Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Check for updates

Human umbilical cord mesenchymal stem cells-derived exosomal microRNA-451a represses epithelial–mesenchymal transition of hepatocellular carcinoma cells by inhibiting ADAM10

Yunxiuxiu Xu[a,b*], Yu Lai[b,c*], Linhui Cao[b,d*], Yanshan Li[e], Guangcheng Chen[b,c], Liang Chen[b,f], Hanqin Weng[g], Tao Chen[a,b], Lingyun Wang[b,c] and Yibiao Ye[a,b]

[a]Department of Hepato-Billiary Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China; [b]Key Laboratory of Malignant Tumor Gene Regulation and Target Therapy of Guangdong Higher Education Institutes, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China; [c]Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China; [d]Department of Traditional Chinese Medicine, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China; [e]Department of Blood Transfusion, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China; [f]Department of Intensive Care Unit, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China; [g]Department of Hepato-Billiary Surgery, Dongguan people's Hospital, Southern Medical University, Guangdong, China

ABSTRACT

Exosomes derived from human umbilical cord mesenchymal stem cells (hucMSCs) expressing microRNAs (miRNAs) have been highlighted in human cancers. However, the detailed molecular mechanism of hucMSCs-derived exosomal miR-451a on hepatocellular carcinoma (HCC) remains further investigation. Our study aims to explore the impact of exosomal miR-451a on the progression of HCC. Expression of miR-451a and a disintegrin and metalloprotease 10 (ADAM10) in HCC tissues and adjacent normal tissues were determined. The exosomes were extracted from hucMSCs and co-cultured with Hep3B and SMMC-7721 cell lines. After the treatment of relative exosomes or exosome inhibitor GW4869 in Hep3B and SMMC-7721 cells, the paclitaxel resistance and malignant phenotypes of HCC cells were measured. Moreover, the effect of hucMSCs-derived exosomes on the expression of miR-451a and ADAM10 in HCC cells was assessed. The targeting relationship between miR-451a and ADAM10 was verified to detect the impact of ADAM10-wild type and ADAM10-mutant type (MUT) on HCC cell processes. Low expression of miR-451a and high expression of ADAM10 indicated a poor prognosis of HCC patients. MiR-451a was up-regulated while ADAM10 was down-regulated in HCC cells after co-culture with HucMSC-derived exosomes. The exosomes elevated miR-451a and inhibited ADAM10 to suppress the paclitaxel resistance, cell cycle transition, proliferation, migration and invasion, and promote apoptosis of HCC cells. ADAM10 was verified to be a target gene of miR-451a. ADAM10-MUT promoted HCC process independent of miR-451a mimic. HucMSC-derived exosomal miR-451a could restrict the epithelial–mesenchymal transition of HCC cells by targeting ADAM10, which might provide new targets for HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is a kind of complex disease with multiple aetiological factors and is the 2nd leading reason for cancer-correlated death all over the world due to multi-focal recurrence [1]. Viral hepatitis B and C infection, chronic alcohol consumption and exposure to aflatoxin B1 are reported to be the main risk factors of HCC [2]. Surgical resection and liver transplantation are the major treatments for HCC patients. Nevertheless, resection is only available for about 20% of the HCC patients [3]. Although the diagnosis and therapy for different stages of HCC have been developed, the overall prognosis of HCC is still unsatisfactory for the reason that there are still 70%-80% of HCC patients diagnosed at the advanced stage due to the inapparent clinical symptoms at early stages [4]. Therefore, novel biomarkers remain to be studied for the further development of strategies on diagnosis and treatment for HCC patients.

The microRNA (miRNA) functions as a modulatory role in gene expression through binding to the 3′-untranslated region (3′-UTR) of their target mRNAs [5]. It has been demonstrated that there are some miRNAs implicated in HCC progression, including miR-2053 [6], miR-548b [7] and miR-466 [8]. MiR-451 is one of the most conservative miRNAs with an essential clinical application value [9]. The evident difference of miR-451 expression has been reported in various cancers, such as renal cell carcinoma [10] and lung cancer [11]. Importantly, the inhibitive effects of miR-451 on HCC progression have also been demonstrated before [12,13]. Furthermore, a disintegrin and metalloprotease (ADAM) could potentially regulate a variety of signalling pathways in tumourthe micro-environment, such as inflammation reaction, angiogenesis,

cell migration and proliferation. As a vital member of the ADAM family, ADAM10 is one of the membrane-anchored ADAM group [14] and its role in HCC has been uncovered by several extant literatures [15,16]. Cheng et al. [17] have demonstrated that overexpression of both miR-144/451 suppresses ADAM10 protein levels in Alzheimer disease. However, Zhang et al. [18] have revealed that only miR-451 but not miR-144 is a negative regulator of the ADAM10 protein in breast cancer and head and neck squamous cell carcinoma (HNSCC) cells. MiR-451 guides the cleavage of endogenous ADAM10 mRNA. Therefore, we only chose miR-451a as the target miRNA regulating ADAM10.

Stem cells are a kind of cells with self-renewal and multi-directional differentiation potential, and mesenchymal stem cells (MSCs) derive from mesoderm and refer to one of the most critical sources of adult stem cells (ASCs). MSCs can proliferate and differentiate into cartilage, bone, tendon, muscle, fat, ligament and other tissues, with weak immunogenicity [19], which are also considered as ideal seed cells for tissue engineering [20]. Human umbilical cord mesenchymal stem cells (hucMSCs) are MSCs isolated from the Wharton's jelly of the umbilical cord and are rich in sources and easy to collect with low immunogenicity and high proliferation and differentiation potential [21–23]. It has also been found that hucMSCs promotes apoptosis of ovarian cancer cells and osteosarcoma cells [24]. Yuan et al. [25] have found that hucMSCs may inhibit the malignant biological behaviours of human lung cancer and hepatocellular cancer cells *in vitro* by activating cell apoptosis and inhibiting the Wnt signalling. Thus, we chose hucMSCs for our experiment. Moreover, exosomes are the smallest extracellular vesicles with a diameter of 30–100 nm and density of 1.13–1.19 g/mL that could be secreted by almost all cells. Exosomes contain mRNA and short RNAs including miRNAs [26]. Hence, exosomes could transfer miRNAs to exert regulatory functions in human diseases. Exosomes can be manufactured in culture by incorporating therapeutic miRNAs into exosome-producing cells, thereby possibly enabling personalized treatment [27]. Among the cell types known to produce exosomes, MSCs are the most prolific producers [28]. Infusion of human MSCs-derived exosomes into an immunocompetent mouse model of acute myocardial ischaemia has been shown to be therapeutically effective and lacking of evident adverse effects [29]. It has been reported that serum-derived exosomes from HCC patients regulate the expression of miR-451a [30], while the role of hucMSC-derived exosomal miR-451a in HCC remains scarcely explored. Based on the above data, we investigated whether the exosome-mediated transfer of miR-451 via miRNA-modified hucMSCs could regulate the biological functions of HCC cells.

This research was aimed to verify the effects of exosomal miR-451a and ADAM10 on the progression of HCC and we supposed that the exosomal miR-451a could act as a tumour repressor in HCC progression by targeting ADAM10.

Materials and methods

Ethics statement

Written informed consents were acquired from all patients before this study. The protocol of this study was confirmed by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University and was in compliance with the guidelines of the *Declaration of Helsink*i.

Study subjects

Ninety-two HCC patients (59 males and 33 females ageing 38–71 years, the mean age of 58 years) that had accepted treatment in Sun Yat-sen Memorial Hospital, Sun Yat-sen University from December 2012 to November 2013 were collected. These patients were all diagnosed as primary HCC by pathological examination and imageological examination. Before HCC resection operation, none patients were treated with any anti-tumour treatments, and there were complete clinical and follow-up data. Patients with secondary metastatic HCC, history of liver transplantation, other malignancies, or serious infection and women during pregnancy or lactation were excluded. Among these patients, there were 36 cases of high differentiation, 41 cases of moderate differentiation and 15 cases of poor differentiation. In tumour size, 54 patients had tumour diameter equal to or more than 5 cm and 38 patients had tumour diameter less than 5 cm. There were 50 patients with portal vein invasion and 42 patients without portal vein invasion. During the resection operation, cancer tissues and adjacent normal tissues (over 3 cm away from tumourthe) from HCC patients were separated. After the operation, patients were followed-up for 60 months, until 30 November 2018.

Immunohistochemical staining

HCC cancer tissues and adjacent normal tissues that obtained from the operation were fixed in formalin for 24 h, embedded by paraffin and sectioned. Dewaxed by xylene and hydrated by gradient ethanol, the sections were added with citric acid antigen retrieval solution for antigen retrieval, blocked by 3% hydrogen peroxide for 10 min and appended with diluted primary antibodies N-cadherin (1:300) and E-cadherin (1:500, both from Abcam Inc., Cambridge, MA, USA) and then incubated for 1 h. Next, the sections were rinsed by phosphate-buffered saline (PBS), added with relative secondary antibody, and incubated for 30 min, then developed by diaminobenzidine (DAB) and stained by haematoxylin. After differentiated by 0.1% hydrochloric acid solution, the sections were dehydrated by gradient ethanol and xylene, sealed by neutral balsam and observed by a microscope. PBS was taken as the negative control (NC), replacing the primary antibody and the results were photographed and observed under a microscope. In the evaluation of E-cadherin and N-cadherin expression, brown-yellow staining in the cytomembrane and/or cytoplasm were considered positive for expression [31].

HucMSC culture

The umbilical cord that aseptically collected from caesarean delivered full-term foetus was washed by PBS containing 3% penicillin-streptomycin for three times with vein, artery and membrane removed, cut into 1 mm^3 and seeded into culture flasks. Subsequently, the cord was incubated in Dulbecco's modified Eagle medium (DMEM)/F12 containing 10% foetal bovine serum (FBS) at 37°C with 5% CO_2 and the medium was changed every 3 d. After cultured for 10 d, there were fibrous cells grew out from the tissues, which were detached and passaged by 0.25% trypsin when the cell confluence reached 80%. Cells from the 3rd to the 6th passage ingood growth were selected for the experiments.

Identification of hucMSC phenotype

Morphology and adherence of hucMSCs were observed under a microscope and hucMSC was identified by flow cytometry: hucMSC in the 3rd passage were subpackaged into flow tubes, with 2×10^5 cells in each tube, and, respectively, supplemented with CD29-phycoerythrin (PE), CD44-PE, CD73-PE, CD90-PE, CD105-PE, CD31-fluorescein isothiocyanate (FITC), CD14-FITC and HLA-DR-FITC antibodies and IgG-PE and IgG-FITC were used as isotype controls (all from Abcam), then incubated at 4°C without light exposure for 30 min and measured by a flow cytometer (Becton, Dickinson and Company, NJ, USA).

Extraction of exosomes

The exosomes were extracted by differential-ultra centrifugation [32]. HucMSCs from the 3rd to the 6th passage that in good growth was paved in a 10 cm culture dish and cultured with DMEM/F12 overnight. Next, the cells became adherent and the medium was replaced by fresh exosome-free serum medium. After 2-d culture, the cells were centrifuged at $2000 \times g$ and 4°C for 10 min to remove dead cells and large fragments and the supernatant was centrifuged at $10,000 \times g$ and 4°C for 30 min to remove organelles and granules. Next, the supernatant was ultra-centrifuged at $110,000 \times g$ and 4°C for 70 min to remove the supernatant. After rinsed by normal saline, the cells were ultra-centrifuged at $110,000 \times g$ and 4°C for 70 min and the sediments were exosomes.

Identification of exosomes

The marker proteins of exosomes were measured by Western blot analysis: the sediments were lysed by protein lysis buffer and the protein concentration was evaluated. Next, the proteins (20 μg) were appended with $5 \times$ sodium dodecyl sulphate (SDS) loading buffer, heated at 99°C in the metal bath for 15 min and centrifuged at $12,000 \times g$ for 5 min, then conducted with SDS-polyacrylamide gel electrophoresis (PAGE), followed by transfer onto membranes. Blocked by 5% skim milk for 1 h, the membranes were supplemented with primary antibody CD63 (1:2,000), Alix (1:500) and TSG101 (1:500, all from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and incubated

overnight at 4°C. Afterwards, the membranes were added with secondary antibodies (1:200, Santa Cruz Biotechnology) for 1.5-h incubation, appended with enhanced chemiluminescent (ECL) solution (Sigma–Aldrich Chemical Company, St Louis, MO, USA) and photographed by the chemiluminescent gel-imaging system.

