Received: 5 February 2024 | Revised: 9 April 2024 | Accepted: 15 April 2024

DOI: 10.1002/ags3.12814

ORIGINAL ARTICLE

AGSurg Annals of Gastroenterological Surgery Open Access WILEY

Lenvatinib-resistant hepatocellular carcinoma promotes malignant potential of tumor-associated macrophages via exosomal miR-301a-3p

Yuhei Waki[1] | Yuji Morine[1] | Yu Saito[1] | Hiroki Teraoku[1] | Shinichiro Yamada[1] | Tetsuya Ikemoto[1] | Tatsuya Tominaga[2] | Mitsuo Shimada[1]

[1]Department of Digestive and Transplant Surgery, Tokushima University, Tokushima, Japan

[2]Department of Bioanalytical Technology, Tokushima University, Tokushima, Japan

Correspondence
Yuji Morine, Department of Digestive and Transplant Surgery, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima, 770-8503, Japan.
Email: ymorine@tokushima-u.ac.jp

Funding information
the Research Program on Hepatitis of the Japanese Foundation for Multidisciplinary Treatment of Cancer; Japan Agency for Medical Research and Development, Grant/Award Number: 23fk0210103h0002 and 23fk0210103s0202; Taiho Pharmaceutical Co., Ltd; Grant-in-Aid for Scientific Research, Grant/Award Number: 23K08072

Abstract

Background: The interactions between cancer cells and tumor-associated macrophages (TAMs) via microRNAs (miRNAs) play crucial roles in malignant potential and drug resistance. However, it remains unclear how lenvatinib-resistant hepatocellular carcinoma (LR HCC) promotes TAM tumor biology. Here we investigated the crosstalk between LR HCC cells and TAMs for cancer progression and lenvatinib resistance, focusing on an exosomal miRNA.

Methods: We used two bioinformatics software programs to identify miRNAs that target PTEN in gastrointestinal cancers, then investigated exosomal miRNA expression in LR HCC conditioned medium (CM). After modifying TAMs with LR HCC CM (LR TAM), macrophage phenotype and PTEN-Nrf2 signaling pathway component expression were analyzed in LR TAMs. The malignant potential and drug resistance were investigated in naïve HCC cells cultured with LR TAM CM.

Results: LR HCC cells highly induced M2-like properties in macrophages compared with naïve HCC cells. Exosomal miR-301a-3p expression was increased in LR HCC CM, with higher activation of the PTEN/PI3K/GSK3β/Nrf2 signaling pathway in LR TAMs. Naïve HCC cells were educated with LR TAM CM to promote malignant potential and lenvatinib resistance. Inhibition of exosomal miR-301a-3p prevented the malignant potential of LR TAMs. Activation of Nrf2 signaling by LR HCC cell-derived exosomal miR-301a-3p skewed the transformation of macrophages to the M2 phenotype.

Conclusion: Our study provides new findings on the role of miR-301a-3p, suggesting it is a promising therapeutic target to improve HCC lenvatinib resistance.

KEYWORDS
drug resistance, exosome, hepatocellular carcinoma, lenvatinib, PTEN

1 | INTRODUCTION

The treatment methods for early-stage hepatocellular carcinoma (HCC) include resection, transplantation, and ablation, but more than 80% of HCC patients are still diagnosed with progressive stage disease with no chance of treatment.[1] Lenvatinib was the second approved first-line drug for advanced HCC, indicating its noninferior survival benefit compared with sorafenib.[2] Recently, combination immune checkpoint blockade therapy using atezolizumab, a programmed death ligand 1 inhibitor, and bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF), has improved patient survival rates compared with sorafenib, and has become the first-line treatment for HCC.[3] Nevertheless, lenvatinib remains widely used in immune-excluded HCC cases, and cytokine-related resistance to tyrosine kinase inhibitor drugs has been reported in HCC.[3] Our previous study reported that the B-cell-activating factor/NF-κB axis in cancer-associated fibroblasts could induce sorafenib resistance in HCC.[4] However, the specific mechanisms of lenvatinib resistance (LR) in the tumor microenvironment (TME) remain largely unclear. Therefore, further study of LR HCC in the TME may lead to the development of new molecular targets to overcome LR.

The TME is mostly composed of extracellular matrix, fibroblasts, vascular endothelial cells, and immune cells and plays a pivotal role in tumor cell survival and malignancy.[5] Tumor-associated macrophages (TAMs) are preexisting tissue resident macrophages or recovered monocytes that were activated by TME-derived cytokines that are inseparable from the TME.[5] Moreover, TAMs, broadly classified as M1 or M2, can receive different microenvironmental signals, resulting in a multifunctional and heterogeneous phenotype.[5] M1 macrophages trigger the inflammatory precancer niche and lead to initial tumorigenic mutations, while M2 macrophages release a variety of growth factors and support an immunosuppressive TME.[6] Additionally, M2 TAMs have been shown to correlate with tumor growth, angiogenesis, and the epithelial-mesenchymal transition (EMT) in HCC.[7]

Exosomes are small extracellular vesicles that contain various molecules as cargo, including microRNAs (miRNAs), messenger RNAs (mRNAs), DNA, and proteins. They contribute to tumor proliferation and drug resistance in intercellular communication within the TME.[8] miRNAs are small endogenous noncoding RNA molecules that negatively regulate gene expression and translation by acting on specific target mRNAs.[9] In the TME, exosomal miRNAs from cancer cells play crucial roles in macrophage polarization.[10] However, the miRNA-specific effects on tumor progression and drug resistance in the TME remain unclear.

The nuclear translocation and upregulation of nuclear factor erythroid-derived 2-like 2 (Nrf2) can promote tumor progression and chemotherapy resistance in HCC.[11] Nrf2 helps maintain the equilibrium of drug and oxidative stress and can enhance cancer cell survival by activating the transcription of several antioxidant genes.[12] Activation of the PI3K/AKT/glycogen synthase kinase-3 beta (GSK3β) signaling pathway increases the nuclear accumulation of Nrf2,[13] and its continuous activation is strongly linked to cell transformation, tumorigenesis, tumor metastasis, and angiogenesis.[14] Meanwhile, PTEN suppressed tumor induction by directly inhibiting the PI3K/AKT signaling pathway and was associated with Nrf2.[10]

Recently, miR-301a-3p was reported as a multifunctional miRNA targeting the PTEN/PI3K/AKT signaling pathway.[10,15] miR-301a-3p contributes to tumor malignancy by regulating the PTEN/AKT pathway and was related to the M2 macrophage polarization in the TME of pancreatic and esophageal cancers.[10,15] Moreover, miR-301a-3p mediated the acquisition of trastuzumab resistance in gastric cancer.[16] Therefore, miR-301a-3p plays an important role in tumor progression, M2 polarization of TAMs, and drug resistance.

In the current study, LR HCC cell lines were established to elucidate the mechanism of malignant potential and drug resistance through TAM regulation, focusing on the interplay between exosomal miRNAs, cytokines, and the PTEN/Nrf2 signaling pathways.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human HCC cell lines Huh-7 (Huh) (RRID: CVCL_0336) and PLC/PRF/5 (PLC) (RRID: CVCL_0485) (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA). The human monocyte cell line THP-1 (RRID: CVCL_0006) (the Japanese Collection of Research Bioresources Cell Bank) was cultured in RPMI-1640 (Thermo Fisher Scientific). All media described above were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution (Thermo Fisher Scientific). All cells were cultured at 37°C in a humidified incubator with 5% CO_2. Experiments were conducted when cells were in the logarithmic growth phase. The THP-1 cells were treated with 150 nM phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich; Merck, Darmstadt, Germany) for 48 h to induce the M0 macrophages (M0). All human cell lines have been authenticated using STR profiling within the last 3 y. All experiments were performed with mycoplasma-free cells.

