Original Article

Hepatocellular carcinoma serum derived exosomal HIF-1α induces phosphoinositide-3 kinase/protein kinase B signaling to induce the angiogenesis and proliferation of HCC

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Background: Tumor exosomes are nanovesicles mostly secreted by tumor cells that play roles of paracrine signaling during tumor progression, including tumor-stromal interactions, activation of proliferative pathways and inducing immunosuppression. The hypoxia-inducible factor (HIF) family serve role of the principal molecular mediators of hypoxia in physiological and pathophysiological course. HIF-1α excreted from tumor and stromal cells serves an important role in tumor angiogenesis, especially in hepatocellular carcinoma (HCC). However, exosomal HIF-1α from HCC serum has not been studied extensively. The present study aimed to determine role of HIF-1α derived from HCC serum exosomes in the development of HCC.

Methods: We extracted exosomes from the serum of 48 patients suffering HCC and 24 healthy individuals. The exosomes were examined by transmission electron microscopy and nanoparticle tracking analysis. The concentrations of exosomal HIF-1α were measured by ELISA. In vitro experiments were performed to testify the exosomal role in human umbilical endothelial cells (HUVECs) and Huh-7 cells by tube formation, cell viability and western blotting analyses. Nude mouse xenograft Huh-7 tumors were generated by injecting HCC patient serum exosomes, healthy individuals' serum exosomes and PBS into nude mice. Tumors *in vivo* were isolated, weighed and subjected to immunohistochemical assays.

Results: HCC patients exhibited a greater capacity to convert HIF-1α in serum exosomes than normal individuals (P=0.0007), and high serum exosomal HIF-1α levels were correlated with Barcelona Clinic Liver Cancer (BCLC) staging (P=0.031) and the phosphoinositide-3 kinase (PI3K)/protein kinase B (AKT) signaling pathway. The results of flow cytometry analysis demonstrated that the HUVECs cocultured with HCC exosomes turned down the percentage of HUVECs in the G1 phase but turned up the percentage of HUVECs in the S phase (P<0.05). HUVECs and Huh-7 cells cultured with HCC serum exosomes enhanced the proliferation of HUVECs and Huh-7 cells, and the tube formation of HUVECs, while exosomes from healthy individuals and the controls did not. The results demonstrated that serum exosomes from patients with HCC appeared to accelerate tumor growth compared to the control group (P<0.05) and immunohistochemical analysis revealed that the nude mice injected with exosomes from HCC patient serum exhibited higher microvessel density (MVD) (P<0.05).

Conclusions: HCC serum derived exosomes could induce angiogenesis and the proliferation of HCC potentially via the HIF-1α-PI3K/AKT signaling pathway, it making the prevention and treatment of HCC angiogenesis and proliferation available.

Keywords: Serum derived exosomes; hepatocellular carcinoma (HCC); hypoxia-inducible factor-1 (HIF-1); phosphoinositide-3 kinase/protein kinase B signaling; angiogenesis; proliferation

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Introduction

Hepatocellular carcinoma (HCC) is the most common malignant tumor of liver, with the third highest incidence and mortality rate in the world (1). The majority of patients with HCC succumb largely died of recurrence and metastases in one year. Hypoxia-inducible factor (HIF) family serve important role in HCC metastasis including epithelial-mesenchymal transition (EMT) (2), invasion of the extracellular matrix, intravasation, extravasation and secondary growth of metastases (3). Highly expression of HIF-1α means poor prognosis for clinical outcome in HCC patients (4) and is prone to resulting in metastasis (5,6). Many reports have described HCC metastasis was closely associated with glucose metabolism, and HIF-1α signaling may serve an essential role (7-9). HIF-1α stabilization may drive aerobic glycolysis in HCC (10). HIF-1α also recruits suppressive and pro-angiogenic T-cell subsets by the selective chemokine secreted from tumor and vascular cells (11,12). Thus, HIF-1α not only exerts effects on HCC tumor but also on the HCC microenvironment.

