnight. After being washed by phosphate-buffered saline, cells were treated with 300 µL RNase/PI at 4°C for 30 min. Stained cells were immediately analyzed on a BD Accuri C6 Flow Cytometer (BD Biosciences).

Cell Migration and Invasion Assay
The migration and invasion assays were performed by using Transwell insert chambers (8 mm pore size, Corning). For the migration assay, 5 × 10^4 SMMC-7721 cells were placed into the upper chamber in a serum free medium after transfection for 24 hr. The lower chamber was filled with 600 µL DMEM with 10% fetal bovine serum as chemoattractant. After incubation for 24 hr, cells on the upper chamber were removed with a cotton swab, and cells on the lower chamber were fixed and stained. For the invasion assay, SMMC-7721 cells were planted into the upper chamber precoat with a layer of diluted basement membrane Matrigel (ECM gel, Sigma) and were harvested after incubation for 48 hr. There were three random fields that were chosen to take pictures and count. The numbers of migrated and invaded cells were counted in four randomly selected microscope fields and presented in a bar graph. A wound healing assay was carried out as formerly described,26 and the relative cell motility rate were presented in a bar graph.

Cell Apoptosis Assay
SMMC-7721 cells were seeded on a 6-well plate and then treated with PBS, free DOX, L-DOX-NPs, L-miR-375-NPs, and L-miR-375/DOX-NPs, respectively. The concentration of miR-375 and DOX was (100 nM, 0.25 µg/mL). Finally, cells were stained with Hoechst 33342/PI (Promoter), specific procedures in accordance with the instructions, and then observed under a fluorescence microscope.

RNA Extraction and Real-Time RT-PCR
Total RNA extraction and real-time RT-PCR were performed as previously described.26 The sequences of the primers are listed in

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**Figure 5. The Anti-tumor Efficiency of L-miR-375/DOX-NPs In Vivo**

(A) Tumors were removed at the end of the experiment (day 14 after the drug administered). (B and C) Growth curves of mouse model and the volume changes of xenograft tumors treated with PBS, free DOX, L-DOX-NPs, L-miR-375-NPs, and L-miR-375/DOX-NPs by intratumoral injection (1 nmol miR-375 per mouse, 1 mg/kg DOX). (D and E) Acute phase changes of liver and renal function after 3 days of drug injection. (F) Hematoxylin and eosin staining analysis of tissues including heart, liver, spleen, lung, and kidney; (B) tumor growth curve (p < 0.05); and (C) tumor volume curve (p < 0.05).

**Figure 6. L-miR-375/DOX-NPs Reversing Drug Resistance of Doxorubicin in SMMC-7721/ADM Cells**

(A and B) Identification of SMMC-7721/ADM cells. (C) The comparison of fluorescence intensity about DOX in SMMC-7721/ADM cells after treated with free DOX, L-DOX-NPs, and L-miR-375/DOX-NPs, respectively, at 1 hr and 20 hr. (D) The release of mature miR-375 of SMMC-7721/ADM cells after treated with L-miR-375/DOX-NPs for 48 hr. (E) Molecular mechanism of L-miR-375/DOX-NPs reversing drug resistance in SMMC-7721/ADM cells. (F) The proliferation inhibitory effect of SMMC-7721/ADM cells after treated with free DOX, L-DOX-NPs, and L-miR-375/DOX-NPs with different concentration gradient of DOX. (G) Migration ability of SMMC-7721/ADM cells was determined by Transwell assay.
Table S2. The expression levels of mature hsa-miR-375 were analyzed by Bulge-Loop miRNA qRT-PCR Primer (Ribobio) and normalized to U6. Data analysis was performed using the 2ΔΔCt method. Each sample was tested in triplicate.

Western Blot
Cell lysates were separated on a 12% polyacrylamide gel and transferred to a PVDF membrane, then the membrane was blocked for 1 hr in 5% BSA, and incubated overnight at 4°C with primary antibodies AEG-1 (1:1,000, Proteintech), YAP1 (1:1,000, Proteintech), ATG7 (1:1,000, Cell Signaling), JNK (1:1,000, Cell Signaling), P38 (1:1,000, Cell Signaling), p-JNK (1:1,000, Cell Signaling), p-p38 (1:1,000, Cell Signaling), p53 (1:1,000, Promoter), Bax (1:1,000, Proteintech), Bcl-2 (1:1,000, Promoter), caspase-3 (1:2,000, Abcam), P-gp (1:1,000, Abcam), and GAPDH (1:2,000, Promoter). Following this, the membrane was washed in TBST three times and then incubated for 1 hr with the anti-mouse or anti-rabbit IgG secondary antibody (1:3,000, Promoter). Then the signals were detected with an ECL assay kit (Juneng Nano Bio).

Histology
Blood, heart, liver, lung, kidney, spleen, and tumor tissues were collected, fixed in 4% paraformaldehyde, and embedded in paraffin after the mice were euthanized and killed. The slides were sectioned and stained with H&E, tumor tissues were also used for immunohistological evaluation. The procedures were performed as previously described.14

Liver and Kidney Toxicity
To check the liver and kidney toxicity of L-miR-375/DOX-NPs, mice were injected with PBS, free DOX, L-DOX-NPs, L-miR-375-NPs, or L-miR-375/DOX-NPs when doxorubicin (1.0 mg/kg) and miR-375 (1 nmol per mouse) every 3 days for four times. Tumor volume (V) was calculated with the formula V = (L × W²) / 2.

Statistic Analyses
All experiments were performed in triplicate unless specified. Results are represented as the mean ± SEM. Statistical analysis was performed using unpaired Student’s t test. Multiple groups were compared by one-way ANOVA with Dunnett’s post test. The p values < 0.05 were considered significant and p < 0.01 was considered highly significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.omtn.2017.03.010.

AUTHOR CONTRIBUTIONS
X.-X.H. and W.-X.T. designed the study. Y.-P.F., J.-Z.L., Y.-Q.L., and P.-X.Z. performed the experiments. F.Y. and G.-Y.X. contributed reagents/materials. Y.-P.F. and X.-X.H. analyzed the data and wrote the paper. Y.-P.F., J.-Z.L., and D.-A.T. contributed to data interpretation and provided intellectual input. All authors reviewed, participated in revision, and approved the final version of the manuscript.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

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