2.2 | The lenvatinib half maximal inhibitory concentration (IC50) of each cell line

Cells (1×10^4) treated with different concentrations of lenvatinib were cultured in 96-well plates for 72 h. Cell viability was examined using a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) by measuring the absorbance values at 450 nm with a microplate reader SoftMax Pro 7 (Molecular Devices, San Jose, CA, USA). Cell viability was represented by the percentage of the absorbance value for the control cells. Lenvatinib IC50 curves of each cell line were analyzed and plotted using GraphPad Prism v. 9.3 software (GraphPad Software, Boston, MA, USA).

2.3 | Establishment of LR cell lines

The LR Huh (Huh^{LR}) and PLC (PLC^{LR}) cell lines were established from naïve Huh and PLC cells according to a protocol of continuous exposure to increasing lenvatinib concentrations and stepwise selection. HCC cells were treated with lenvatinib, with concentrations gradually increasing by 0.5–2.0 μM per week.[17] Approximately 4 mo later, the LR HCC cell lines were established and cultured in DMEM with 26.0 μM lenvatinib (FC71772; Carbosynth, Compton, UK), which was first dissolved in dimethyl sulfoxide (DMSO.) The resulting lenvatinib IC50 values of Huh^{LR} and PLC^{LR} cells were approximately three to four times higher than that of naïve HCC cells (27.0 vs 6.0 μM and 46.7 vs 11.9 μM, respectively) (Figure 1A).

2.4 | Conditioned medium (CM) and TAM culturing

The naïve HCC, LR HCC, and M0 cells were cultured to 80% to 90% confluency, washed twice with phosphate-buffered saline (PBS), then incubated with DMEM without FBS for 48 h at 37°C. The supernatant was collected, centrifuged (2000 *g*; 10 min) at room temperature, and filtered using a 0.2 μm filter. The CM was stored at −80°C, avoiding repeated freeze–thaw cycles. The M0 cells were treated with HCC- or LR HCC-derived CM for 48 h at 37°C to obtain TAMs or LR TAMs, respectively. Next, the CM from M0, TAMs, and LR TAMs were collected in the same manner as described above, then mixed with complete medium (1:1) for cell proliferation, wound healing, and migration assays. Cell morphology was captured using a light microscope (CKX41; Olympus, Tokyo, Japan).

2.5 | RNA interference

The miR-301a-3p inhibitor negative controls (INC) (4464076) or inhibitors (MH10978, Thermo Fisher Scientific) were transfected at a final concentration of 100 nM into Huh^{LR} cells with lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 24 h after transfection the medium was replaced with DMEM without FBS for collecting the CM of INC and inhibited Huh^{LR} cells. Thereafter, the CM from INC and inhibited TAMs (Huh^{LR}) were collected in the same manner as described above and were mixed with complete medium (1:1) for further experiments.

2.6 | Exosome isolation and identification

The cell lines were cultured in complete growth medium until 80% to 90% confluent, then washed twice with PBS. Thereafter, they were incubated with DMEM without FBS for 48 h at 37°C. Then, 30 mL of supernatant was collected and centrifuged at 2000 *g* for 30 min, then transferred to a new tube without disturbing the pellet. The exosomes were also isolated by a precipitation method using Total Exosome Isolation (from cell culture media) (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, CM samples were incubated with the Total Exosome Isolation reagent for over 12 h, centrifuged at 10000 *g* for 1 h, and exosomes were resuspended and stored at −80°C for further use.

Subsequently, the size and concentration of exosomes were evaluated by nanoparticle tracking analysis system (Nanosight NS300, Malvern Panalytical, UK) according to company protocols.

2.7 | Cell proliferation assay

The tumor cells were seeded at a density of 1×10^4 cells/well in 96-well plates and cultured with complete growth medium at 37°C. After cell adhesion, the cells were cultured with the indicated CM at 37°C. Cell proliferation was analyzed from day 0 to day 4 using CCK-8 (Dojindo Molecular Technologies,) by measuring the absorbance values at 450 nm with SoftMax Pro 7.

2.8 | Wound-healing assay

Wound-healing assays were performed as previously described.[18] Briefly, the cancer cells were seeded and grown to 90% confluency in 6-well plates. The cells were cultured with indicated CM in each group for 12 h at 37°C. The images of the wound area were captured using a light microscope (40× magnification, DP22-CU; Olympus) at 0 and 12 h after scratching. The cell migration rates were calculated using Image J v1.46r software (National Institutes of Health, Bethesda, MD, USA).

2.9 | Migration assay

Migration assays were performed as previously described.[18] Briefly, serum-starved cancer cells were seeded at a density of 2.0×10^4 cells in the upper chamber of a 24-well Transwell system with 8.0 μm pores (Corning, Corning, NY, USA). The CM FBS concentrations were 1% in the upper chambers and 5% in the lower chambers. After incubation for 24 h at 37°C, the cancer cells were fixed and stained with 100% methanol and 0.1% crystal violet. The number of migrated cells was counted in three random fields of view under a light microscope (100× magnification, BX43; Olympus).

2.10 | RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as previously described.[18] Briefly, the following TaqMan assays (assay identification number) and primers were used: CD163 (Hs00174705_ml), CD206 (Hs00267207_ml), PTEN (Hs002621230_sl), NRE2L2 (Hs00975961_g1), E-cadherin (Hs00170423_ml), N-cadherin (Hs00169953_ml), Snail (Hs00195591_ml), and vimentin (Hs00185584_ml). GAPDH (Hs02786624_g1) was