Exosomes are small lipid-bilayer vesicles secreted by many different cell types, including tumor cells. They play role in tumor development and metastasis (13,14). DNA, mRNAs, noncoding RNAs, and proteins take exosome as a cargo to make phenotypic changes in target cells (15-17). He *et al*. (18) revealed that exosomes isolated from metastatic HCC cell lines were apt to increase the migratory and invasive abilities in normal hepatocytes. However few studies have been reported of the effects of HCC serum-derived exosomes on HCC cells.

The HCC serum-derived exosomes with HIF-1α expressed highly were identified and co-incubated with human umbilical endothelial cells (HUVECs) and Huh7 cells. HCC serum-derived exosomes may promote angiogenesis and proliferation via the phosphoinositide-3 kinase (PI3K)/protein kinase B (AKT) signaling pathway in *in vivo* and *in vitro* experiments. It may offer novel potential therapeutic strategies targeting HCC angiogenesis and proliferation.

Methods

Patients

Serum samples from patients with HCC (n=48) and healthy individuals (n=24) were obtained from Jiang Su Cancer Hospital (Nanjing, China). Research procedures were approved by the Ethical Committee of Jiang Su Cancer Hospital, for clinical research and all patients signed written informed consent for biomedical research. The diagnosis of HCC was performed using histological samples and/or by combining clinical, biochemical and radiological analyses according to the European Association for the study of the liver guidelines, and staging was determined according to the Barcelona Clinic Liver Cancer (BCLC) system.

Cell lines

The hepatic tumor cell lines Huh-7 and HUVECs were cultured with 37 ℃ under a 5% CO_2 humidified environment in Dulbecco's modified Eagle's medium (DMEM) with high glucose (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and 10% fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), or DMEM low glucose media with 10% FCS respectively. The two cell lines were purchased from Wuhan Institute of Cell Biology, China Center (Wuhan, China). Mycoplasma was tested negative in all cells prior to the experiments.

Exosome isolation and purification

The present study used Total Exosome Isolation reagent (Thermo Fisher Scientific, Inc.) to precipitate exosomes. The purified exosome samples were washed thrice with PBS. To analyze the protein in exosome, samples were suspended in exosome-depleted PBS and then assessed using a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China).The exosomes were kept at −80 ℃ and used no more than 7 days following isolation.

Assessment of exosome size and distribution in serum samples

Serum exosomes were resuspended in 100 μL of pre-filtered PBS and analyzed by Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) equipped with a monochromatic 465 nm laser. Datas were processed by the particle-tracking software (Zetasizer Ver 7.03, Malvern Instruments, Ltd).

Transmission electron microscopy

Prepared exosomes were immobilized with 2.5% glutaraldehyde for 2 h. Twenty μL exosomes were put on a formvar/carbon-coated grid. Purified exosomes were negatively stained with 3% aqueous phosphotungstic acid for 2 min and viewed with transmission electron microscope at 80 kV (JEM-1230; JEOL, Ltd., Tokyo, Japan).

Exosome labeling

Exosomes from HCC patients were labeled using PKH67 (green) membrane-binding fluorescent labels (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Labeled exosomes were washed with DMEM, collected by ultracentrifugation and resuspended in DMEM with low glucose. HUVECs were incubated at 37 ℃ with 200 μg/mL of PKH67-labeled exosomes for 6 h. The samples were fixed with 4% paraformaldehyde, washed three times with PBS. Nuclei were stained with Hoechst in 20 min and fixed with PBS glycerol. Image collection was performed with TCS SP-5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were collected with scanning rate of 400 Hz with resolution of 512×512 pixels, image analysis was conducted using Leica Application Suite 2.02 (Leica Microsystems GmbH).

Protein analysis

A total of 50 or 100 μL of serum-derived exosomes from HCC patients or healthy individuals were lysed in 50 μL of Radioimmunoprecipitation Assay (RIPA) buffer (Nanjing KeyGen BioTech Co., Ltd., Nanjing, China).We quantified exosomal protein concentration by the BCA Protein Assay kit (Beyotime Institute of Biotechnology).