Morphology of exosomes was observed by a transmission electronic microscope (TEM): the exosomes (20 μL) were supplemented into a copper network (diameter of 2 mm) and placed for 5 min, then negatively stained by uranyl acetate for 2 min. After dried under an incandescent lamp, the exosomes were photographed by a TEM (Hitachi, Ltd., Tokyo, Japan).

Nanoparticle tracking analysis (NTA): the exosome suspension (0.1 mL) that resuspended by PBS was diluted by PBS at 4°C (1:10) and placed in a cuvette, which was put into the particle size analyser, thereby the particle size of the exosomes was determined.

Transfection and grouping of hucMSCs

According to lipofectamine^{TM} 2000 reagent (Invitrogen Inc., CA, USA), hucMSCs in 3rd to 6th passages were transfected with miR-451a mimic (oligonucleotide sequence: 5′-AAACCGUUACCAUUACUGAGUU-3′), mimic NC (5′-UUGUACUACACAAAAGUACUG-3′), miR-451a inhibitor (oligonucleotide sequence: 5′-AACUCAGUAAUGGUAACGGUUU-3′), miR-451a inhibitor NC (5′-CAGUACUUUUGUGUAGUACAA-3′), miR-451a mimic + overexpressed (Oe)-NC (NC of ADAM10 overexpressed plasmid), miR-451a mimic + Oe-ADAM10 (ADAM10 overexpressed plasmid), miR-451a inhibitor + short hairpin RNA (sh)-NC (NC of ADAM10 down-regulated plasmid and its sequence was 5′-TTCTCCGAACGTGTCACGTTTC-3′) and miR-451a inhibitor + sh-ADAM10 (ADAM10 down-regulated plasmid and its sequence was 5′-GGTCTCATGTACCTCCCAAAG-3′). The above oligonucleotides and plasmids were all designed and constructed by GenePharma Co., Ltd. (Shanghai, China). Cultured for 48 h, the hucMSCs was performed with ultra-centrifugation (the ultracentrifuge was obtained from Thermo Fisher Scientific Inc., Waltham, MA, USA) to extract the exosomes in hucMSC of each group.

HCC cell culture

Human normal liver cell line L-02 and human HCC cell lines Hep3B, MHCC-97 H, HB611, HepG2 and SMMC-7721 that obtained from American type culture collection (Rockefeller, MD, USA) were cultured in DMEM containing 10% FBS (both from Gibco, Grand Island, NY, USA) at 37°C with 5% CO_2. The medium was changed every 2 d and the cells were passaged when cell confluence reached 80%-90%. After 2–3 passages, cells in the logarithmic growth phase were conducted with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to assess miR-451a and ADAM10 expression in each cell line. Moreover, Western blot analysis was employed to measure the protein expression of ADAM10 in the cells. Cells with the largest and least differences of miR-

451a and ADAM10 expression from the L-02 cells were selected for the subsequent experiments.

Exosome uptake experiment

Hep3B and SMMC-7721 cells were seeded onto 24-well plates at 3×10^4 cells/well and hucMSC-derived exosomes (hucMSC-Exo, 80 µg/mL) that had been stained by PKH26 were added into the plates [33]. The cell slides were fixed in 4% paraformaldehyde for 20 min at 0 h and 48 h of the co-culture, which were then stained by 4′,6-diamidino-2-phenylindole 2hci (DAPI) for 1 h, sealed with anti-fluorescence quenching reagent and photographed under a laser confocal microscope (Olympus, Tokyo, Japan).

Co-culture of hucMSC-Exo with Hep3B and SMMC-7721 cells

To observe the effect of hucMSC-Exo on HCC cells, we divided Hep3B and SMMC-7721 cells into the control group (no treatment), the Exo group (cells was co-incubated with 200 µg hucMSC-Exo for 48 h) and the Exo + GW4869 group (cells were co-incubated with hucMSC-Exo and 5 µM exosome inhibitor GW4869 [exosome inhibitor, HY-19,363, MCE, Monmouth Junction, NJ, USA]).

Next, to further observe the impact of hucMSC-Exo carrying miR-451a on Hep3B cells, the cells were classified into the mimic-NC-Exo group (hucMSC-Exo that had been transfected with miR-451a mimic NC were co-cultured with Hep3B cells), the miR-451a mimic-Exo group (hucMSC-Exo that had been transfected with miR-451a mimic were co-cultured with Hep3B cells), the sh-NC-Exo group (hucMSC-Exo that had been transfected with sh-ADAM10 NC was co-cultured with Hep3B cells), the sh-ADAM10-Exo group (hucMSC-Exo that had been transfected with sh-ADAM10 were co-cultured with Hep3B cells), the miR-451a mimic + Oe-NC-Exo group (hucMSC-Exo that had been transfected with miR-451a mimic and NC of Oe-ADAM10 were co-cultured with Hep3B cells), and the miR-451a mimic + oe-ADAM10-Exo group (hucMSC-Exo that had been transfected with miR-451a mimic and overexpressed ADAM10 were co-cultured with Hep3B cells).

In order to explore the impact of hucMSC-Exo carrying miR-451a on SMMC-7721 cells, the cells were separated into the inhibitor-NC-Exo group (hucMSC-Exo that had been transfected with miR-451a inhibitor NC were co-cultured with SMMC-7721 cells), the miR-451a inhibitor-Exo group (hucMSC-Exo that had been transfected with miR-451a inhibitor were co-cultured with SMMC-7721 cells), the Oe-NC-Exo group (hucMSC-Exo that had been transfected with NC of overexpressed ADAM10 were co-cultured with SMMC-7721 cells), the Oe-ADAM10-Exo group (hucMSC-Exo that had been transfected with ADAM10 overexpressed plasmids were co-cultured with SMMC-7721 cells), the miR-451a inhibitor + sh-NC-Exo group (hucMSC-Exo that had been transfected with miR-451a inhibitor and sh-ADAM10 NC was co-cultured with SMMC-7721 cells), the miR-451a inhibitor + sh-ADAM10-Exo group (hucMSC-Exo that had been transfected with miR-451a inhibitor and sh-ADAM10 were co-cultured with SMMC-7721 cells).

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay

Hep3B and SMMC-7721 cells were detached by 0.25% trypsin when the cell confluence reached 80% and then were seeded onto 24-well plates at 5.0×10^3 cells/well. After incubated for 24 h, the cells were incubated with 50 mL of 1 mg/mL MTT (Sigma) in PBS for 3 h. The formed formazan was then solubilized by adding 150 mL of dimethyl sulphoxide. The optical density value was read at 570 nm with a FLUOstar Galaxy plate reader (BMGLabtech, Offenburg, Germany).

Propidium iodide (PI) single staining

Hep3B and SMMC-7721 cells of each group were seeded onto 12-well plates, incubated by serum-free medium for 12 h after adherence, then detached by trypsin. Next, the cells were fixed by 70% ethanol overnight, incubated by RNase A (prepared by PBS containing 0.2% Triton-X100, with the final concentration of 20 mg/L) at 37°C for 30 min and added with PI (50 mg/L) for 5 min, then evaluated by a flow cytometer. A total of 20,000 cells were analysed by ModFit software (Verity Software House, ME, USA).

Annexin V-FITC/PI double staining

Hep3B and SMMC-7721 cells were detached by trypsin when cell confluence reached 80% and was seeded onto 6-well plates at 4×10^4 cells/well for 72-h culture, then centrifuged at 1,000 r/min for 5 min with supernatant discarded. Afterwards, the cells were rinsed by pre-cooled PBS twice, centrifuged with supernatant discarded and suspended by 5,000 µL binding buffer, then appended with Annexin V-FITC and PI, each for 5 µL. The blank group and single staining control group were set. After 10-min incubation without light exposure, the cells were measured by a flow cytometer.

Transwell assay

Cell migration assay: Transwell chambers (Millipore Inc., MA, USA) with polycarbonate membrane (aperture of 0.8 µm) were put in 24-well plates, Hep3B and SMMC-7721 cell suspension (3×10^5 cells/mL) was appended into the apical chambers at 200 µL/well and 0.5% bovine serum albumin (BSA) was also added into the apical chambers. The basolateral chambers were supplemented with medium containing 10% serum at 600 µL/well and incubated at 37°C with 5% CO_2 for 24 h and the apical chambers were fixed in 4% paraformaldehyde for 20 min; then, stained by 0.1% crystal violet dye for 30 min. Five fields of view were randomly photographed under a microscope. The cells were counted by ImageJ software.

Cell invasion assay: Transwell chambers coated by matrigel were put into 24-well plates. Hep3B and SMMC-7721 cell suspension (3×10^5 cells/mL) was appended into the apical chambers at 200 µL/well and the basolateral chambers were supplemented with medium containing 10% serum at 600 µL/well and incubated at 37°C with 5% CO_2 for 48 h. Subsequently, the apical chambers were fixed by 4%

paraformaldehyde, dried, stained by crystal violet dye and decolourized. The cells were photographed and counted.

Effect of miR-451a and ADAM10 on paclitaxel sensitivity

Hep3B and SMMC-7721 cells were transiently transfected for 24 h with miR-451a mimic (or its NC), miR-451a inhibitor (or its NC), miR-451a mimic + Oe-NC, miR-451a mimic + Oe-ADAM10, miR-451a inhibitor + sh-NC or miR-451a inhibitor + sh-ADAM10. Paclitaxel (0, 5, 10 and 20 nM) was then added to the transfected cells and reacted for 72 h. Cell viability was measured using MTT assay as described above and the survival rate was calculated.

Hep3B and SMMC-7721 cells were treated with 20 nM paclitaxel for 72 h after incubation with an equal number of exosomes for 48 h. Cell viability was measured using MTT assay as described above and the survival rate was calculated.

RT-qPCR

Total RNA from tissues, cells and exosomes was isolated using TRIzol extractions (Invitrogen). RNA concentration was estimated using a Nanodrop spectrophotometer and RNA quality was examined using denaturing formaldehyde agarose gel electrophoresis. For miRNA, RNA was reversely transcribed to synthesize cDNA using Hairpin-it^{TM} miRNAs quantitative detection kit (GenePharma; in this kit, there was a specific miRNA reverse transcription primer with stem-loop structure, which could combine with the 3′UTR of miRNA so that the reverse transcription could avoid the interference of miRNA precursor). The mRNA was performed with RNA reverse transcription by GoldScript one-step RT-PCR Kit (Applied Biosystems, USA) to synthesize the cDNA. SYBR premix Ex Taq^{TM} II PCR Kit (TaKaRa Biotechnology Co., Ltd., Liaoning, China) was used to conduct PCR on the ABI7500 fluorescent quantitative PCR. The PCR primers (Table 1) were designed and synthesized by GenePharma. U6 was used as the loading control of miR-451a, miR-144-3p and miR-144-5p and β-actin was used as the loading control of ADAM10. The data were analysed by $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

The total protein of the tissues, cells and exosomes were extracted in strict accordance with efficient radio-immunoprecipitation assay (RIPA) lysis buffer (R0010, Solarbio Science & Technology Co. (Beijing, China)). The protein concentration was evaluated by bicinchoninic acid assay and the proteins were supplemented with prepared SDS-PAGE loading buffer, conducted with electrophoresis and transferred onto the polyvinylidene fluoride membranes. The membranes were blocked by skim milk for 2 h, added with diluted primary antibodies against ADAM10 (1:300; Sangon Biotech, Shanghai, China), N-cadherin, E-cadherin and β-actin (all 1:1,000 and from Abcam Inc., Cambridge, UK) and incubated at 4°C overnight. Subsequently, the membranes were supplemented with horseradish peroxidase-labelled secondary antibody (1:2,000, Abcam) for 2-h incubation. ECL reagent (Thermo Scientific, Rockford, IL, USA) was used for development. The relative protein levels were determined by the density of Western blot bands as measured by Image J software and normalized against the internal control β-actin.