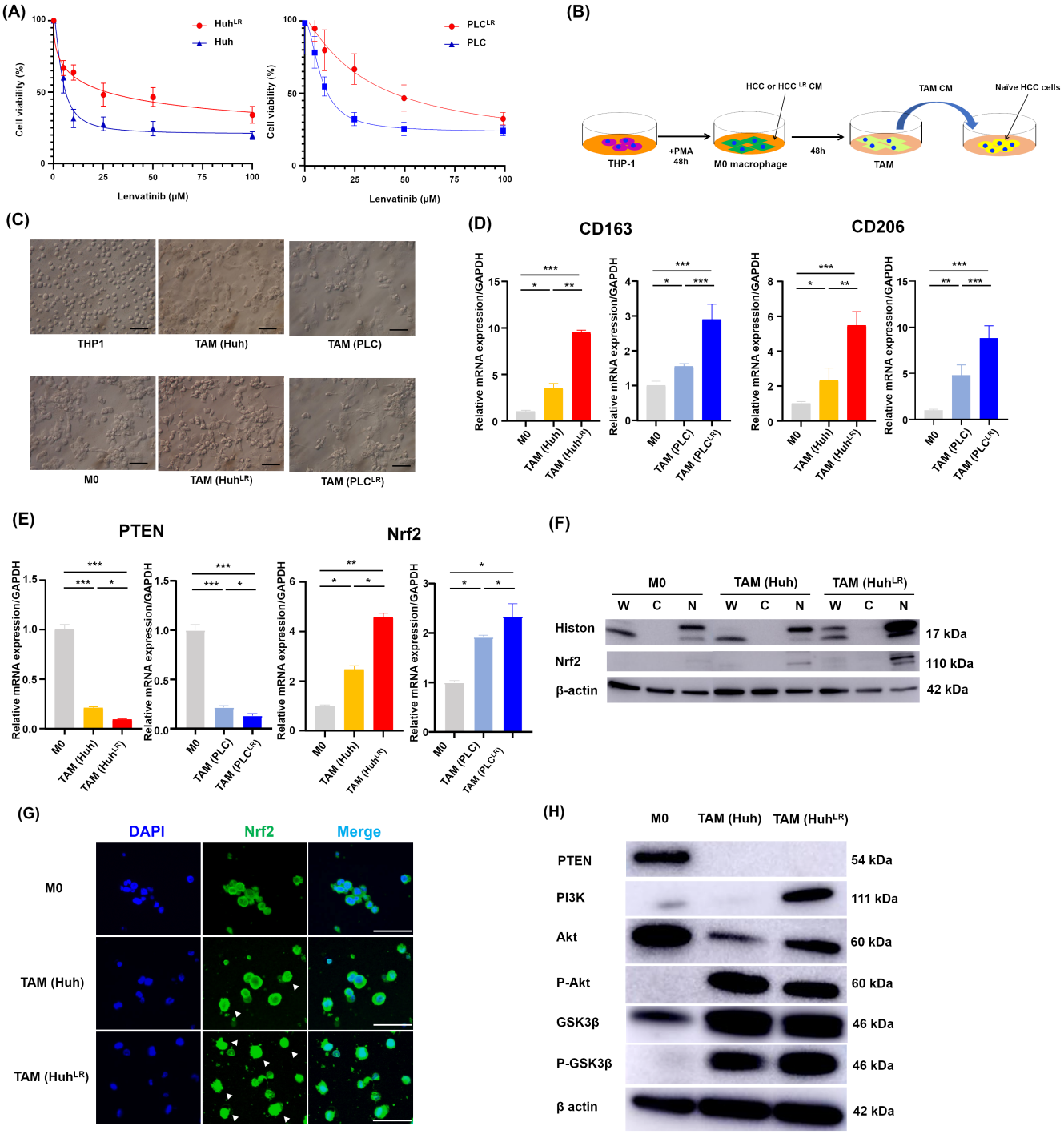


FIGURE 1 Characteristics of LR HCC cells cocultured with TAMs. (A) The cell viabilities of HCC and LR HCC cells were examined by CCK-8 assays, then cultured with complete growth medium with increasing doses of lenvatinib. (B) TAMs were stimulated with the CM of naïve HCC or LR HCC cells. (C) Morphology of TAMs cultured with the CM of M0, HCC, or LR HCC cells. (D) CD163 and CD206 expression levels were evaluated by qRT-PCR in each TAM group. (E) PTEN and Nrf2 expression levels in TAMs were detected by qRT-PCR after being stimulated with the CM of M0, HCC, or LR HCC cells. (F) Intranuclear Nrf2 protein expression levels in TAMs were detected by western blot analysis (W; whole, C; cytoplasm, N; nuclear). (G) TAMs stimulated with the CM of M0, Huh-, or Huh^{LR} cells were stained with Nrf2 (green) and DAPI (blue). White arrowheads show the enhanced intranuclear Nrf2 signals. (H) Protein expression levels of signaling pathway components downstream of PTEN were detected in TAMs by western blot analysis after being stimulated with the CM of M0, Huh, or Huh^{LR} cells. CM, conditioned medium; HCC, hepatocellular carcinoma; LR, lenvatinib-resistant; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TAM, tumor-associated macrophage. The data are shown as mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Bar = 100 μm.

used as an internal control for normalization. Relative expression values of all genes were calculated as ratios to GAPDH expression. All primers were purchased from Thermo Fisher Scientific. The $2^{-\Delta\Delta Ct}$ method was used to analyze the data. Fold changes as relative mRNA expression levels in each experimental group were compared with the control group.

The exosomal RNAs in the CM were extracted using the miRNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Reverse transcription and qRT-PCR for exosomal miRNAs, miR-301a-3p, and internal reference miR-16-5p were performed using the miRNA RT-PCR Quantitation Kit (Qiagen). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression values of exosomal miRNAs.

2.11 | Analysis of miR-301a-3p expression levels and patient survival rates

The miR-301a-3p expression levels and survival outcomes in HCC patients were obtained from a public database, Genome Data Commons, The Cancer Genome Atlas (GDC-TCGA) Liver Cancer via the University of California, Santa Cruz Xena platform (https://gdc.xenahubs.net). In total, 372 human HCC samples and 50 human liver samples from the GDC-TCGA Liver Cancer dataset of stem loop expression—miRNA Expression Quantification were analyzed. During the analysis of HCC patient overall survival rates, six patients were excluded who had no recorded survival data. The median value of miR-301a-3p was used as the cutoff value to divide the patients into two groups.

2.12 | Western blot analysis

Western blot analysis was performed according to the method previously reported.[18] Briefly, RIPA buffer (Thermo Fisher Scientific) containing both a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and PhosSTOP phosphatase inhibitor cocktail (Roche, Tokyo, Japan) was used for total protein extraction. Additionally, lysis or extraction buffer (Thermo Fisher Scientific) containing protease inhibitor cocktail, PhosSTOP phosphatase inhibitor cocktail, and 0.1 M dithiothreitol (Thermo Fisher Scientific) was used to extract intranuclear or cytoplasm proteins. The proteins were detected with chemiluminescence (GE Healthcare, Little Chalfont, UK). The primary antibodies for western blotting are listed in Table 1.

2.13 | Immunofluorescence staining

The iPGell (Geno staff, Tokyo, Japan) was used to gelatinize the indicated cells according to the manufacturer's protocol. Then, the thinly sliced gelatinized cell slides were incubated with an anti-Nrf2 primary antibody (Abcam, ab137550; Cambridge, UK) overnight at 4°C. After fluorophore-conjugated second antibody incubation, the cells were counterstained with DAPI (P306931; Thermo Fisher Scientific). Finally, the slides were observed under a fluorescence microscope (Keyence, Itasca, IL, USA).

2.14 | Enzyme-linked immunoassay (ELISA) analysis

The CMs of indicated cells were collected in the same manner as aforementioned for the measurement of cytokines concentration. The human ELISA kit for VEGF (R&D Systems, Minneapolis, MN, USA) was utilized according to the manufacturer's protocol.

2.15 | Statistical analysis

All data are presented as the mean ± standard deviation. Statistical analysis and graphing were conducted using GraphPad Prism v.9.3 software, ImageJ software, and R v.4.2.0 software. Comparisons

TABLE 1 Primary antibodies used in western blot analysis.

Name	Product number	Company	Dilution ratio
Histone H3 antibody	#9649	Cell Signaling	1:1000
β-Actin antibody	#4970	Cell Signaling	1:3000
Nrf2 antibody	ab137550	Abcam	1:500
PTEN antibody	#9188	Cell Signaling	1:1000
AKT antibody	#4691	Cell Signaling	1:1000
pAKT antibody	#4060	Cell Signaling	1:500
GSK3β antibody	#12456	Cell Signaling	1:500
pGSK antibody	#9336	Cell Signaling	1:500
PI3K antibody	#4249	Cell Signaling	1:500
N-cadherin antibody	#13116	Cell Signaling	1:1000
E-cadherin antibody	#3195	Cell Signaling	1:1000
Vimentin antibody	#5741	Cell Signaling	1:1000
CD63 antibody	#EXOAB-CD63A-1	System Biosciences	1:1000

between two groups were analyzed using the Mann–Whitney test. Differences among multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Survival analysis data were plotted using the Kaplan–Meier method. All experiments were repeated at least three times. *P*-values <0.05 (two-sided) were considered statistically significant.