Western blot assay

Equal quantities of proteins were separated by SDS-PAGE, transferred to PVDF membranes and blocked with 5% non-fat milk. Cells were collected and washed twice with chilled PBS, and dissolved in RIPA buffer (Sigma-Aldrich; Merck KGaA). For western blot analysis, anti-cluster of differentiation CD-63, anti-CD9 (1:1,000; Sangon Biotech Co., Ltd., Shanghai, China), anti-HIF-1α (1:1,000; Abcam, Cambridge, MA, USA), anti-Flotillin-1, anti-AKT and anti-phosphorylated -AKT (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used. β-actin (1:2,000; Santa Cruz Biotechnology, Inc.) was used as house-keeping genes in Western blot assays. The expressed proteins were checked and analyzed by SDS-PAGE and Gel-Pro Analyzer (Media Cybernetics, USA). HRP-conjugated Donkey Anti-Rabbit IgG, HRP-conjugated Donkey Anti-Mouse IgG (1:1,000; Sangon Biotech Co., Ltd., Shanghai, China) were used as secondary antibodies.

Flow cytometric cell cycle analysis

HUVECs were collected at 48 hours with different types of exosomes in 6 cm plates. Cells were harvested using cold PBS and fixed with 70% cold ethanol for 24 hours at –20 ℃ and treated with 1 ng/mL RNaseA for 20 minutes at 4 ℃. A total of 0.5 mg/mL propidium iodide (50 mg/mL; Becton, Dickinson and Company, Franklin, Lakes, NJ, USA) was used to stain the cellular DNA in the dark for 20 minutes at 4 ℃. HUVECs were categorized by FACSCalibur Flow Cytometer (Becton, Dickinson and Company) and CellQuest acquisition and analysis programs (Becton, Dickinson and Company). All procedures were repeated three times.

Tube formation assay

A total of 2×10^4 HUVECs were plated in 96-well plates on top of matrigel, treated with exosomes cultured for 24 hours. Tube networks were quantified with per pixel length acquired with image analysis of 5 random microscopic fields by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell viability assay

Cell viability was obtained by Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology). Cells were embedded into 96-well plates, grown to 50–60% confluence for 12 hours. Following treatment under different conditions, we mixed 10 μL of CCK-8 reagent in each well culture

medium containing 100 µL and incubated cells for 1.5 hours at 37 ℃. We used Fluorescence Spectrophotometer (Hitachi, Ltd., Tokyo, Japan) to read the optical density (OD) of each well at 450 nm.

Wound-healing assays

For wound-healing assays, we monitored the cell migration for 24 h by using the 100 µL micropipette tip to scratch the confluent HUVECs monolayers incubated with or without exosomes. We calculated the widths of the wound by ImageJ software (http://rsbweb.nih.gov/ij/), measured and the percentage of wound healing by the formula: 100% – (width after 24 hours/width at the beginning) × 100%.

Detection of HIF-1α by ELISA

The concentration of exosomal HIF-1α was acquired by ELISA kits (HIF-1 ELISA kit, cat. No. CSB-E12112h; CusAbio, Wuhan, China). The OD of each well was read in a Fluorescence Spectrophotometer (Hitachi, Ltd.) at 450 nm. Analyses were repeated in duplicate.

Animal xenograft tumor model

Experiments mice were used with the permission and consent of the Animal Care and Use Committee and Animal Center of Nanjing Medical University (Nanjing, China). 20 BALB/c nude mice (4–6 weeks old) were bought from Shanghai Animal Center (Shanghai, China). The nude mice were kept at a temperature of 20–25 ℃ and housed in a relative humidity of 30–60% with standard rodent chow and water for freedom in the light controlled vivarium (14 hours light and 10 hours darkness). About 6-weeks-old male nude mice were implanted subcutaneously near the forepaw with Huh-7 cells (1×10^6, 100 µL) and were randomly assigned to three groups (7 animals in HCC-ex or normal-ex group and 6 animals in control group). Exosome therapy was carried out following 1 week of tumor cell inoculation; 20 µg of exosome protein was dissolved in PBS and injected into the area around the tumor 3 times a week for 1 month. When primary visible tumors appeared, the tumor size, mice weight and health were measured daily. Mice were sacrificed 1 month following tumor injection, *in vivo* tumors were isolated, weighed and subjected to immunohistochemical assays using the anti-CD31 antibody (1:40; Sangon Biotech Co., Ltd.).