Dual-luciferase reporter gene assay

A bioinformatics software (http://www.microrna.org/microrna/home.do) was used to predict the potential binding sites between ADAM10 and miR-451a. To further identify the targeting relationship between miR-451a and ADAM10, a sequence containing binding sites of miR-451a in ADAM10 3′UTR was amplified and cloned to pmirGLO luciferase plasmid (Promega, WI, USA) to construct wild type (WT) ADAM10 recombinant plasmid (ADAM10-WT). Site mutation kits (TaKaRa) were used to establish mutant type (MUT) ADAM10 recombinant plasmid (ADAM10-MUT) by mutating the binding site of ADAM10 and miR-451a (plasmids were designed and synthesized by TaKaRa). Cultured Hep3B and SMMC-7721 cells were seeded onto 24-well plates and transfected with ADAM10-WT or ADAM10-MUT reporter plasmid together with miR-451a mimic and mimic NC based on instructions of Lipidosome 2000 reagent

Table 1. Primer sequence.

Gene	Primer
miR-451a	Reverse transcription: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTCA-3′ F: 5′-CGCGCAAACCGTTACCATTAC-3′ R: 5′-GTGCAGGGTCCGAGGT-3′
miR-144-3p	Reverse transcription: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTACA-3′ F: 5′-GCGCGCTACAGTATAGATGA-3′ R: 5′-GTGCAGGGTCCGAGGT-3′
miR-144-5p	Reverse transcription: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTACA-3′ F: 5′-GCGCGCGGATATCATCATATAC-3′ R: 5′-GTGCAGGGTCCGAGGT-3′
U6	Reverse transcription: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGG-3′ F: 5′-GCAAATTCGTGAAGCGTT-3′ R: 5′-GTGCAGGGTCCGAGGT-3′
ADAM10	F: 5′-GCTGATGAGAAGGACCCTACAA-3′ R: 5′-ATGCTCCAACCCAAGCCAGAC-3′
β-actin	F: 5′-AGCGAGTATCCCCCAAAGTT-3′ R: 5′-GGGCACGAAGGCTCATCATT-3′

Note: F, forward; R, reverse; miR-451a, microRNA-451a; miR-144-3p, microRNA-144-3p; miR-144-5p, microRNA-144-5p; ADAM10, a disintegrin and metalloprotease 10.

(Invitrogen) when the cell confluence reached 85%. After 48-h transfection, the Dual-Luciferase® Reporter Assay System (Promega) was used to determine the luciferase activity of ADAM10-WT or ADAM10-MUT reporter plasmid. ADAM10 gene ID: 102. ADAM10-WT sequence: 5′-uuaUCAAAAUAUUUAAACGGUUu-3′, ADAM10-MUT sequence: 5′-uuaUCAAAAUAUUUAAACGGUUu-3′.

Statistical analysis

All data analyses were conducted using SPSS 21.0 software (IBM Corp. Armonk, NY, USA). The measurement data conforming to the normal distribution were expressed as mean ± standard deviation. The unpaired t-test was performed for comparisons between two groups and one-way analysis of variance (ANOVA) was used for comparisons among multiple groups and the Tukey's multiple comparison test was used for pairwise comparisons after one-way ANOVA. Pearson test was used to analyse the relationship between expression of miR-451a and ADAM10 in HCC tissues. Chi–square test was employed to identify the correlations of miR-451a and ADAM10 with clinicopathological features of the HCC patients and the prognosis of HCC patients was analysed by Kaplan-Meier. P value <0.05 was indicative of a statistically significant difference.

Results

Expression of miR-451a, ADAM10, E-cadherin and N-cadherin in HCC patients; association between miR-451a/ADAM10 expression and clinicopathological characteristics and prognosis of HCC patients

In order to observe the expression of ADAM10, miR-451a and its corresponding gene cluster miR-144 in HCC cancer tissues, RT-qPCR and Western blot analysis was used to determine the expression of miR-451a, miR-144-3p, miR-
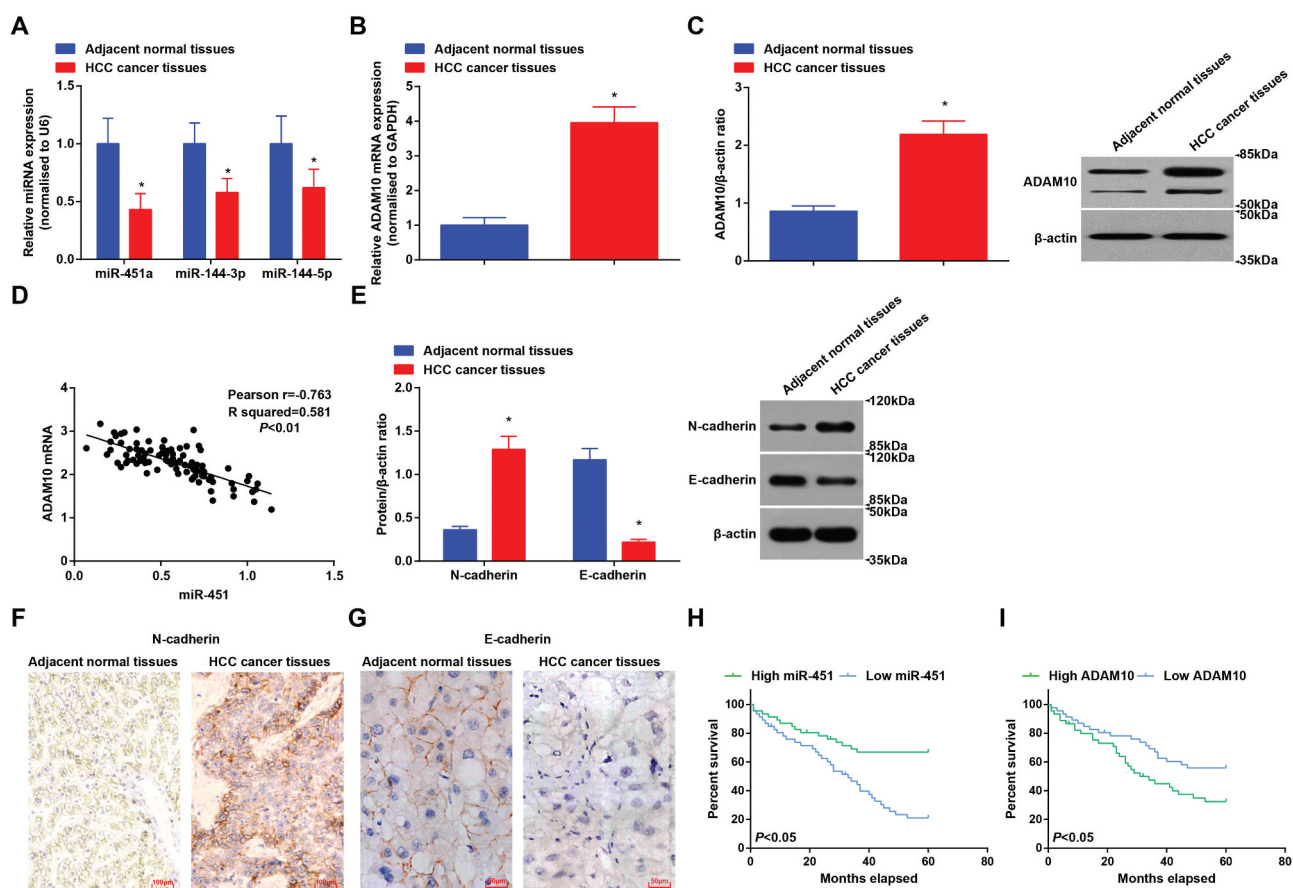

Figure 1. Expression of miR-451a, ADAM10, E-cadherin and N-cadherin in HCC patients, association between miR-451a/ADAM10 expression and clinicopathological characteristics and prognosis of HCC patients. A, expression of miR-451a, miR-144-3p and miR-144-5p in HCC cancer tissues and adjacent normal tissues detected by RT-qPCR; B, ADAM10 mRNA expression in HCC cancer tissues and adjacent normal tissues detected by RT-qPCR; C, protein expression of ADAM10 in HCC cancer tissues and adjacent normal tissues detected by Western blot analysis; D, relation between miR-451a and ADAM10 in HCC tissues analysed by Pearson test; E, protein expression of N-cadherin and E-cadherin in HCC cancer tissues and adjacent normal tissues detected by Western blot analysis; F, N-cadherin level in HCC cancer tissues and adjacent normal tissues was assessed by immunohistochemical staining; G, E-cadherin level in HCC cancer tissues and adjacent normal tissues was assessed by immunohistochemical staining; H, effect of miR-451a on prognosis of HCC patients analysed by Kaplan-Meier; I, effect of ADAM10 on prognosis of HCC patients analysed by Kaplan-Meier; n = 92. The measurement data were expressed as mean ± standard deviation, * P< 0.05 *vs* the adjacent normal tissues. HCC, hepatocellular cancer; miR-451a, microRNA-451a; ADAM10, a disintegrin and metalloprotease 10.

144-5p and ADAM10 in HCC cancer and adjacent normal tissues. It could be found that relative to the adjacent normal tissues, miR-451a, miR-144-3p and miR-144-5p were down-regulated and ADAM10 was up-regulated in HCC cancer tissues (all $P < 0.05$). Among the three down-regulated miRNAs, the expression of miR-451a was the lowest (Fig. 1A, B, C).

Pearson test was employed to analyse the relationship between expression of miR-451a and ADAM10 and it was found that miR-451a was negatively related to ADAM10 ($r = -0.763$; $P < 0.01$; Fig. 1D).

To investigate the expression and distribution of epithelial–mesenchymal transition (EMT)-related proteins, Western blot analysis was used to detect the protein expression of N-cadherin and E-cadherin in HCC cancer tissues and adjacent normal tissues. The outcomes suggested that in contrast to the adjacent normal tissues, protein expression of N-cadherin was elevated and that of E-cadherin was reduced in HCC cancer tissues (both $P < 0.05$, Fig. 1E). Expression of N-cadherin and E-cadherin in HCC cancer tissues and adjacent normal tissues were assessed by immunohistochemical staining, it came out that the brownish-yellow particles of N-cadherin were thinly distributed in the cytoplasm of adjacent normal tissues, and broadly distributed in the cytoplasm of HCC cancer tissues (Fig. 1F); as for E-cadherin, its positive expression was commonly distributed in cytomembrane of adjacent normal tissues, while scarcely distributed in HCC cancer tissues (Fig. 1G).

With the aim to observe the relation between miR-451a/ADAM10 expression and clinicopathological characteristics of HCC patients, the 92 patients were classified into the miR-451a low expression group (n = 46) and the miR-451a high expression group (n = 46) based upon the median miR-451a

expression level. According to the median ADAM10 mRNA expression, the 92 patients were divided into the ADAM10 mRNA low expression group (n = 46) and the ADAM10 mRNA high expression group (n = 46). The results indicated that patients with larger tumour diameter, no tumour capsule, advanced TNM stage, high differentiation and portal vein injury had lower expression of miR-451a and higher expression of ADAM10, suggesting that expression of miR-451a and ADAM10 was correlated to tumour diameter, tumour capsule, TNM stage, tumour differentiation and portal vein injury of HCC patients (all $P < 0.05$), while was not associated with age, gender and hepatitis B surface antigen (HBsAg) of HCC patients (all $P > 0.05$, Table 2).

Kaplan–Meier method was used to analyse the effect of miR-451a/ADAM10 expression on prognosis of HCC patients, and we found that there existed a significant difference in survival time of HCC patients between the miR-451a low expression group and the miR-451a high expression group, and also between the ADAM10 mRNA low expression group and the ADAM10 mRNA high expression group (both $P < 0.05$). Namely, lower miR-451a expression or higher ADAM10 mRNA expression indicated a poor prognosis of HCC patients (Fig. 1H, I).