3 | RESULTS

3.1 | Characteristics of TAMs cultured in LR HCC CM

The THP-1 cells were exposed to PMA for 48 h to stimulate their differentiation to the M0 phenotype, as previously reported.[17] CM from the HCC cells were used as culture medium for the M0 macrophages for 48 h. To imitate the interactions between HCC and TAMs in the TME, the M0 macrophages were exposed to cancer cell-derived CM to obtain TAMs (Huh/PLC) and LR TAMs (Huh^{LR}/PLC^{LR}), respectively (Figure 1B). LR TAMs exhibited an elongated and spindle-shaped morphology, similar to that of M2-polarized macrophages[19] (Figure 1C). After activation by the HCC CM, expression levels of M2 macrophage markers CD163 and CD206 were significantly upregulated in LR TAMs, indicating that the M0 cells transdifferentiated into M2 polarized TAMs (Figure 1D). PTEN is the primary regulator of the procancer metastatic switch via the PI3K/AKT signaling pathways.[15] In TAMs cultured with LR HCC CM, PTEN mRNA expression levels were significantly decreased, while Nrf2 mRNA expression levels were significantly increased compared with M0 CM and HCC CM ($P < 0.05$, Figure 1E). Additionally, the protein expression levels of intranuclear Nrf2 were promoted in TAMs (Huh^{LR}) (Figure 1F). Moreover, fluorescent immunostaining showed enhanced intranuclear Nrf2 signals (Figure 1G). Additionally, enhanced nuclear Nrf2 signal was observed in TAMs around the residual tumors in patients who underwent hepatic resection after an effective response to lenvatinib compared with TAMs around normal HCC (Figure S1). For the PTEN signaling pathway, PI3K and p-GSK3β protein expression levels were upregulated in the TAMs (Huh^{LR}) (Figure 1H).

3.2 | Enhancement of the malignant potential and drug resistance of naïve HCC cells educated by LR TAM CM

All three types of CM, from M0, TAMs, and LR TAMs, were collected and added to naïve HCC cells. Compared with CM from M0 and TAMs, LR TAM CM significantly increased the proliferation and migration capabilities of the cancer cells ($P < 0.05$, Figure 2A–C). The lenvatinib IC50 value of naïve HCC cells cultured with TAM (Huh^{LR}) CM was twice as high as that of the cells cultured with TAM (Huh) CM (14.0 vs 7.0 μM) (Figure 2D). VEGF concentration was higher in LR TAM CM than in TAM CM and CM of M0 macrophages (Figure 2E). These data suggest that the cancer cells cultured with TAM (Huh^{LR}) CM acquired lenvatinib drug resistance. In the cancer cells cultured with TAM (Huh^{LR}) CM, the mRNA expression levels of EMT markers N-cadherin and vimentin were significantly increased, while E-cadherin mRNA expression was significantly decreased ($P < 0.05$, Figure 2F). The same expression patterns were observed at the protein level (Figure 2G). These data suggest that the naïve HCC cells cultured with TAM (Huh^{LR}) CM promoted the EMT ability.

3.3 | The roles of exosomal miR-301a-3p in HCC

To identify a specific miRNA that can regulate Nrf2 signaling by directly targeting PTEN, two bioinformatics software programs, Target Scan[20] and miDIP,[21] were used to predict miRNAs that could possibly target PTEN mRNA. Ten miRNAs were identified, among which miR-301a-3p expression levels were significantly upregulated in HCC tissues compared with nontumoral tissues in the miRNA expression data from GDC-TCGA Liver Cancer (Figure 3A). Reports have indicated interactions between cancer cells and TAMs in gastrointestinal cancers involving miR-301a-3p.[10,15] Furthermore, miR-301a-3p upregulation was significantly associated with poor prognosis in HCC patients from GDC-TCGA Liver Cancer data (Figure 3B). The size and particle concentration of exosomes from HCC-derived CMs were evaluated (Figure 3C), and CD63, an exosome marker, was enriched in HCC-derived CMs (Figure 3D). Moreover, qRT-PCR results demonstrated that the exosomal miR-301a-3p levels were significantly upregulated both in Huh^{LR} and PLC^{LR} cells compared with parental naïve HCC cells (Figure 3E). These results demonstrate that miR-301a-3p is enriched in exosomes derived from LR HCC cells.

3.4 | Inhibition of huh LR-derived exosomal miR-301a-3p decreases malignant potential by downregulating TAM M2 polarization

We next investigated the effects of inhibiting exosomal miR-301a-3p. Huh^{LR} cells were transfected with the INC or miR-301a-3p inhibitor, then the transfection efficiencies were evaluated using qRT-PCR (Figure 4A). Further qRT-PCR analysis demonstrated downregulation of M2 markers (CD206 and CD163) and Nrf2, as well as upregulation of PTEN, in TAMs cultured with CM from Huh^{LR} cells transfected with the miR-301a-3p inhibitor (Figure 4B). Additionally, western blotting demonstrated increased protein expression levels of PTEN and decreased protein expression levels of Nrf2 in TAMs cultured with CM from Huh^{LR} cells transfected with the miR-301a-3p inhibitor (Figure 4C,D). These results demonstrate that exosomal miR-301a-3p could induce M2 macrophage polarization and upregulate components of the PTEN/Nrf2 pathway. We then examined the effects of inhibiting Huh^{LR}-derived miR-301a-3p on TAMs. CM from macrophages cocultured with exosomes derived from miR-301a-3p inhibitor-transfected Huh^{LR} cells decreased the