Microvessel density (MVD) assessment

We stained vascular endothelial cells with CD31 (PECAM-1), and calculated the CD31-positive MVD. We selected five areas with the highest number of hot spots, and counted the number of vessels at a high-power magnification (×200).

Statistical analysis

We performed each experiment at least three times. Results were recorded as the mean ± standard deviation. We analyzed the differences between groups by ANOVA analysis and Student's *t*-test. All P values were based on a two-sided statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

Results

Characterization and concentration of serum exosomes from patients undergoing HCC and healthy individuals

Serum exosomes were observed by transmission electron micrographs. These vesicles exhibited cup-shaped morphology (*Figure 1A*) and a high concentration of the exosome protein markers CD9, CD63 and Flotillion-1 was observed following western blot analysis (*Figure 1B*). Nanoparticle tracking analysis of isolated serum HCC exosomes revealed that the size distribution was in accordance with the expected size range of exosomes (~58 to 164 nm; number mean: 83.16 nm; PDI: 0.467, *Figure 1C*). In addition, the exosome concentration was higher in patients suffering HCC when compared with controlled group (*Figure 1D*). *Tables S1* and *S2* summarize the characteristics of the healthy individuals and patients.

HCC-exosomes promote tumor angiogenesis and proliferation in vitro

The present study demonstrated the transport of exosomes from HCC patients into HUVECs using the method modified from Umezu's reports (19). Following incubation with PKH67-labeled exosomes from HCC patients, the PKH67 signals were colocalized in the cytoplasm of HUVECs (*Figure 2A*). Flow cytometric analysis demonstrated that the HCC exosomes decreased the percentage of cells in the G1 phase (50.50%±1.91% and