Identification of hucMSCs and exosomes

In order to identify the extracted hucMSCs, hucMSCs was cultured by 10% exo-free DMEM/F12 containing FBS, and it was found that the cells were in good morphology after adherence under a microscope, and the cells were fibroblast-like and in even long fusiform, and grew in whirl manner with abundant cytoplasm, clear nuclei and fewer impurity. Cells in other morphology could not be observed (Fig. 2A). Surface

Table 2. The relation between miR-451a/ADAM10 expression and clinicopathological characteristics of HCC patients.

Clinicopathological characteristics	n	miR-451a expression		P	ADAM10 mRNA expression		P
		Low (n = 46)	High (n = 46)		Low (n = 46)	High (n = 46)	
Age (year)							
≥ 56	62	29	33	0.374	34	28	0.182
< 56	30	17	13		12	18	
Gender							
Male	59	32	27	0.277	32	27	0.277
Female	33	14	19		14	19	
HBsAg							
Negative	29	16	13	0.501	13	16	0.501
Positive	63	30	33		33	30	
Tumour diameter							
< 5 cm	37	11	26	0.001	13	24	0.019
≥ 5 cm	55	35	20		33	22	
Tumour capsule							
Yes	38	13	25	0.011	26	12	0.003
No	54	33	21		20	34	
TNM stage							
I–II	42	16	26	0.036	28	14	0.003
III–IV	50	30	20		18	32	
Differentiation							
Poor	36	11	25	0.007	25	11	0.003
Moderate	41	24	17		18	23	
High	15	11	4		3	12	
Portal vein injury							
Yes	50	30	20	0.036	19	31	0.012
No	42	16	26		27	15	

Note: HCC, hepatocellular carcinoma; miR-451a, microRNA-451a; ADAM10, a disintegrin and metalloprotease 10; HBsAg, hepatitis B surface antigen; TNM, tumour, node and metastasis. Data in this table are enumeration data and analysed using chi-square test.
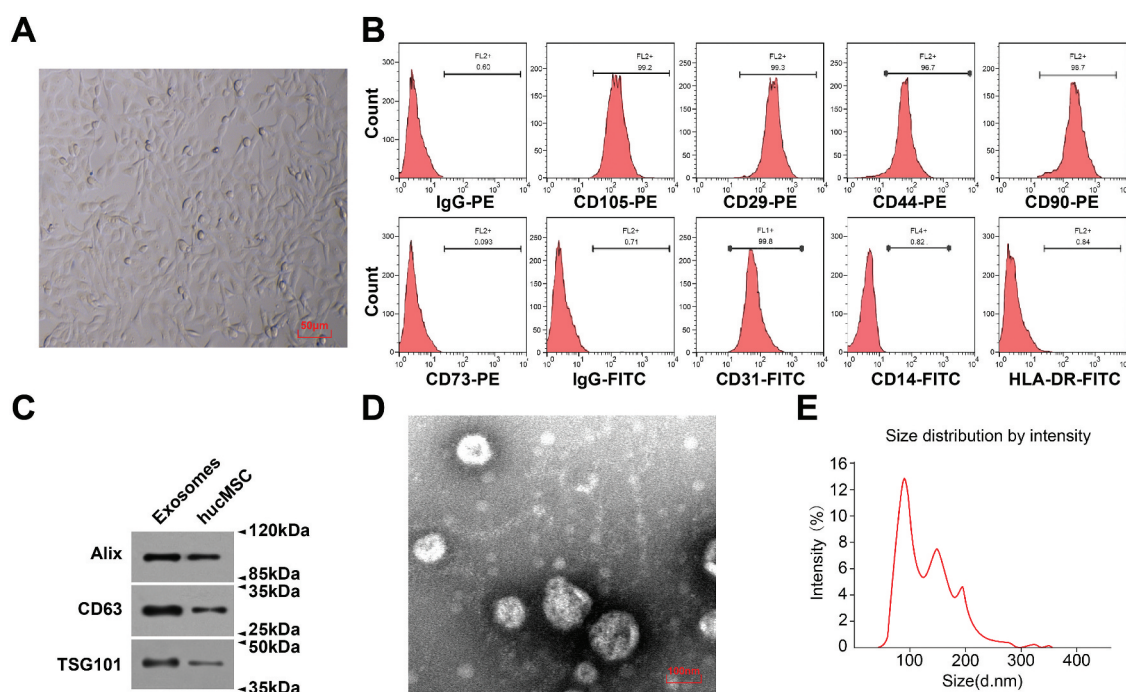
Figure 2. Identification of hucMSCs and exosomes. A, morphological observation of hucMSCs; B, surface markers of hucMSCs were detected by flow cytometry; C, surface markers of hucMSC-Exo were detected by Western blot analysis; D, morphology of hucMSC-Exo was observed by a TEM; E, NTA of hucMSC-Exo; N = 3. hucMSCs, human umbilical cord mesenchymal stem cells; Exo, exosome; TEM, transmission electronic microscope; NTA, nanoparticle tracking analysis.

markers of the cells were evaluated by flow cytometry, and the results indicated that CD29, CD44, CD73, CD90 and CD105 in cells were positively expressed, while CD14, CD31 and HLA-DR were negatively expressed (Fig. 2B).

To identify the exosomes, hucMSC culture solution was collected to extract the hucMSC-Exo using the differential-ultracentrifugation method. HucMSC-Exo was identified by TEM observation, NTA and Western blot analysis. It was measured by Western blot analysis that the surface marker Alix, CD63 and TSG101 of hucMSC-Exo were positively expressed (Fig. 2C). As observed by a TEM, there were circular or oval membranous vesicles in a different size, and membranous structure could be found around the vesicles, in which there were low-density substances with obvious heterogeneity (Fig. 2D). Furthermore, NTA reflected that the diameter of exosomes was between 80 and 150 nm, and mainly concentrated at 94 nm (Fig. 2E).

Exosome uptake and expression of miR-451a and ADAM10 in HCC cells and exosomes; upregulated miR-451a or downregulated ADAM10 reduces paclitaxel resistance in HCC cells

Expression of miR-451a and ADAM10 in L-02, Hep3B, MHCC-97 H, HepG2 and SMMC-7721 cells was evaluated by RT-qPCR and Western blot analysis to screen the target HCC cell lines. The outcomes indicated that in comparison to L-02 cells, the level of miR-451a was declined and ADAM10 was amplified in HCC cell lines (all $P < 0.05$). Among the HCC cell lines, miR-451a expression was the lowest and ADAM10 expression was the highest in Hep3B cells, which had the maximum difference

from L-02 cells; miR-451a expression was the highest and ADAM10 expression was the lowest in SMMC-7721 cells, which had the minimum difference from L-02 cells (Fig. 3A, B). Therefore, Hep3B and SMMC-7721 cells were selected for the subsequent experiments.

In order to observe that whether hucMSC-Exo could enter Hep3B and SMMC-7721 cells, hucMSC-Exo pre-stained by PKH26 (80 μg/mL) were co-cultured for 0 h and 24 h with Hep3B and SMMC-7721 cells in a 24-well plate. The results observed by a fluorescence microscope indicated that at the 0 h of co-transfection, the exosomes did not enter Hep3B and SMMC-7721 cells, and there were few cells, after co-cultured for 24 h, exosomes entered Hep3B and SMMC-7721 cells, and gathered along the nuclear membranes. Red fluorescence could be found around the nuclei (Fig. 3C).

Exosomes were extracted from hucMSCs after relative transfection and the expression of miR-451a and ADAM10 in hucMSC-Exo was determined using RT-qPCR. We discovered that miR-451a mimic up-regulated miR-451a while down-regulated ADAM10 in hucMSC-Exo; miR-451a inhibitor had an opposite effect on miR-451a and ADAM10 expression in hucMSC-Exo; Oe-ADAM10 plasmid overexpressed ADAM10 while sh-ADAM10 suppressed ADAM10 expression in hucMSC-Exo; Oe-ADAM10 reversed the effect of miR-451a mimic on ADAM10 expression, and sh-ADAM10 also reversed the impact of miR-451a inhibitor on ADAM10 expression (Fig. 3D, E).

In order to observe the effect of altered miR-451a and ADAM10 expression on paclitaxel resistance of HCC cell lines, MTT assay was conducted. We found that in Hep3B cells that had been treated with different concentrations of
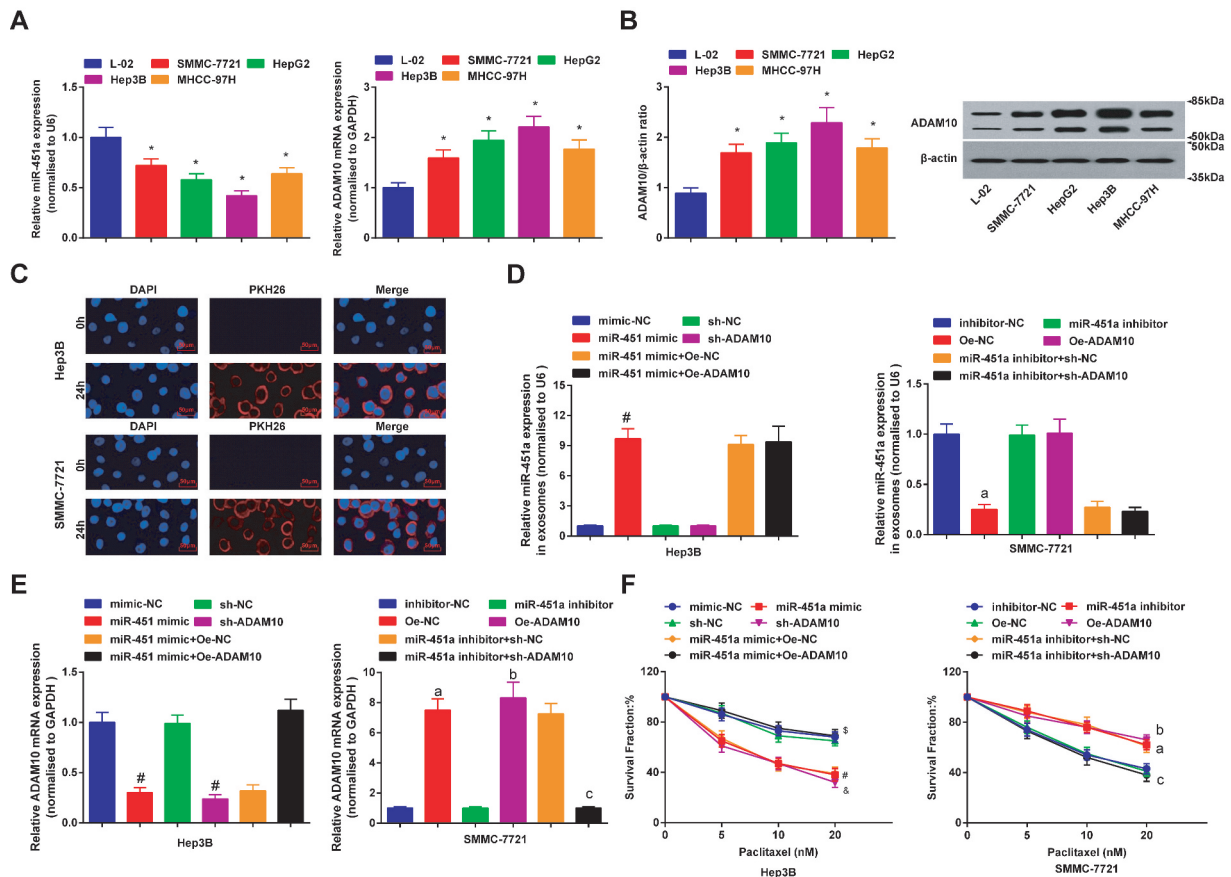
Figure 3. Exosome uptake and expression of miR-451a and ADAM10 in HCC cells and exosomes; upregulated miR-451a or downregulated ADAM10 reduces paclitaxel resistance in HCC cells. A, expression of miR-451a and ADAM10 in cells of each group was detected by RT-qPCR; B, protein expression of ADAM10 in cells of each group was detected by Western blot analysis; C, uptake of hucMSC-Exo in Hep3B and SMMC-7721 cells; D, expression of miR-451a in hucMSC-Exo was assessed using RT-qPCR; E, expression of ADAM10 in hucMSC-Exo was assessed using RT-qPCR; F, survival rate of Hep3B and SMMC-7721 cells under different concentrations of paclitaxel was estimated by MTT assay; N = 3. The measurement data were expressed as mean ± standard deviation, * $P < 0.05$ *vs* the L-02 cells, # $P < 0.05$ *vs* the mimic-NC group, & $P < 0.05$ *vs* the sh-NC group; $ $P < 0.05$ *vs* the miR-451a mimic + Oe-NC group, a $P < 0.05$ *vs* the inhibitor-NC group, b $P < 0.05$ *vs* the Oe-NC group, c $P < 0.05$ *vs* the miR-451a inhibitor + sh-NC group. HCC, hepatocellular cancer; miR-451a, microRNA-451a; ADAM10, a disintegrin and metalloprotease 10; hucMSCs, human umbilical cord mesenchymal stem cells; Exo, exosome; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.

paclitaxel, miR-451a mimic or sh-ADAM10 suppressed the survival rate of Hep3B cells; Oe-ADAM10 reversed the effect of miR-451a mimic on the survival rate of Hep3B cells. In SMMC-7721 cells that had been treated with different concentrations of paclitaxel, miR-451a inhibitor or Oe-ADAM10 promoted the survival rate of SMMC-7721 cells; sh-ADAM10 eliminated the impact of miR-451a inhibitor on SMMC-7721 cell survival. The falling range of cell survival rate increased with the paclitaxel concentration grew (Fig. 3F).