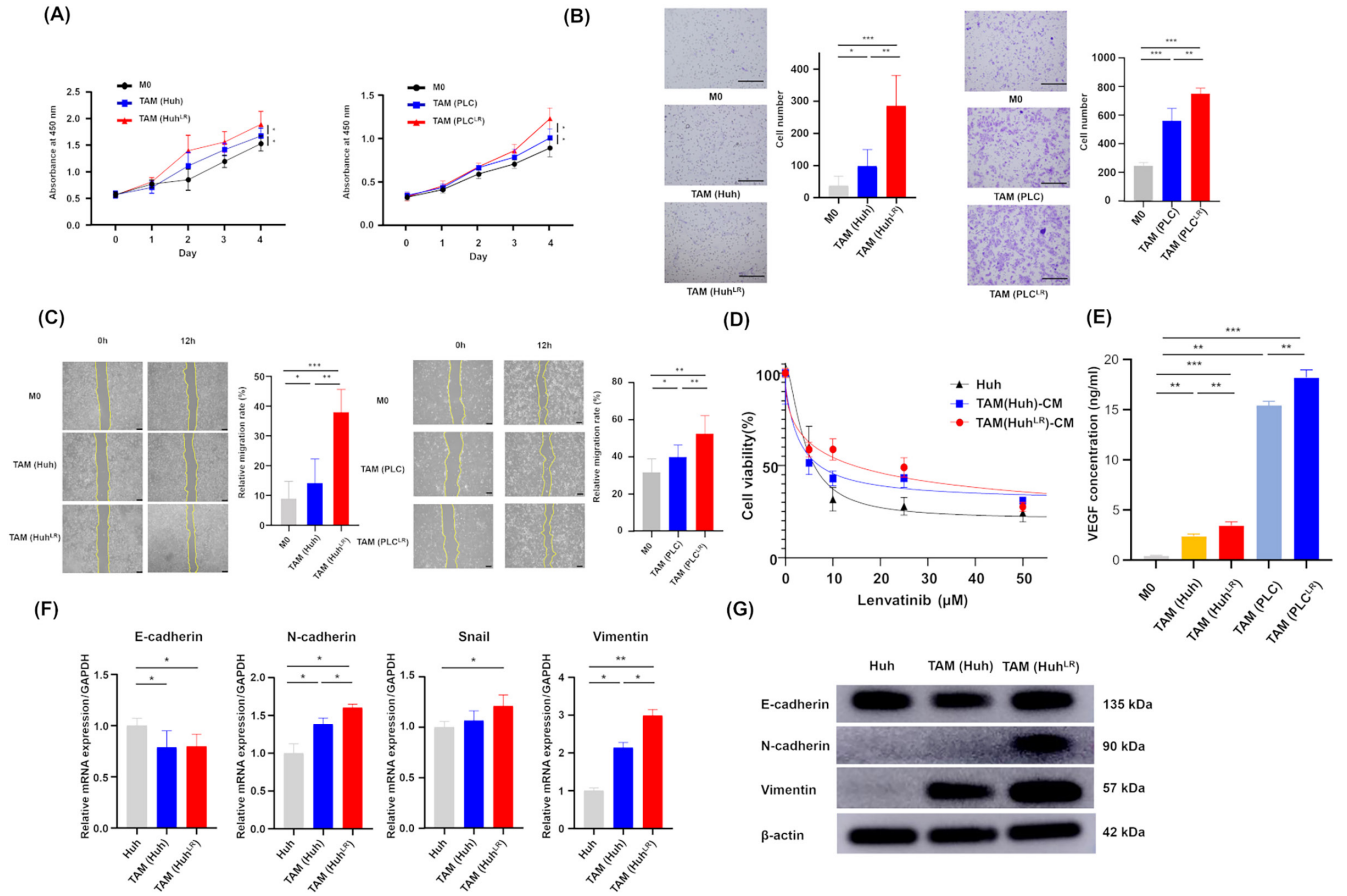


FIGURE 2 Enhancement of the malignant potential and drug resistance of naïve HCC cells cocultured with LR TAM CM. (A–C) The proliferation and migration capabilities of naïve HCC cells cultured with the CM of M0, HCC, or LR HCC cells were detected. (D) Cell viability was detected by CCK-8 assays in naïve HCC cells cultured with the CM of TAM (Huh) or TAM (Huh^{LR}) cells for 24 h. (E) VEGF concentration was higher in LR TAM CM than in TAM CM and CM of M0 macrophages. (F) E-cadherin, N-cadherin, Snail, and vimentin mRNA expression levels in naïve HCC cells were detected by qRT-PCR after being stimulated with the CM of M0, TAM (Huh), or TAM (Huh^{LR}) cells for 24 h. (G) E-cadherin, N-cadherin, and vimentin protein expression levels were detected by western blot analysis in naïve HCC cells cultured with the CM of M0, TAM (Huh), or TAM (Huh^{LR}) cells for 24 h. CM, conditioned medium; HCC, hepatocellular carcinoma; LR, lenvatinib-resistant; TAM, tumor-associated macrophage. The data are shown as mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Bar = 100 μm.

cell proliferation and migration abilities (Figure 4E–G). The IC50 values for lenvatinib of naïve Huh cell lines after culturing with the indicated CMs for 24 h showed a nearly 2-fold increase in TAM (Huh^{LR})-CM compared to the normal culture, while in TAM-CM from Huh^{LR} cells transfected with the miR-301a-3p inhibitor, the values were similar to those of the normal culture (normal culture: 6.5 μM, TAM (Huh^{LR})-CM: 12 μM, TAM transfected with INC-CM: 10.0 μM, inhibited TAM-CM; 7.3 μM, respectively) (Figure 4H). These results demonstrate that miR-301a-3p inhibition in exosomes derived from LR HCC cells downregulated the tumor malignancy and drug resistance via regulating the M2 polarization of TAMs.

4 | DISCUSSION

The present study revealed that the LR HCC cells had upregulated exosomal miR-301a-3p expression levels, and CM from these cells could stimulate M2-polarlized TAMs and activate the PTEN/PI3K/GSK3B/Nrf2 pathway, leading to increased malignant potential and drug resistance of naïve HCCs via EMT. This study also demonstrated that inhibiting exosomal miR-301a-3p from LR HCC cells could suppress HCC cell proliferation and migration abilities and drug resistance. The possible mechanisms of the current study are shown in Figure 5.

Peripheral blood monocytes are activated to generate a broad spectrum of TAMs that are responsive to various chemokines and growth factors in the TME.[22] TAMs are mainly divided into two different polarized types: M1 or M2. Activated M1 macrophages have antitumor and proinflammatory properties, specifically through secretion of cytokines such as interleukin (IL)-1 and IL-6. However, activated M2 macrophages have antiinflammatory and protumorigenic features and primarily express CD163 and CD206.[22] In the present study, LR HCC cells had significantly higher CD163 and CD206 expression levels compared with the naïve HCC cells. M2 macrophages have been demonstrated to induce malignant potential, including through increased cell proliferation, migration, invasion, and angiogenesis in HCC.[23]