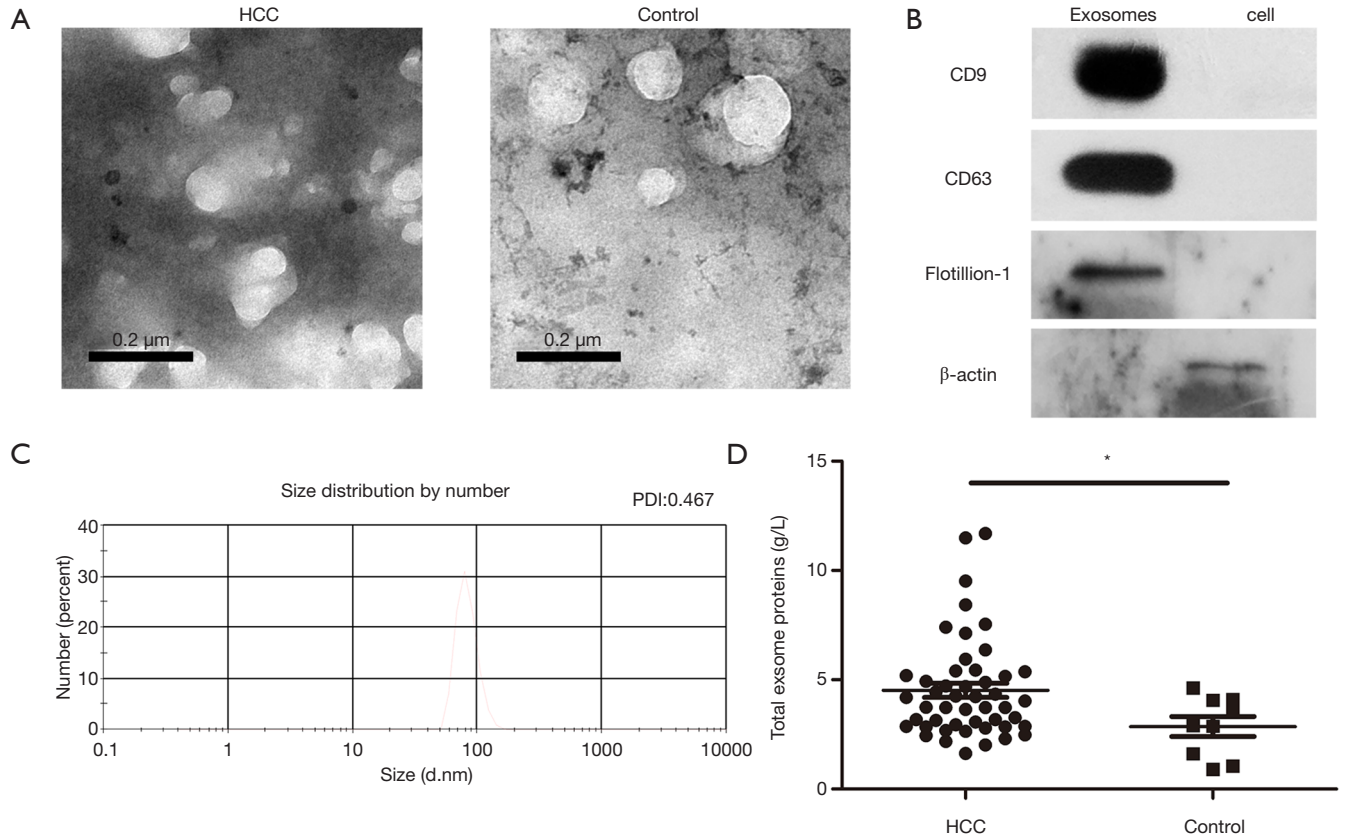

Figure 1 Comparative analysis of serum exosomes from HCC and healthy individuals. (A) Transmission electron micrographs of blood serum exosomes derived from HCC patients and healthy individuals; (B) immunoblotting of CD63, CD9, flotillin-1 and β-actin in exosomes and Huh-7 cells; (C) nanoparticle tracking analysis of serum exosomes from HCC isolated by total exosome isolation reagent precipitation; (D) increased concentration of serum exosomes was observed in HCC (n=48) when compared with the healthy individuals (n=9). *P<0.05, as indicated. HCC, hepatocellular carcinoma; CD, cluster of differentiation.

43.99%±4.81% *vs.* 66.55%±0.79% and 69.11%±1.67%, respectively; P=0.0005) whereas there was a rising percentage of cells in the S phase (24.75%±3.99% and 40.09%±3.26% *vs.* 12.69%±0.47% and 12.20%±1.11%, respectively; P=0.0002; *Figure 2B,C*). HCC exosomes especially high HIF-1α exosomes could promote cell proliferation and cell cycle arrest. In addition, HUVECs cocultured with high HIF-1α exosomes exhibited an increase in the percentage of cells in the S phase and a decrease percentage of cells in the G1 phase when compared with the low HIF-1α exosomes from HCC patients (40.09%±3.26% *vs.* 24.75%±3.99%; 43.99%±4.81% *vs.* 50.50%±1.91%, respectively; P<0.05; *Figure 2B,C*). Wound healing was significantly increased by HCC-exosomes compared to the control group following 24 h (*Figure 2D,E*). HUVECs cultured with HCC-exosomes promoted the tube formation of HUVECs while healthy individual exosomes and controls did not (*Figure 2F*). We found HCC-exosomes (200 μg protein) increased the proliferation of HUVECs and Huh-7 cells when compared with the control or normal exosomes (*Figure 2G,H*) by CCK-8 assays.