HucMSC-Exo up-regulates miR-451a, down-regulates ADAM10, promotes apoptosis and suppresses paclitaxel resistance, proliferation and cell cycle progression of HCC cells

Expression of miR-451a and ADAM10 in HCC cells was assessed using RT-qPCR and Western blot analysis after co-transfection of hucMSC-Exo and HCC cells. We discovered that in Hep3B and SMMC-7721 cells, treatment of hucMSC-Exo up-regulated miR-451a while down-regulated ADAM10; treatment of GW4869 reversed the

effect of hucMSC-Exo on miR-451a and ADAM10 expression (both $P < 0.05$ (Fig. 4A, B).

To measure the impact of hucMSC-Exo on paclitaxel resistance and biological functions of HCC cells, MTT assay was performed to detect the survival rate and viability of Hep3B and SMMC-7721 cells under 20 nM paclitaxel, and flow cytometry was employed to assess the cell cycle distribution and apoptosis. The results reflected that the treatment of hucMSC-Exo promoted apoptosis and suppressed paclitaxel resistance, proliferation and cell cycle progression of HCC cells; GW4869 reversed the impact of hucMSC-Exo on paclitaxel resistance, proliferation, cell cycle distribution and apoptosis of HCC cells (all $P < 0.05$; Fig. 4C, D, E, F).

HucMSC-Exo represses migration, invasion and EMT of HCC cells

Transwell assay was used to detect migration and invasion abilities of HCC cells, and Western blot analysis was conducted to assess EMT-related protein expression to determine the role of hucMSC-Exo in migration, invasion and
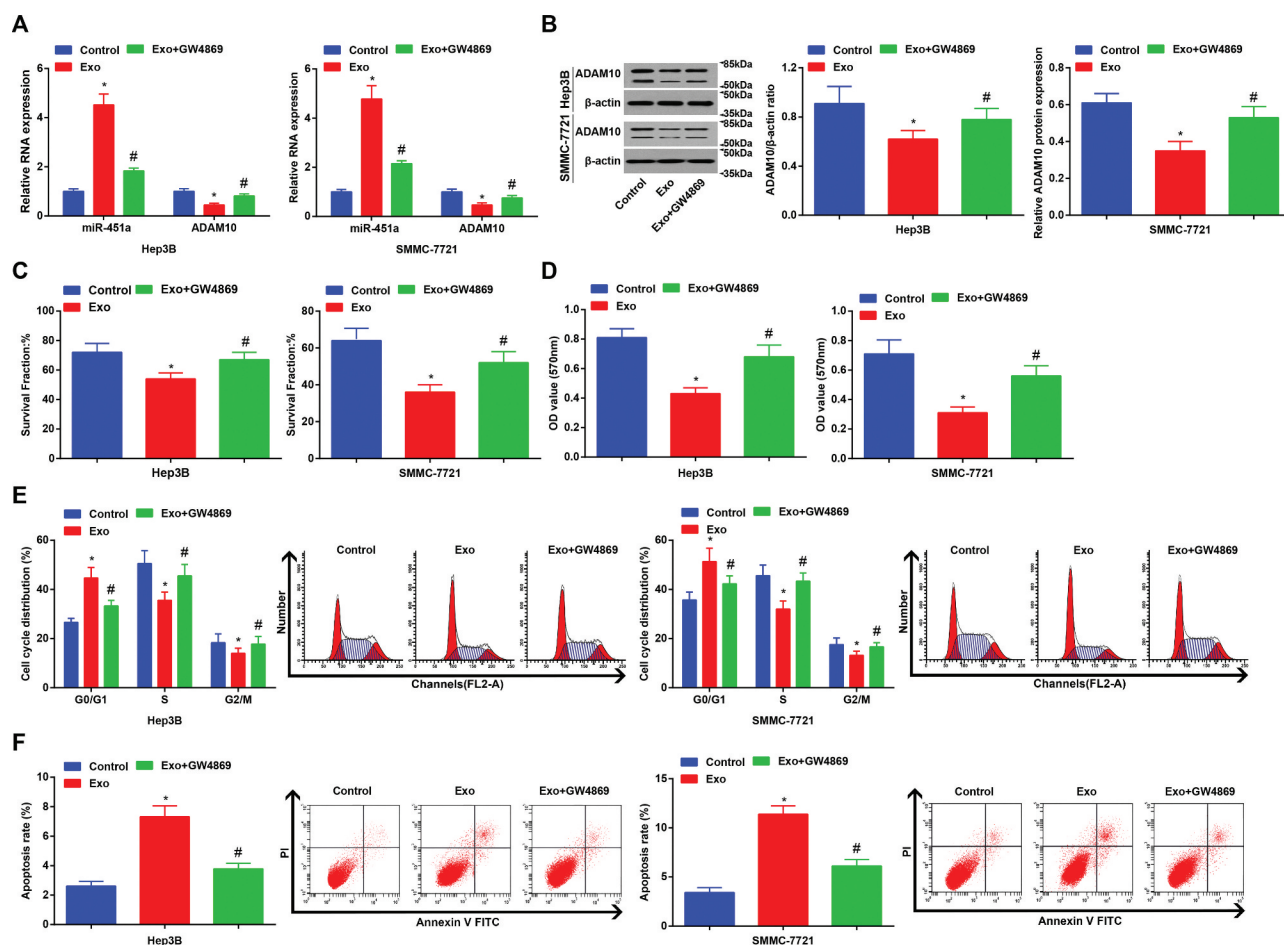
Figure 4. HucMSC-Exo up-regulates miR-451a, down-regulates ADAM10, promotes apoptosis and suppresses paclitaxel resistance, proliferation and cell cycle progression of HCC cells. A, expression of miR-451a and ADAM10 in Hep3B and SMMC-7721 cells after exosome treatment was determined using RT-qPCR; B, protein expression of ADAM10 in Hep3B and SMMC-7721 cells after exosome treatment was determined using Western blot analysis; C, survival rate of paclitaxel-treated Hep3B and SMMC-7721 cells after exosome treatment was evaluated by MTT assay; D, viability of Hep3B and SMMC-7721 cells after exosome treatment was evaluated by MTT assay; E, cell cycle distribution of Hep3B and SMMC-7721 cells after exosome treatment was evaluated by flow cytometry; F, apoptosis of Hep3B and SMMC-7721 cells after exosome treatment was evaluated by flow cytometry; N = 3. The measurement data were expressed as mean ± standard deviation; * $P < 0.05$ vs the control group, # $P < 0.05$ vs the Exo group. HCC, hepatocellular cancer; miR-451a, microRNA-451a; ADAM10, a disintegrin and metalloprotease 10; hucMSCs, human umbilical cord mesenchymal stem cells; Exo, exosome; GW4869, exosome inhibitor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.

EMT of HCC cells. Our results showed that in Hep3B and SMMC-7721 cells, hucMSC-Exo restricted migration and invasion abilities and N-cadherin expression, whereas promoted E-cadherin expression; treatment of GW4869 promoted migration, invasion and EMT of HCC cells (all $P < 0.05$ (Fig. 5A, B, C, D)).

HucMSC-Exo overexpressing miR-451a or silencing ADAM10 accelerates apoptosis and constrains paclitaxel resistance, proliferation and cell cycle progression of HCC cells

To assess the role of hucMSC-Exo expressing miR-451a or ADAM10 in paclitaxel resistance and biological functions of HCC cells, MTT assay was used to detect survival rate and viability, and flow cytometry was used to measure the cell cycle arrest and apoptosis of HCC cells under 20 nM paclitaxel. It was found that in Hep3B cells, hucMSC-Exo up-regulating miR-451a or down-regulating ADAM10 suppressed survival rate, viability and cell cycle progression, and

promoted apoptosis of HCC cells; hucMSC-Exo overexpressing ADAM10 abolished these effect induced by hucMSC-Exo up-regulating miR-451a. In SMMC-7721 cells, hucMSC-Exo down-regulating miR-451a or up-regulating ADAM10 promoted survival rate, viability and cell cycle progression, and restrained apoptosis of HCC cells; hucMSC-Exo inhibiting ADAM10 reversed these effects induced by hucMSC-Exo down-regulating miR-451a (all $P < 0.05$; Fig. 6A, B, C, D).

HucMSC-Exo overexpressing miR-451a or silencing ADAM10 restricts migration, invasion and EMT of HCC cells

Transwell assay was performed to determine the effect of hucMSC-Exo expressing miR-451a or ADAM10 on migration and invasion abilities of HCC cells, and the expression of N-cadherin and E-cadherin was evaluated using Western blot analysis. It came out that in Hep3B cells, hucMSC-Exo overexpressing miR-451a or silencing ADAM10 suppressed migration, invasion and N-cadherin expression, but enhanced
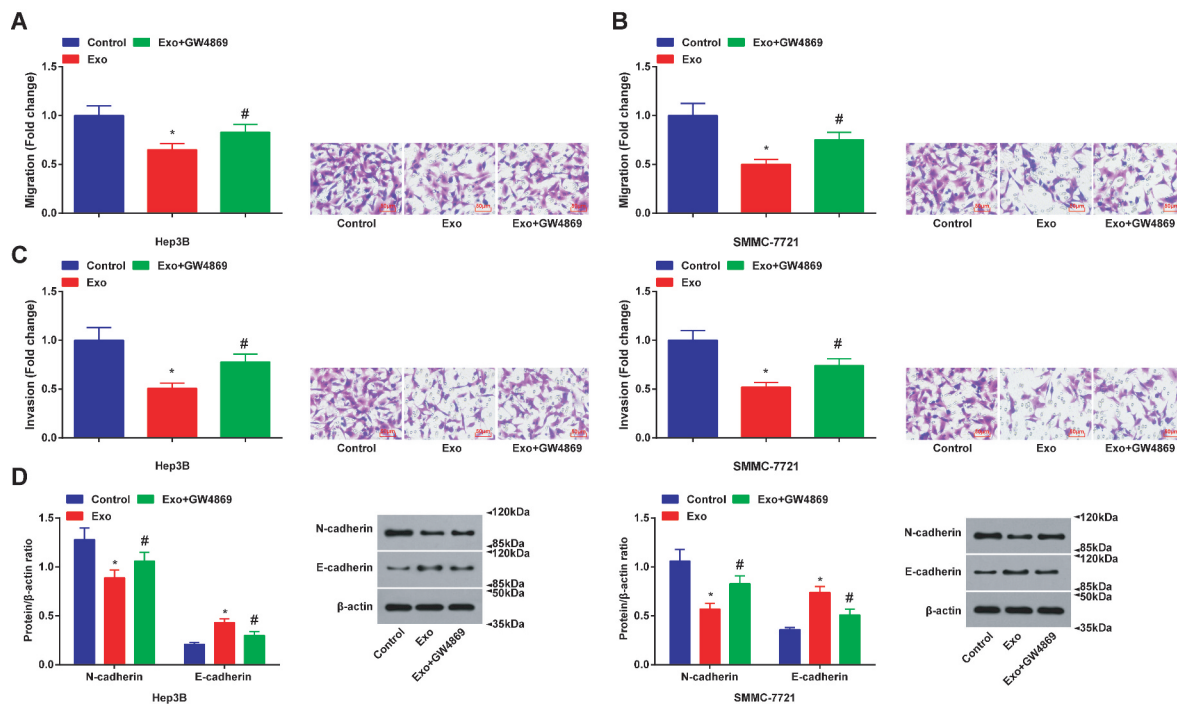
Figure 5. HucMSC-Exo represses migration, invasion and EMT of HCC cells. A&B, membrane-based Transwell assay was conducted to measure the migration ability of Hep3B and SMMC-7721 cells after exosome treatment; C, Matrigel based Transwell assay was conducted to measure the invasion ability of Hep3B and SMMC-7721 cells after exosome treatment; D, protein expression of N-cadherin and E-cadherin in Hep3B and SMMC-7721 cells after exosome treatment was determined by Western blot analysis; N = 3. The measurement data were expressed as mean ± standard deviation; * $P < 0.05$ *vs* the control group, # $P < 0.05$ *vs* the Exo group. HCC, hepatocellular cancer; hucMSCs, human umbilical cord mesenchymal stem cells; Exo, exosome; EMT, epithelial-mesenchymal transition.