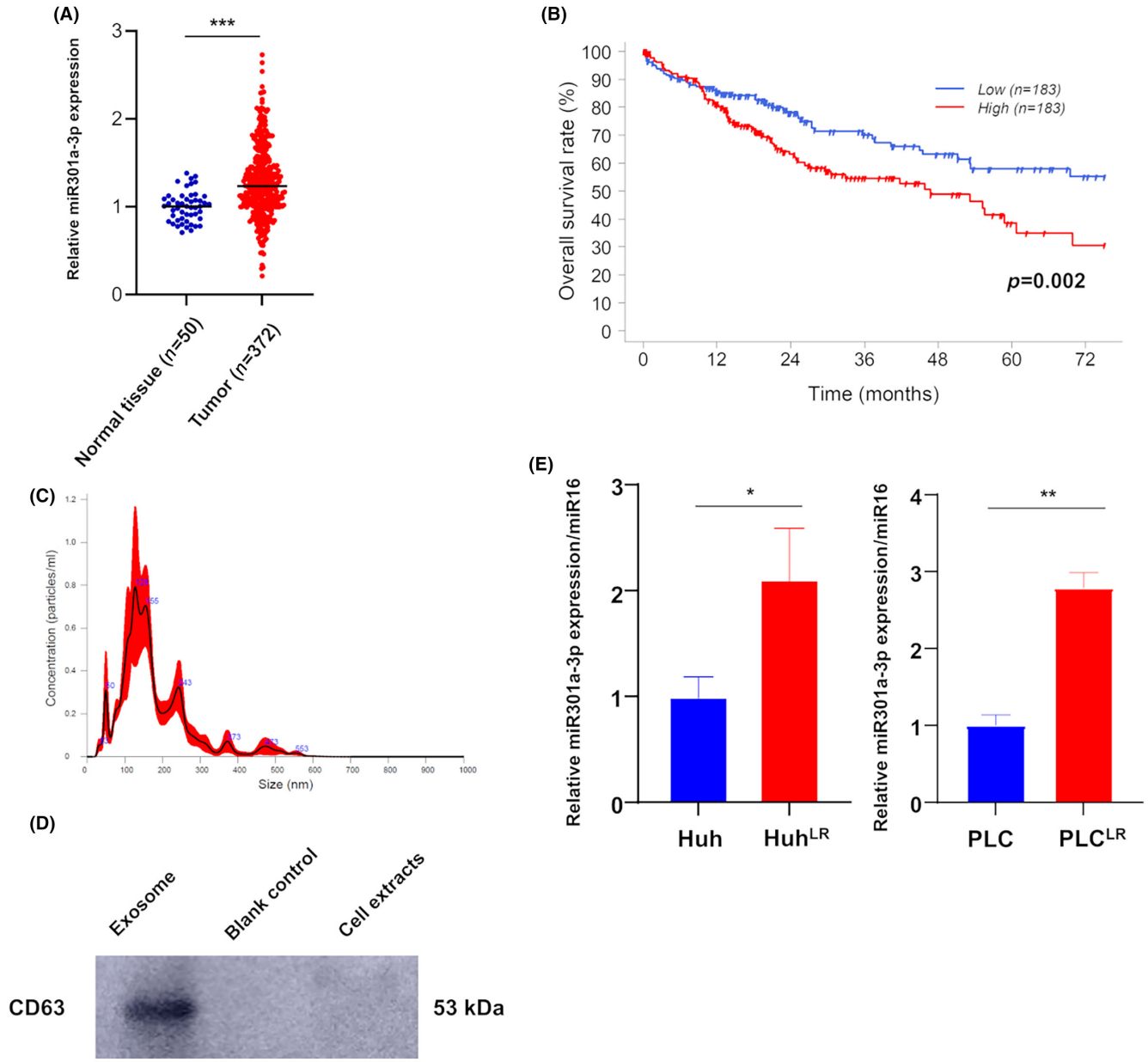
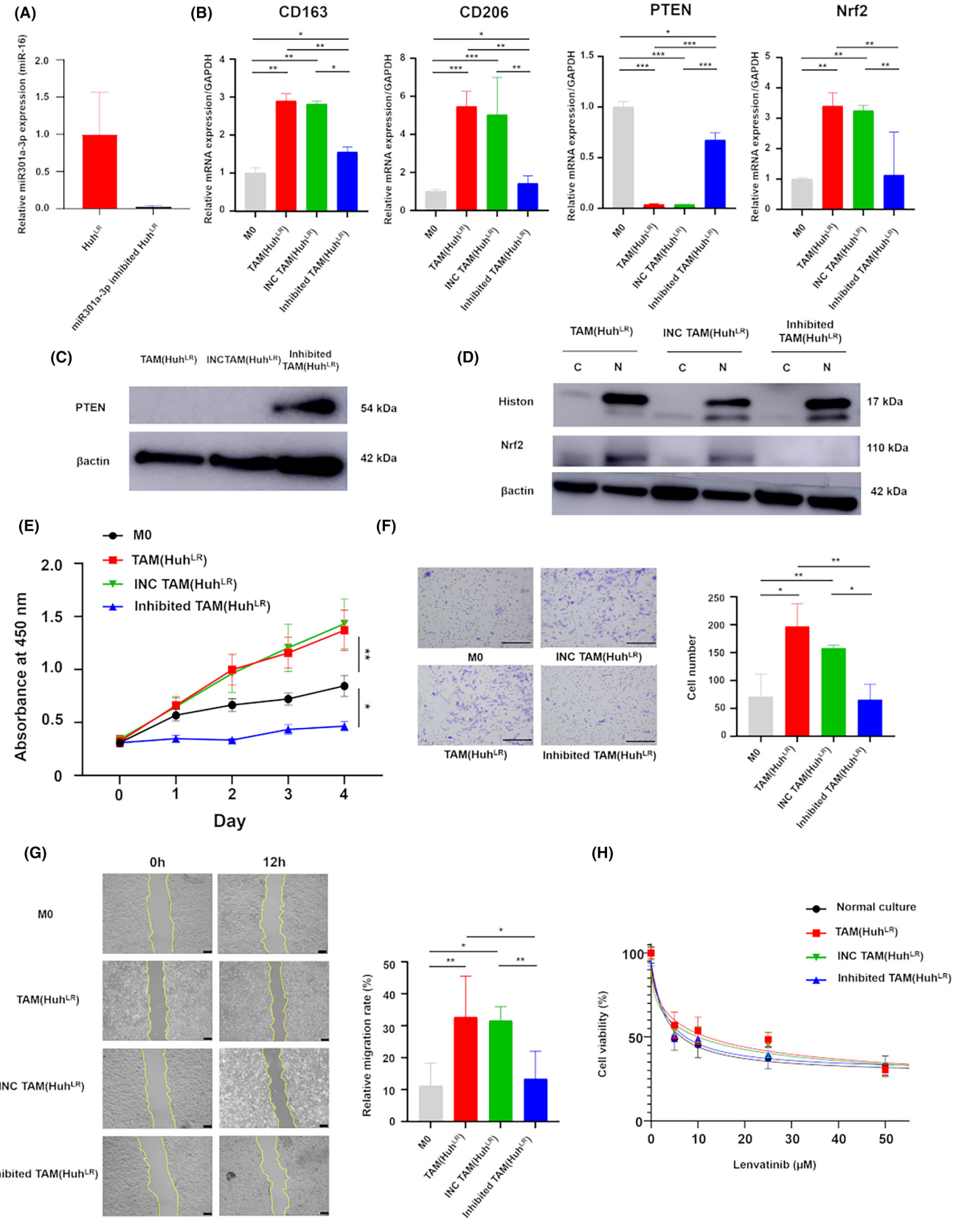


FIGURE 3 The exosomal miR-301a-3p expression levels in HCC. (A) The expression patterns of miR-301a-3p in the HCC tissues and nontumoral tissues in microRNA expression data from the Genome Data Commons the Cancer Genome Atlas Liver Cancer. (B) High miR-301a-3p expression levels were significantly associated with poor survival in HCC patients from the GDC TCGA Liver Cancer dataset ($P=0.002$). (C) Nanosight particle tracking was used to analyze the size and particle concentration secreted by HCC cells. (D) CD63 protein expression in HCC-derived exosomes was detected by western blot analysis. (E) Quantification of exosomal miR-301a-3p expression in HCC and LR HCC cells. HCC, hepatocellular carcinoma; LR, lenvatinib-resistant. The data are shown as mean ± standard deviation (SD). $^{*}P<0.05$. $^{**}P<0.01$. $^{***}P<0.001$.

FIGURE 4 Decreased malignant potential by downregulating M2 polarization of TAMs through inhibition of Huh^{LR}-derived exosomal miR-301a-3p. (A) Efficiency of miR-301a-3p inhibitor transfection into Huh^{LR} cells was assessed using qRT-PCR for exosomal miR-301a-3p expression. (B) The mRNA expression levels of CD163, CD206, PTEN, and Nrf2 were evaluated by qRT-PCR in the M0, Huh^{LR}, INC Huh^{LR}, and inhibited Huh^{LR} cocultured TAMs. (C,D) PTEN and Nrf2 protein expression levels in the M0, Huh^{LR}, INC Huh^{LR}, and inhibited Huh^{LR} cocultured TAMs were detected by western blot analysis. (E,F,G) The proliferation and migration capabilities of naïve HCC cells stimulated with the CM of M0, TAM (Huh^{LR}), INC TAM (Huh^{LR}), or inhibited TAM (Huh^{LR}) cells. (H) The IC50 values for lenvatinb of naive Huh cell lines stimulated with the CMs of TAM (Huh^{LR}), INC TAM (Huh^{LR}) or inhibited TAM (Huh^{LR}) for 24h". HCC, hepatocellular carcinoma; INC, inhibited negative control; LR, lenvatinib-resistant; TAM, tumor-associated macrophage. The data are shown as mean ± standard deviation (SD). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Bar = 100 μm.