HIF-1α level in exosomes is upregulated in HCC and exosomal HIF-1α induces PI3K/AKT signaling to induce angiogenesis

HIF-1α level in exosomes was higher in HCC patients (332.38 pg/mL) compared with the healthy individuals (253.9 pg/mL; P=0.0007; *Table 1*). Upon clinical features correlation analysis, exosomal HIF-1α overexpression

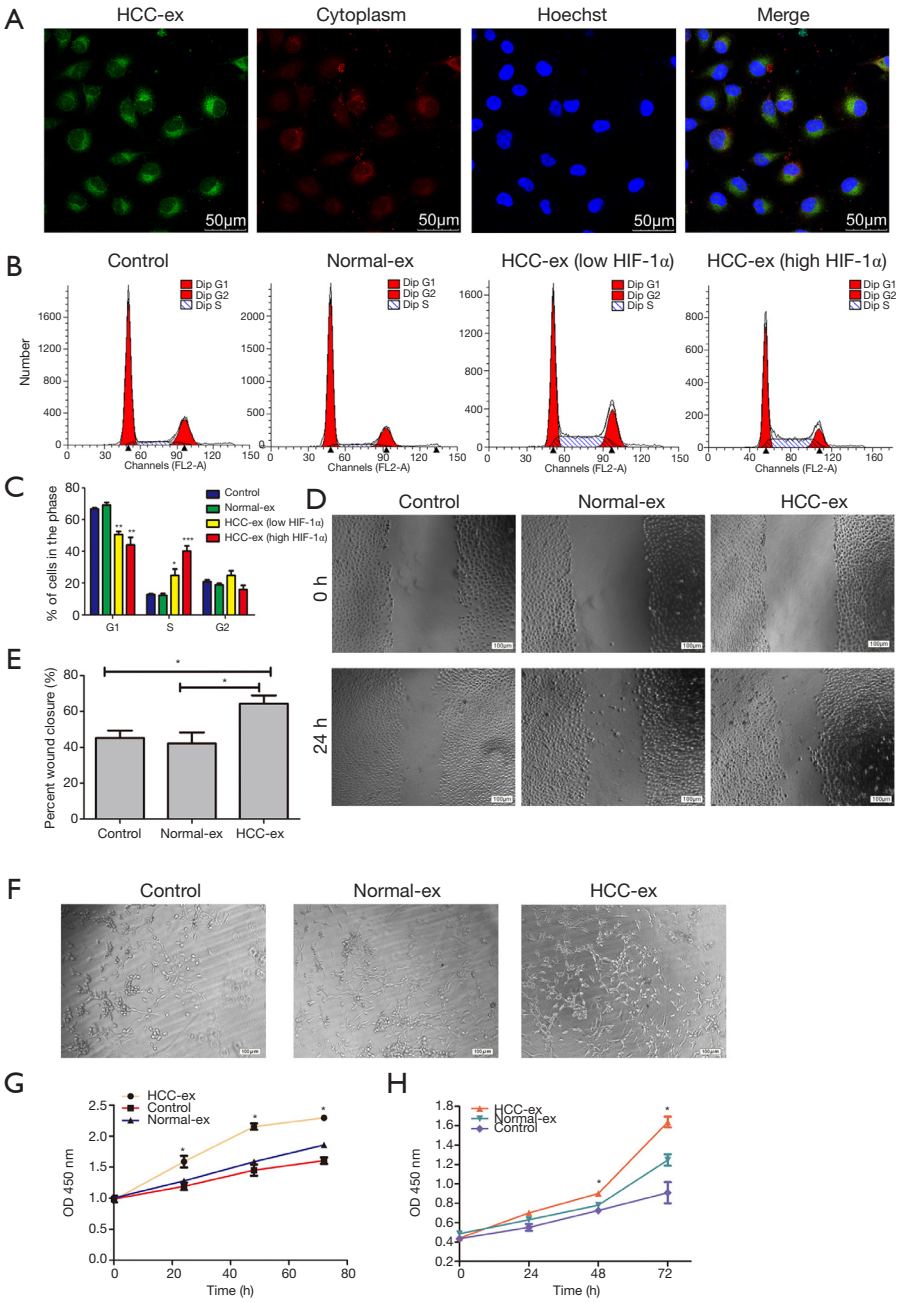

Figure 2 HCC-exosomes promote tumor angiogenesis *in vitro*. (A) HUVECs were cultured with PKH67-labeled exosomes derived from HCC patients. The signals were detected in PKH67-labeled exosomes (green), the cytoplasm of HUVECs (red) and via nuclear counterstaining (blue). (B) Flow cytometry was conducted to (C) analyze the cell cycle in HUVEC cells. (D and E) Migration of HUVEC cells was detected by wound-healing assay following culture with exosomes or control. (F) HUVECs were cultured for 24 h in the absence (PBS) or presence of normal and HCC exosomes and then grown on Matrigel. Representative photomicrographs of tubes from the different treatment groups was presented. (G and H) Proliferation analysis was performed following exosomes being combined with (G) HUVECs or (H) Huh-7 cells. Data were expressed as the mean ± standard deviation. *P<0.05. HCC, hepatocellular carcinoma; HIF, hypoxia inducible factor; HCC-ex, exosomes derived from HCC patients; Normal-ex, exosomes derived from healthy individuals; HCC-ex (low HIF-1α), exosomes derived from HCC patients with a low level of HIF-1α protein; HCC-ex (high HIF-1α), exosomes derived from HCC patients with a high level of HIF-1α protein. HCC, hepatocellular carcinoma; HUVEC, human umbilical endothelial cell.