E-cadherin expression; effect of hucMSC-Exo up-regulating miR-451a was reversed by hucMSC-Exo overexpressing ADAM10. In SMMC-7721 cells, hucMSC-Exo down-regulating miR-451a or up-regulating ADAM10 promoted migration, invasion and N-cadherin expression, while inhibited E-cadherin expression; the impact of hucMSC-Exo down-regulating miR-451a could be reversed by hucMSC-Exo silencing ADAM10 (all $P < 0.05$; Fig. 7A, B, C).

MiR-451a targets ADAM10, and ADAM10-MUT promoted the occurrence and development of HCC independent of miR-451a mimic

In order to assess the role of hucMSC-Exo intervening miR-451a or ADAM10 in the expression of miR-451a and ADAM10 in HCC cells, we used RT-qPCR and Western blot analysis to determine the miR-451a and ADAM10 expression in HCC cells. It was observed that in Hep3B cells, hucMSC-Exo overexpressing miR-451a or silencing ADAM10 down-regulated ADAM10; hucMSC-Exo overexpressing ADAM10 rescued the down-regulation of ADAM10 induced by hucMSC-Exo up-regulating miR-451a. In SMMC-7721 cells, hucMSC-Exo down-regulating miR-451a or up-regulating ADAM10 promoted ADAM10 expression; hucMSC-Exo inhibiting ADAM10 reversed the impact of hucMSC-Exo down-regulating miR-451a on ADAM10 expression in SMMC-7721 cells (Fig. 8A, B, C).

An online analysis software (http://www.microrna.org/microrna/home.do) was used to detect the targeting relationship

between miR-451a and ADAM10, and it was found that there existed a particular binding region between miR-451a gene sequence and ADAM10 sequence (Fig. 8D). It was further confirmed by dual-luciferase reporter gene assay that in Hep3B and SMMC-7721 cells, the co-transfection of ADAM10-WT and miR-451a mimic suppressed the luciferase activity ($P < 0.05$); while the co-transfection of ADAM10-MUT and miR-451a mimic did not affect the luciferase activity in Hep3B and SMMC-7721 cells ($P > 0.05$; Fig. 8E). These data indicated that miR-451a mimic particularly bound to ADAM10.

In order to reveal the effect of miR-451a mimic on ADAM10 mutant, the established ADAM10-WT and ADAM10-MUT plasmids were co-transfected with miR-451a mimic into Hep3B cells to perform the migration, invasion and apoptosis assays. The results of our experiments implied that versus the miR-451a mimic + ADAM10-WT group, the migration and invasion abilities were facilitated and the number of apoptotic cells was declined in the miR-451a mimic + ADAM10-MUT group (all $P < 0.05$) revealing the promotive role of ADAM10-MUT in HCC occurrence and development independent of miR-451a mimic (Fig. 8F, G, H).

Discussion

HCC is one of the major malignancies all over the world with a high prevalence, which leads to a significant rate of death as well [34]. It has been unravelled that the aberrant expression of miRNAs is correlated to the procedures and tumorigenesis of human cancers [35]. Our research was conducted to
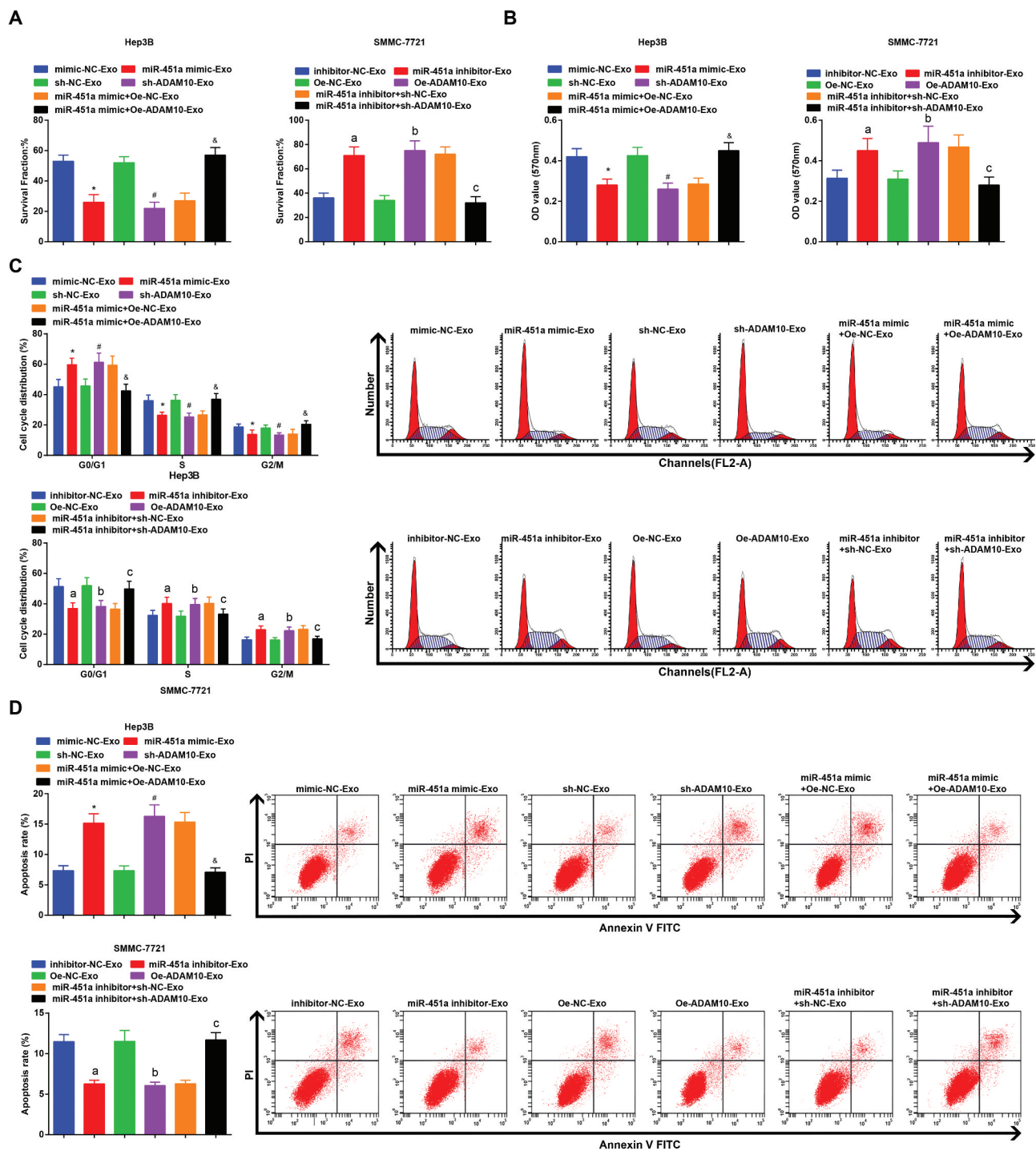
Figure 6. HucMSC-Exo overexpressing miR-451a or silencing ADAM10 accelerate apoptosis and constrain paclitaxel resistance, proliferation and cell cycle progression of HCC cells. A, survival rate of paclitaxel-treated Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was evaluated by MTT assay; B, viability of paclitaxel-treated Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was evaluated by MTT assay; C, cell cycle distribution of Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was evaluated by flow cytometry; D, apoptosis of Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was evaluated by flow cytometry. N = 3. The measurement data were expressed as mean ± standard deviation. In Hep3B cells, * $P < 0.05$ *vs* the mimic-NC-Exo group, # $P < 0.05$ *vs* the sh-NC-Exo group, & $P < 0.05$ *vs* the miR-451a mimic + oe-NC-Exo group; in SMMC-7721 cells, a $P < 0.05$ *vs* the inhibitor-NC-Exo group, b $P < 0.05$ *vs* the oe-NC-Exo group, c $P < 0.05$ *vs* the miR-451a inhibitor + sh-NC-Exo group. HCC, hepatocellular cancer; miR-451a, microRNA-451a; ADAM10, a disintegrin and metalloprotease 10; hucMSCs, human umbilical cord mesenchymal stem cells; Exo, exosome; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.
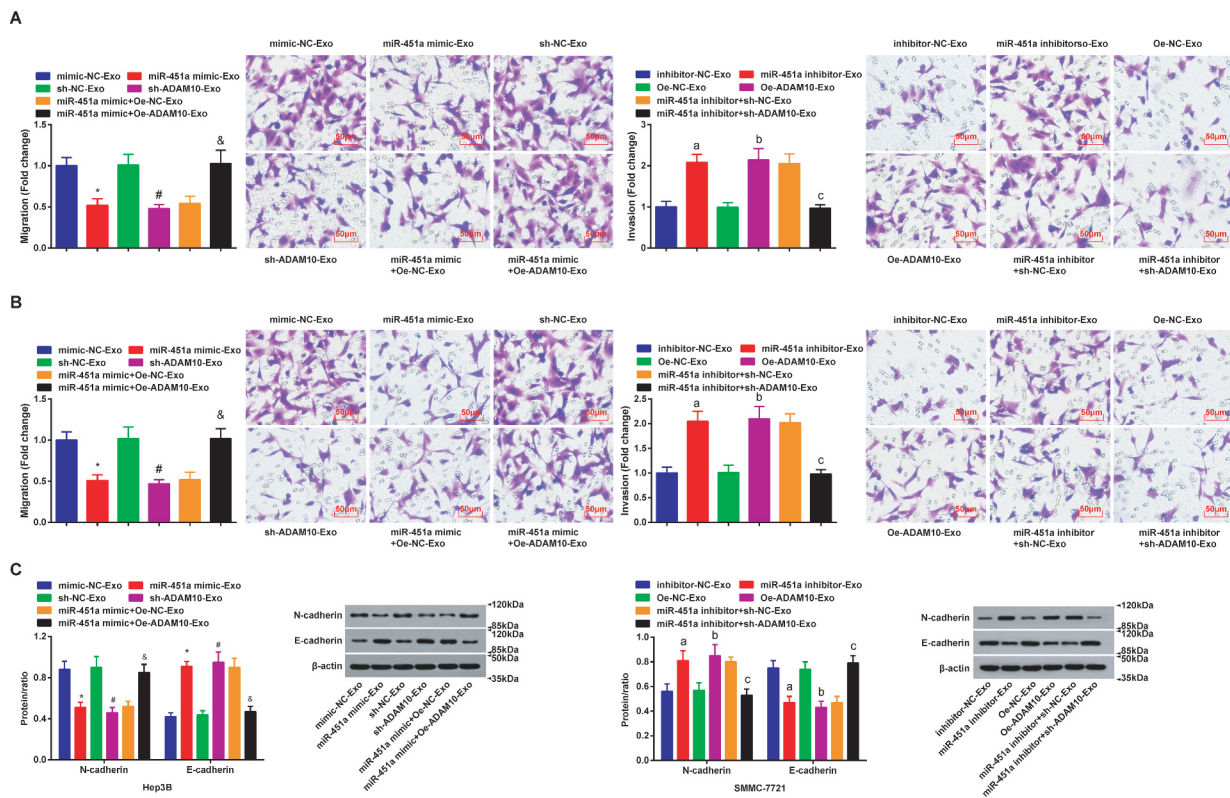
Figure 7. HucMSC-Exo overexpressing miR-451a or silencing ADAM10 restrict migration, invasion and EMT of HCC cells. A, migration ability of Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was assessed by membrane-based Transwell assay; B, invasion ability of Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was assessed by Matrigel based Transwell assay; C, protein expression of N-cadherin and E-cadherin in Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was determined by Western blot analysis; N = 3. The measurement data were expressed as mean ± standard deviation. In Hep3B cells, * $P < 0.05$ vs the mimic-NC-Exo group, # $P < 0.05$ vs the sh-NC-Exo group, & $P < 0.05$ vs the miR-451a mimic + oe-NC-Exo group; in SMMC-7721 cells, a $P < 0.05$ vs the inhibitor-NC-Exo group, b $P < 0.05$ vs the oe-NC-Exo group, c $P < 0.05$ vs the miR-451a inhibitor + sh-NC-Exo group. HCC, hepatocellular cancer; miR-451a, microRNA-451a; ADAM10, a disintegrin and metalloprotease 10; hucMSCs, human umbilical cord mesenchymal stem cells; Exo, exosome, EMT, epithelial-mesenchymal transition.