Lenvatinib was approved for the treatment of unresectable HCC cases and was demonstrated to be noninferior to sorafenib as a first-line agent.[2] Nevertheless, many patients with advanced HCC failed to obtain any long-term benefit from systemic therapy after developing drug resistance.[24] Many studies have investigated the mechanisms of LR, with PDGF-AA, EGFR, multidrug resistance protein 1 and breast cancer resistance protein transporters, and STAT3/ABCB1 signaling having been implicated in LR HCC.[25–28] Although

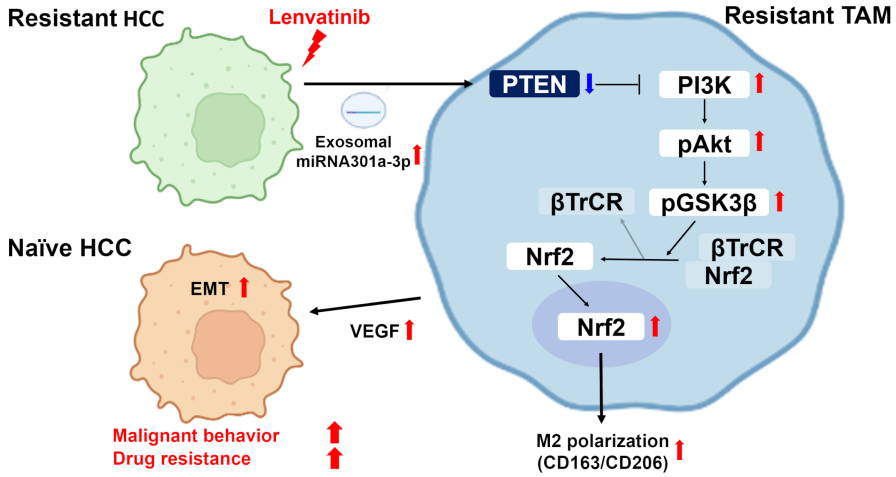


FIGURE 5 Schematic representation of the proposed interactions between LR HCC cells and TAMs. TAM, tumor-associated macrophage; HCC, hepatocellular carcinoma; LR, lenvatinib-resistant.

few studies have examined LR in HCC with a focus on the TME, this study demonstrated that miR-301a-3p in the TME was associated with the LR mechanisms. Therefore, miRNAs may be an important mediator involved in LR in the TME. Exosomal miR-301a-3p is a multifunctional miRNA that can promote tumor growth, modulate inflammatory responses, and mediate drug tolerance in gastrointestinal cancers.[10,15] Additionally, exosomal miR-301a-3p from esophageal cancer cells have been found to induce M2 polarization of macrophages through upregulating phosphorylated AKT by targeting the PTEN/PI3K signaling pathway in TAMs.[10] Our study demonstrated that exosomal miR-301a-3p secreted by LR HCC cells could induce M2 polarization through the PTEN/PI3K/AKT/GSK3β signaling pathway. Our findings indicated that exosomal miR-301a-3p is possibly an important messenger for intercellular signaling between LR HCC cells and TAMs in the TME.

PI3K is partially implicated in myeloid cell infiltration into tumors and the regulation of tumor growth by enhancing the polarization of neutrophils and controlling immune suppression.[29,30] Recently, PI3K was found to regulate macrophage M2 polarization by inhibiting immune reactions and enhancing the invasion of pancreatic ductal adenocarcinoma cells.[31] Our study showed that activation of exosomal miR-301a-3p/PI3K signaling promoted polarization of M2 macrophages, leading to increased HCC cell migration and EMT. Degradation of PTEN was inversely associated with constitutive activation of the PI3K/AKT signaling pathway.[15] The PI3K/AKT/mTOR signaling pathway was strongly associated with activation and polarization of macrophages, as well as expression regulation of GSK3β and Nrf2/HO-1.[29,30] Then, PTEN downregulation resulted in increased PI3K, p-AKT, p-GSK3β, and Nrf2 expression levels. Accordingly, our findings demonstrated that miR-301a-3p plays an important role in enhancing M2 polarization by regulating the PTEN/PI3K/GSK3β/Nrf2 pathway.

Previous studies have demonstrated that Nrf2 protects cancer cells and enhances cancer progression by reducing the endogenous reactive oxygen species levels.[32] Nrf2 overexpression can enhance the resistance of cancer cells to chemotherapeutic drugs, such as cisplatin, doxorubicin, and etoposide.[33] Additionally, Nrf2 could evoke macrophage polarization toward the M2 phenotype via NF-κB genes.[4] Similarly, our research demonstrated that Nrf2 overexpression in macrophages elevated the expression levels of M2 markers. In addition, M2-phenotype TAMs have been reported to promote progression and cancer stemness in breast cancer via the VEGF secretion.[34] Likewise, our previous studies supported that Nrf2 overexpression in TAMs promoted VEGF secretion and was associated with tumor malignancy of HCCs by inducing the EMT.[18,35] Although EMT acquisition is known as one of the mechanisms of drug resistance in cancer cells, and the present study suggested that EMT may promote malignant behaviors and lenvatinib drug resistance via VEGF in the naïve HCC cells cultured with LR TAM-CM. Taken together, Nrf2 upregulation in TAMs led to M2 polarization and could be associated with malignant potential and drug resistance via EMT in LR HCC cells.

The present study has certain limitations. The results were based on in vitro experiments using miRNA inhibitor transfection in HCC cell lines. In vivo animal models and further investigation into the effects of miRNA inhibition on the crosstalk between LR HCC cells and TAMs are necessary.

In conclusion, we found that exosomal miR-301a-3p from LR HCC cells could stimulate the polarization of macrophages to the M2 phenotype through activation of the PTEN/PI3K/GSK3β/Nrf2 signaling pathway. These findings not only demonstrate that exosomal miRNA-mediated crosstalk between HCC cells and TAMs in the TME can contribute to TAM M2 polarization, but also provide new insights into the possible roles of miR-301a-3p in HCC progression and lenvatinib drug resistance.

AUTHOR CONTRIBUTIONS

Y.M. and M.S. conceived the study and supervised the project. Y.W. performed the experiments, analyzed and interpreted the data, and wrote the original draft of the article. Y.M., Y.S., H.T., S.Y., T.I., T.T., and M.S. were also involved in data analysis and final article preparation. All authors reviewed and agreed to the published version of the article. The work reported in the article was performed by the authors, unless clearly specified in the text.

ACKNOWLEDGMENTS

We thank J. Iacona, Ph.D., from Edanz (https://jp.edanz.com/ac) for editing a draft of this article.

FUNDING INFORMATION

This study was partly supported by the Research Program on Hepatitis of the Japanese Foundation for Multidisciplinary Treatment of Cancer, the Japan Agency for Medical Research and Development (AMED) grant numbers 23fk0210103s0202 and 23fk0210103h0002, and a Grant-in-Aid for Scientific Research (grant no. 23K08072). This study was also funded by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan).

CONFLICT OF INTEREST STATEMENT

M.S. received research grants from Taiho Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., and EPS Holdings, and scholarship donations from Taiho Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., AbbVie G.K., Johnson & Johnson K.K., Eisai Co., Ltd., Bayer Yakuhin, Ltd., Ono Pharmaceutical Co., Ltd., and Takeda Pharmaceutical Co., Ltd. The other authors have no competing interests related to this study.

DATA AVAILABILITY STATEMENT

Data sources and handling of the publicly available datasets used in this study are described in the Materials and Methods. Further details and other data that support the findings of this study are available from the corresponding authors upon request.

ETHICS STATEMENTS

Approval of the research protocol: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All procedures followed the ethical standards of the responsible committee on human experimentation (institutional and national) and the Helsinki Declaration of 1964 and later versions.