explore the impacts of hucMSC-derived exosomal miR-451a and its target gene ADAM10 in HCC development, and we found that miR-451a together with ADAM10 played a predictive role in HCC prognosis, and the elevation of exosomal miR-451a could inhibit EMT in HCC cells by modulatingADAM10 expression, thus exerting a repressive effect on HCC.

We have concluded several results in our study, and one of them implied that the expression of miR-451a was decreased while the expression of ADAM10 was increased in HCC tissues and cell lines, respectively, in contrast to the adjacent normal tissues and human normal liver cell line L-02. According to previous studies, Lv et al. have found that the level of miR-451 is downregulated in HCC tissues [13]. ADAM10 has been found to be expressed in all prostate tumour samples [36], and its expression is up-regulated by androgen stimulation. Additionally, a recent study has provided evidence that ADAM10 is up-regulated in both HCC tissues and cells [15], and it has been revealed by Wu et al. that the expression of ADAM10 is upregulated in HCC tissues, which was relative to non-tumourous liver tissues [16]. Another result of our research suggested that the expression of miR-451 and ADAM10 was related to tumour diameter, tumour capsule, TNM stage, tumour differentiation, and

portal vein injury of HCC patients. Similar to this outcome, a previous document has discovered that the expression of miR-451 is correlated to histological differentiation degree and TNM stage of patients with bladder cancer [37]. Wang et al. have pointed out that the expression of ADAM10 is associated with tumour size and TNM stage of patients with gastric cancer [38]. What is more, we have found that the low expression of miR-451 together with the high expression of ADAM10 indicates a poor prognosis of HCC patients. Consistent with this result, Ren et al. have proved from the reverse side that the expression of miR-451 is positively related to the prognosis of patients with gastric cancer [39], and it has also been revealed that the high expressed ADAM10 may be of predictive effect on HCC prognosis [40]. The targeting relationship between miR-451a and ADAM10 was confirmed by dual-luciferase reporter gene assay in our research, and it came out that ADAM10 was targeted by miR-451a. Zhang *et al.* [18] confirmed that miR-451 is a negative regulator of the ADAM10 protein in breast cancer and HNSCC cells. The target relation has also been identified in Alzheimer disease [17]. Furthermore, we used the Pearson test to detect the relation between miR-451a and ADAM10 mRNA in HCC tissues, and we found that miR-451a and ADAM10 were in a negative relation.
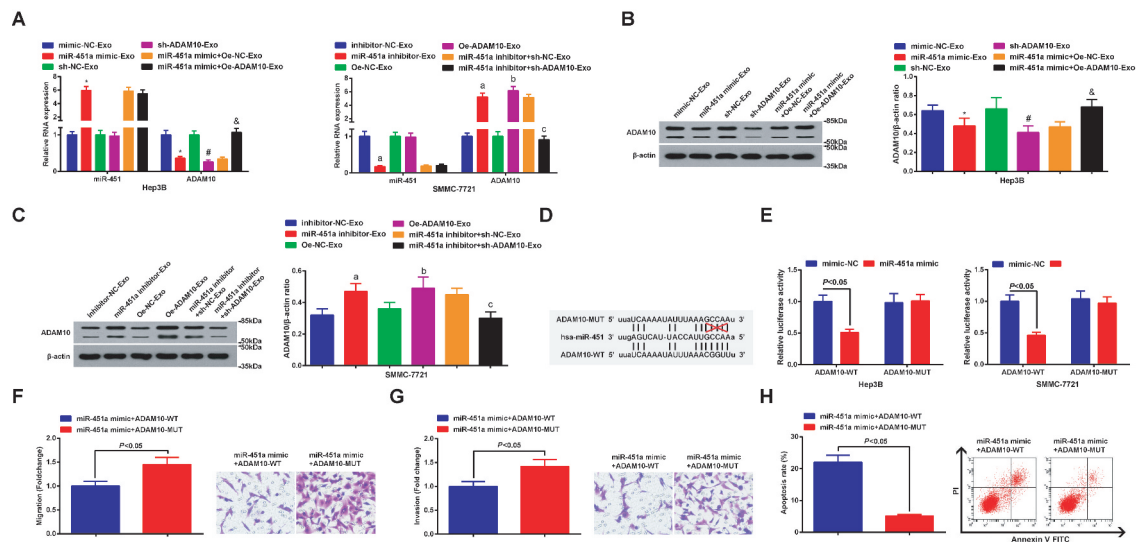
Figure 8. MiR-451a targets ADAM10. A, expression of miR-451a and ADAM10 in Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was assessed by RT-qPCR; B&C, protein expression of ADAM10 in Hep3B and SMMC-7721 cells with treatment of hucMSC-Exo intervening miR-451a or ADAM10 was assessed by Western blot analysis; D, particular binding region of miR-451a and ADAM10 was predicted by an online prediction software; E, regulatory relation between miR-451a and ADAM10 in Hep3B and SMMC-7721 cells was confirmed by dual-luciferase reporter gene assay; F, membrane-based Transwell assay was employed to detect Hep3B cell migration of each group; G, Matrigel based Transwell assay was used to detect Hep3B cell invasion of each group; H, apoptosis of Hep3B cells in each group was determined using flow cytometry; N = 3. The measurement data were expressed as mean ± standard deviation. In Hep3B cells, * $P < 0.05$ vs the mimic-NC-Exo group, # $P < 0.05$ vs the sh-NC-Exo group, & $P < 0.05$ vs the miR-451a mimic + oe-NC-Exo group; in SMMC-7721 cells, a $P < 0.05$ vs the inhibitor-NC-Exo group, b $P < 0.05$ vs the oe-NC-Exo group, c $P < 0.05$ vs the miR-451a inhibitor + sh-NC-Exo group. miR-451a, microRNA-451a; ADAM10, a disintegrin and metalloprotease 10; hucMSCs, human umbilical cord mesenchymal stem cells; Exo, exosome, RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
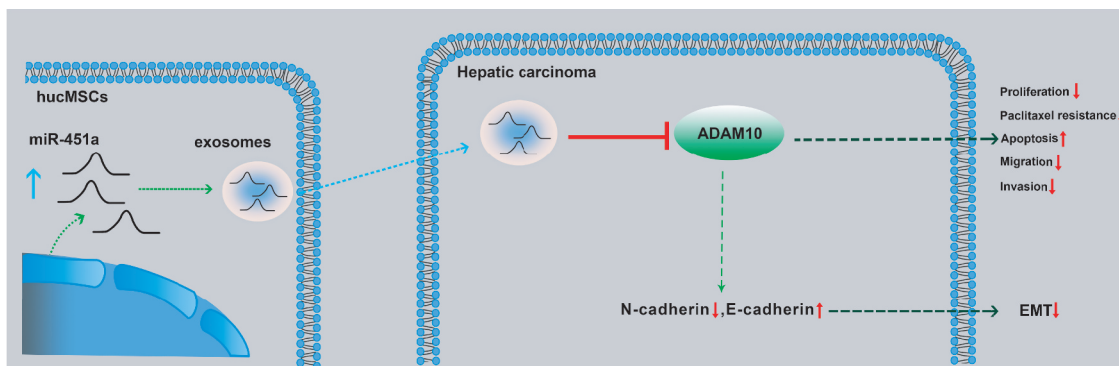

Figure 9. The mechanistic diagram depicts that hucMSC-derived exosomal miR-451a functions as a suppressor in HCC by delaying EMT progression via suppressing ADAM10. HCC, hepatocellular cancer; miR-451a, microRNA-451a; ADAM10, a disintegrin and metalloprotease 10; hucMSCs, human umbilical cord mesenchymal stem cells; EMT, epithelial-mesenchymal transition.

In addition, we have discovered that hucMSC-Exo up-regulated miR-451a and down-regulated ADAM10 to prohibit the proliferation, migration and invasion of HCC cells. Similar to this outcome, Li et al. have elucidated that hucMSC-Exo inhibits the proliferation and migration of endometrial cancer cells [41], Liu et al. have unravelled that miR-451a suppressed the proliferation and migration of non-small cell lung cancer cells [42], and the suppressive impact of overexpressed miR-451a in migration and invasion of bladder cancer cells has been uncovered by a recent study as well [43]. For ADAM10, Woods et al. have mentioned that the proliferation and invasion of pancreatic cancer cells are restricted by down-regulating the expression of ADAM10 [44], and a previous research has validated that the overexpression of ADAM10 is able to promote the proliferation, migration and invasion of HCC cells [40].

Moreover, our study has clarified that hucMSC-Exo up-regulating miR-451a or down-regulating ADAM10 accelerates the apoptosis of HCC cells and arrest HCC cells at the G0/G1 phase. In accordance with this finding, Yuan *et al.* have found that hucMSC is able to arrest tumour cells in particular phases and induces apoptosis of tumour cells [25]. It has been previously demonstrated that the overexpression of miR-451a has the ability to promote the apoptosis of osteosarcoma cells [45], and Li *et al.* have testified that the miR-451a mimics induced osteosarcoma cell arrest in G0/G1 phase [46]. Additionally, it has been unearthed that the down-regulation of ADAM10 induces tumour apoptosis in HCC *in vitr*o [47], and Armanious et al. have provided evidence that the reduction of ADAM10 reduces cell cycle arrest in mantle cell lymphoma [48]. Importantly, we have also identified that hucMSC-Exo

promoted miR-451a and repressed ADAM10 to inhibit EMT by restraining the expression of N-cadherin expression and promoting the expression of E-cadherin. In line with this outcome, an existing literature has revealed that hucMSC reduces EMT *in vivo* and *in vitro* [49], Huang et al. has reported that miR-451a serves as a suppressor of EMT in HCC [12], and the similar function of inhibited ADAM10 in human non-small cell lung carcinoma has been verified as well [50]. In addition, we validated that hucMSC-Exo up-regulating miR-451a or down-regulating ADAM10 reduced the paclitaxel resistance of HCC cells. It has been elucidated that miR-451 is decreased in paclitaxel-resistant breast cancer cells and is considered as a potential target in paclitaxel-resistant breast cancer treatment [51]. Furthermore, Yang et al. has mentioned that ADAM10 overexpression confers resistance to doxorubicin-induced apoptosis in HCC [52]. All these data provide a theoretical basis for investigation on hucMSC-derived exosomal miR-451a or ADAM10 in HCC.

In conclusion, this research suggests that hucMSC-derived exosomal miR-451a could function as a suppressor in HCC by delaying EMT progression via suppressing ADAM10 (Fig. 9), and miR-451a has a prognostic effect on HCC patients, which may contribute to the therapy and prognosis of HCC. However, the role of miR-451a targeting other genes during the development of HCC has not been further discussed in this research, and we would study the relative mechanisms in future works.

Acknowledgments

Disclosure statement

The authors declare that they have no conflicts of interest.

Funding

References

[1] Shimada S, Mogushi K, Akiyama Y, et al. Comprehensive molecular and immunological characterization of hepatocellular carcinoma. EBioMedicine. 2019;40:457–470.

[2] Li Y, Chen B, Yang X, et al. S100a8/a9 signaling causes mitochondrial dysfunction and cardiomyocyte death in response to Ischemic/Reperfusion injury. Circulation. 2019;140(9):751–764. .

[3] Liu Y, Sun L, Gao F, et al. A new scoring model predicting macroscopic vascular invasion of early-intermediate hepatocellular carcinoma. Medicine (Baltimore). 2018;97(49):e13536.

[4] Cheng S, Minshan C, Jianqiang C, et al. Chinese expert consensus on multidisciplinary diagnosis and treatment of hepatocellular carcinoma with portal vein tumor thrombus: 2016 edition. Oncotarget. 2017;8 (5):8867–8876.

[5] Zhu X, Zhang C, Fan Q, et al. Inhibiting microRNA-503 and microRNA-181d with Losartan Ameliorates diabetic nephropathy in KKAy Mice. Med Sci Monit. 2016;22:3902–3909.

[6] Song T, Ma K, Zhao C, et al. MicroRNA-2053 overexpression inhibits the development and progression of hepatocellular carcinoma. Oncol Lett. 2019;18(2):2043–2049.

[7] Yun Z, Meng F, Jiang P, et al. microRNA-548b suppresses aggressive phenotypes of hepatocellular carcinoma by directly targeting high-mobility group box 1 mRNA. Cancer Manag Res. 2019;11:5821–5834.

[8] Ai N, Li B, Li L, et al. MicroRNA-466 inhibits cancer cell migration and invasion in hepatocellular carcinoma by indirectly mediating the down-regulation of ROCK2. Exp Ther Med. 2019;18(2):1493–1499.

[9] Li Z, Li Y, Fu, J, et al. Clinical utility of microRNA-451 as diagnostic biomarker for human cancers. Biosci Rep. 2019;39(1): BSR20180653.

[10] Tang Y, Wan W, Wang L, et al. microRNA-451 inhibited cell proliferation, migration and invasion through regulation of MIF in renal cell carcinoma. Int J Clin Exp Pathol. 2015;8 (12):15611–15621.

[11] Cheng D, Xu Y, Sun C, et al. MicroRNA-451 sensitizes lung cancer cells to cisplatin through regulation of Mcl-1. Mol Cell Biochem. 2016;423(1–2):85–91. .

[12] Huang JY, Zhang K, Chen D-Q, et al. MicroRNA-451: epithelial-mesenchymal transition inhibitor and prognostic biomarker of hepatocellular carcinoma. Oncotarget. 2015;6(21):18613–18630. .

[13] Lv G, HU Z, TIE YI, et al. MicroRNA-451 regulates activating transcription factor 2 expression and inhibits liver cancer cell migration. Oncol Rep. 2014;32(3):1021–1028. .

[14] Huang J, Pan Y, Hu G, et al. SRC fine-tunes ADAM10 shedding activity to promote pituitary adenoma cell progression. Febs J. 2019;287(1):190-204.

[15] Gao J, Yin X, Yu X, et al. Long noncoding LINC01551 promotes hepatocellular carcinoma cell proliferation, migration, and invasion by acting as a competing endogenous RNA of microRNA-122-5p to regulate ADAM10 expression. J Cell Biochem. 2019;120(10):16393–16407.

[16] Wu G, Zheng K, Xia S, et al. MicroRNA-655-3p functions as a tumor suppressor by regulating ADAM10 and beta-catenin pathway in Hepatocellular Carcinoma. J Exp Clin Cancer Res. 2016;35(1):89. .

[17] Cheng C, Li W, Zhang Z, et al. MicroRNA-144 is regulated by activator protein-1 (AP-1) and decreases expression of Alzheimer disease-related a disintegrin and metalloprotease 10 (ADAM10). J Biol Chem. 2013;288(19):13748–13761. .

[18] Zhang J, Qin X, Sun Q, et al. Transcriptional control of PAX4-regulated miR-144/451 modulates metastasis by suppressing ADAMs expression. Oncogene. 2015;34(25):3283–3295.

[19] Uccelli A, Moretta L, Pistoia V. Immunoregulatory function of mesenchymal stem cells. Eur J Immunol. 2006;36 (10):2566–2573.

[20] Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005;105 (4):1815–1822.

[21] Zeddou M, Briquet A, Relic B, et al. The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. Cell Biol Int. 2010;34(7):693–701.

[22] Raio L, Cromi A, Ghezzi F, et al. Hyaluronan content of Wharton's jelly in healthy and down syndrome fetuses. Matrix Biol. 2005;24(2):166–174. .

[23] Prasanna SJ, Gopalakrishnan D, Shankar SR, et al. Pro-inflammatory cytokines, IFNgamma and TNFalpha, influence immune properties of

human bone marrow and Wharton jelly mesenchymal stem cells differentially. PLoS One. 2010;5(2):e9016.

[24] Gauthaman K, Yee FC, Cheyyatraivendran S, et al. Human umbilical cord Wharton's jelly stem cell (hWJSC) extracts inhibit cancer cell growth in vitro. J Cell Biochem. 2012;113 (6):2027–2039.

[25] Yuan Y, Zhou C, Chen X, et al. Suppression of tumor cell proliferation and migration by human umbilical cord mesenchymal stem cells: A possible role for apoptosis and Wnt signaling. Oncol Lett. 2018;15 (6):8536–8544.

[26] Yamashita T, Takahashi Y, Takakura Y. Possibility of exosome-based therapeutics and challenges in production of exosomes eligible for therapeutic application. Biol Pharm Bull. 2018;41(6):835–842.

[27] Katakowski M, Buller B, Zheng X, et al. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. Cancer Lett. 2013;335(1):201–204.

[28] Yeo RW, Lai RC, Zhang B, et al. Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. Adv Drug Deliv Rev. 2013;65(3):336–341.

[29] Lai RC, Arslan F, Lee MM, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res. 2010;4(3):214–222. .

[30] Zhao S, Li J, Zhang G, et al. Exosomal miR-451a functions as a tumor suppressor in hepatocellular carcinoma by targeting LPIN1. Cell Physiol Biochem. 2019;53(1):19–35.

[31] Fei F, Zhang D, Yang Z, et al. The number of polyploid giant cancer cells and epithelial-mesenchymal transition-related proteins are associated with invasion and metastasis in human breast cancer. J Exp Clin Cancer Res. 2015;34:158.

[32] Thery C, Amigorena S, Raposo G, et al. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. 2006;30:**Chapter 3**: p. Unit 3 22. DOI:10.1002/0471143030.cb0322s30.

[33] Fang S, Xu C, Zhang Y, et al. Umbilical cord-derived mesenchymal stem cell-derived exosomal micrornas suppress myofibroblast differentiation by inhibiting the transforming growth factor-beta/SMAD2 pathway during wound healing. Stem Cells Transl Med. 2016;5(10):1425–1439. .

[34] El-Serag HB. Hepatocellular carcinoma. N Engl J Med. 2011;365 (12):1118–1127.

[35] Mao XH, Chen M, Wang Y, et al. MicroRNA-21 regulates the ERK/NF-kappaB signaling pathway to affect the proliferation, migration, and apoptosis of human melanoma A375 cells by targeting SPRY1, PDCD4, and PTEN. Mol Carcinog. 2017;56(3):886–894.

[36] Karan D, Lin F, Bryan M, et al. Expression of ADAMs (a disintegrin and metalloproteases) and TIMP-3 (tissue inhibitor of metalloproteinase-3) in human prostatic adenocarcinomas. Int J Oncol. 2003;23(5):1365–1371.

[37] Zeng T, Peng L, Chao C, et al. miR-451 inhibits invasion and proliferation of bladder cancer by regulating EMT. Int J Clin Exp Pathol. 2014;7(11):7653–7662.

[38] Wang YY, Ye Z-Y, Li L, et al. ADAM 10 is associated with gastric cancer progression and prognosis of patients. J Surg Oncol. 2011;103(2):116–123.

[39] Ren C, Chen H, Han C, et al. High expression of miR-16 and miR-451 predicating better prognosis in patients with gastric cancer. J Cancer Res Clin Oncol. 2016;142(12):2489–2496. .

[40] Yuan S, Lei S, Wu S. ADAM10 is overexpressed in human hepatocellular carcinoma and contributes to the proliferation, invasion and migration of HepG2 cells. Oncol Rep. 2013;30 (4):1715–1722.

[41] Liu S, Wang L, Li Y, et al. Long non-coding RNA CHRF promotes proliferation and mesenchymal transition (EMT) in prostate cancer cell line PC3 requiring up-regulating microRNA-10b. Biol Chem. 111(2019):1036-1045.

[42] Liu Y, Li H, Li L-H, et al. Mir-451 inhibits proliferation and migration of non-small cell lung cancer cells via targeting LKB1/AMPK. Eur Rev Med Pharmacol Sci. 2019;23(3 Suppl):274–280.

[43] Wang J, Zhao X, Shi J, et al. miR-451 suppresses bladder cancer cell migration and invasion via directly targeting c-Myc. Oncol Rep. 2016;36(4):2049–2058.

[44] Woods N, Trevino J, Coppola D, et al. Fendiline inhibits proliferation and invasion of pancreatic cancer cells by interfering with ADAM10 activation and beta-catenin signaling. Oncotarget. 2015;6(34):35931–35948.

[45] Liu W, Liu S, He Y, et al. MiR-451 suppresses proliferation, migration and promotes apoptosis of the human osteosarcoma by targeting macrophage migration inhibitory factor. Biomed Pharmacother. 2017;87:621–627.

[46] Li Z, Wu S, Lv S, et al. Suppression of liver receptor homolog-1 by microRNA-451 represses the proliferation of osteosarcoma cells. Biochem Biophys Res Commun. 2015;461(3):450–455.

[47] Zhang W, LIU S, LIU K, et al. Knockout of ADAM10 enhances sorafenib antitumor activity of hepatocellular carcinoma in vitro and in vivo. Oncol Rep. 2014;32(5):1913–1922.

[48] Armanious H, Gelebart P, Anand M, et al. Constitutive activation of metalloproteinase ADAM10 in mantle cell lymphoma promotes cell growth and activates the TNFalpha/NFkappaB pathway. Blood. 2011;117(23):6237–6246.

[49] Liu B, et al. Human umbilical cord mesenchymal stem cell conditioned medium attenuates renal fibrosis by reducing inflammation and epithelial-to-mesenchymal transition via the TLR4/NF-kappaB signaling pathway in vivo and in vitro. Stem Cell Res Ther. 2018;9(1):7.

[50] Meng H, Huang Q, Zhang X, et al. MiR-449a regulates the cell migration and invasion of human non-small cell lung carcinoma by targeting ADAM10. Onco Targets Ther. 2019;12:3829–3838.

[51] Wang W, Zhang L, Wang Y, et al. Involvement of miR-451 in resistance to paclitaxel by regulating YWHAZ in breast cancer. Cell Death Dis. 2017;8(10):e3071.

[52] Yang CL, Jiang F-Q, Xu F, et al. ADAM10 overexpression confers resistance to doxorubicin-induced apoptosis in hepatocellular carcinoma. Tumour Biol. 2012;33(5):1535–1541. .