ORCID

Yuhei Waki https://orcid.org/0000-0001-5217-0659
Yuji Morine https://orcid.org/0000-0002-5889-9288
Yu Saito https://orcid.org/0000-0001-6349-1669
Hiroki Teraoku https://orcid.org/0000-0001-5730-9808
Shinichiro Yamada https://orcid.org/0000-0003-3847-751X
Tetsuya Ikemoto https://orcid.org/0000-0001-9800-1359

REFERENCES

1. El-Serag HB. Hepatocellular carcinoma. N Engl J Med. 2011;365(12):1118–27.
2. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet. 2018;391(10126):1163–73.
3. Zhu AX, Abbas AR, de Galarreta MR, Guan Y, Lu S, Koeppen H, et al. Molecular correlates of clinical response and resistance to atezolizumab in combination with bevacizumab in advanced hepatocellular carcinoma. Nat Med. 2022;28(8):1599–611.
4. Gao L, Morine Y, Yamada S, Saito Y, Ikemoto T, Tokuda K, et al. The BAFF/NFkappaB axis is crucial to interactions between sorafenib-resistant HCC cells and cancer-associated fibroblasts. Cancer Sci. 2021;112(9):3545–54.
5. Nasrollahzadeh E, Razi S, Keshavarz-Fathi M, Mazzone M, Rezaei N. Pro-tumorigenic functions of macrophages at the primary, invasive and metastatic tumor site. Cancer Immunol Immunother. 2020;69(9):1673–97.
6. Komohara Y, Fujiwara Y, Ohnishi K, Takeya M. Tumor-associated macrophages: potential therapeutic targets for anti-cancer therapy. Adv Drug Deliv Rev. 2016;99(Pt B):180–5.
7. Li Z, Wu T, Zheng B, Chen L. Individualized precision treatment: targeting TAM in HCC. Cancer Lett. 2019;458:86–91.
8. Dai J, Su Y, Zhong S, Cong L, Liu B, Yang J, et al. Exosomes: key players in cancer and potential therapeutic strategy. Signal Transduct Target Ther. 2020;5(1):145.
9. Hausser J, Zavolan M. Identification and consequences of miRNA–target interactions—beyond repression of gene expression. Nat Rev Genet. 2014;15(9):599–612.
10. Shou Y, Wang X, Chen C, Liang Y, Yang C, Xiao Q, et al. Exosomal miR-301a-3p from esophageal squamous cell carcinoma cells promotes angiogenesis by inducing M2 polarization of macrophages via the PTEN/PI3K/AKT signaling pathway. Cancer Cell Int. 2022;22(1):153.
11. Gao L, Morine Y, Yamada S, Saito Y, Ikemoto T, Tokuda K, et al. Nrf2 signaling promotes cancer stemness, migration, and expression of ABC transporter genes in sorafenib-resistant hepatocellular carcinoma cells. PLoS One. 2021;16(9):e0256755.
12. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol. 2007;47:89–116.
13. Pérez-Sen R, Queipo MJ, Morente V, Ortega F, Delicado EG, Miras-Portugal MT. Neuroprotection mediated by P2Y13 nucleotide receptors in neurons. Comput Struct Biotechnol J. 2015;13:160–8.
14. Mayer IA, Arteaga CL. The PI3K/AKT pathway as a target for cancer treatment. Annu Rev Med. 2016;67:11–28.
15. Wang X, Luo G, Zhang K, Cao J, Huang C, Jiang T, et al. Hypoxic tumor-derived Exosomal miR-301a mediates M2 macrophage polarization via PTEN/PI3Kγ to promote pancreatic cancer metastasis. Cancer Res. 2018;78(16):4586–98.
16. Guo J, Zhong X, Tan Q, Yang S, Liao J, Zhuge J, et al. miR-301a-3p induced by endoplasmic reticulum stress mediates the occurrence and transmission of trastuzumab resistance in HER2-positive gastric cancer. Cell Death Dis. 2021;12(7):696.
17. Chen W, Yang J, Zhang Y, Cai H, Chen X, Sun D. Regorafenib reverses HGF-induced sorafenib resistance by inhibiting epithelial-mesenchymal transition in hepatocellular carcinoma. FEBS Open Bio. 2019;9(2):335–47.
18. Okikawa S, Morine Y, Saito Y, Yamada S, Tokuda K, Teraoku H, et al. Inhibition of the VEGF signaling pathway attenuates tumor-associated macrophage activity in liver cancer. Oncol Rep. 2022;47(4):71.
19. Dahlem C, Siow WX, Lopatniuk M, Tse WKF, Kessler SM, Kirsch SH, et al. Thioholgamide a, a new anti-proliferative anti-tumor agent, modulates macrophage polarization and metabolism. Cancers (Basel). 2020;12(5):1288.
20. McGeary SE, Lin KS, Shi CY, Pham T, Bisaria N, Kelley GM, et al. The biochemical basis of microRNA targeting efficacy. Science. 2019;366(6472):eaav1741.
21. Tokar T, Pastrello C, Rossos AEM, Abovsky M, Hauschild A-C, Tsay M, et al. mirDIP 4.1—integrative database of human microRNA target predictions. Nucleic Acids Res. 2017;46(D1):D360–D370.
22. Franklin RA, Liao W, Sarkar A, Kim MV, Bivona MR, Liu K, et al. The cellular and molecular origin of tumor-associated macrophages. Science. 2014;344(6186):921–5.
23. Dong P, Ma L, Liu L, Zhao G, Zhang S, Dong L, et al. CD86^{+}/CD206^{+}, diametrically polarized tumor-associated macrophages,

predict hepatocellular carcinoma patient prognosis. Int J Mol Sci. 2016;17(3):320.
24. Ladd AD, Duarte S, Sahin I, Zarrinpar A. Mechanisms of drug resistance in HCC. Hepatology. 2023;79:926–40.
25. Ao J, Chiba T, Shibata S, Kurosugi A, Qiang N, Ma Y, et al. Acquisition of mesenchymal-like phenotypes and overproduction of angiogenic factors in lenvatinib-resistant hepatocellular carcinoma cells. Biochem Biophys Res Commun. 2021;549:171–8.
26. Hu B, Zou T, Qin W, Shen X, Su Y, Li J, et al. Inhibition of EGFR overcomes acquired lenvatinib resistance driven by STAT3-ABCB1 signaling in hepatocellular carcinoma. Cancer Res. 2022;82:3845–57.
27. Jin H, Shi Y, Lv Y, Yuan S, Ramirez CFA, Lieftink C, et al. EGFR activation limits the response of liver cancer to lenvatinib. Nature. 2021;595(7869):730–4.
28. Sun D, Liu J, Wang Y, Dong J. Co-administration of MDR1 and BCRP or EGFR/PI3K inhibitors overcomes lenvatinib resistance in hepatocellular carcinoma. Front Oncol. 2022;12:944537.
29. Kaneda MM, Messer KS, Ralainirina N, Li H, Leem CJ, Gorjestani S, et al. PI3Kγ is a molecular switch that controls immune suppression. Nature. 2016;539(7629):437–42.
30. Rocher C, Singla DK. SMAD-PI3K-Akt-mTOR pathway mediates BMP-7 polarization of monocytes into M2 macrophages. PLoS One. 2013;8(12):e84009.
31. Kaneda MM, Cappello P, Nguyen AV, Ralainirina N, Hardamon CR, Foubert P, et al. Macrophage PI3Kγ drives pancreatic ductal adenocarcinoma progression. Cancer Discov. 2016;6(8):870–85.
32. Namani A, Li Y, Wang XJ, Tang X. Modulation of NRF2 signaling pathway by nuclear receptors: implications for cancer. Biochim Biophys Acta. 2014;1843(9):1875–85.
33. Wang X-J, Sun Z, Villeneuve NF, Zhang S, Zhao F, Li Y, et al. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. Carcinogenesis. 2008;29(6):1235–43.
34. Wang L, Zhang L, Zhao L, Shao S, Ning Q, Jing X, et al. VEGFA/NRP-1/GAPVD1 axis promotes progression and cancer stemness of triple-negative breast cancer by enhancing tumor cell-macrophage crosstalk. Int J Biol Sci. 2024;20(2):446–63.
35. Feng R, Morine Y, Ikemoto T, Imura S, Iwahashi S, Saito Y, et al. Nrf2 activation drive macrophages polarization and cancer cell epithelial-mesenchymal transition during interaction. Cell Commun Signal. 2018;16(1):54.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Waki Y, Morine Y, Saito Y, Teraoku H, Yamada S, Ikemoto T, et al. Lenvatinib-resistant hepatocellular carcinoma promotes malignant potential of tumor-associated macrophages via exosomal miR-301a-3p. Ann Gastroenterol Surg. 2024;8:1084–1095. https://doi.org/10.1002/ags3.12814