Table 1 Expression of HIF-1α in exosomes in a cohort of 33 cases of HCC patients

Clinical features	No. of cases without exosomal HIF-1α overexpression	No. of cases with exosomal HIF-1α overexpression	P value
Sex			0.699
Male	13	12	
Female	5	3	
BCLC stage			0.031*
B	14	6	
C	4	9	
Tumor size (cm)			0.166
<5	11	5	
≥5	7	10	
Venous invasion			0.239
Absent	15	6	
Present	3	6	

*, statistical analyses were performed by the Pearson χ^2 test. P<0.05 was considered significant. HIF, hypoxia-inducible factor; HCC, hepatocellular carcinoma.

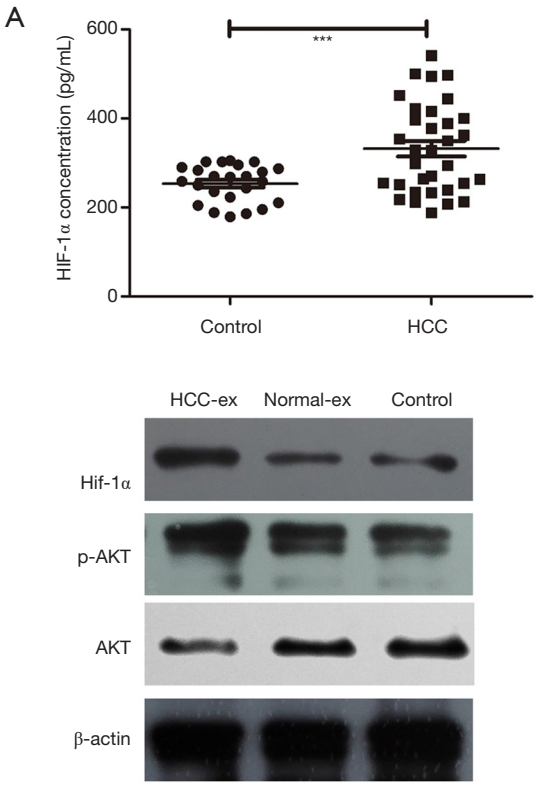

Figure 3 HIF-1α in exosomes is upregulated in HCC and exosomal HIF-1α induces AKT signaling to induce angiogenesis. (A) Levels of HIF-1α in exosomes in a cohort of 33 cases of HCC and 24 healthy individuals (t test). ***P<0.001, as indicated. (B) The expression level of HIF-1α, AKT and p-AKT was detected by western blot analysis in HUVECs cells following coculture with exosomes. HCC, hepatocellular carcinoma; HIF, hypoxia inducible factor; AKT, protein kinase B; p-, phosphorylated; HCC-ex, exosomes derived from HCC patients; normal-ex, exosomes derived from healthy individuals; HUVEC, human umbilical endothelial cell.

was closely associated with BCLC stage (P=0.031; *Figure 3A*). *Figure 3B* and *Tables S1,S2* summarize the patients' characteristics. Exosomes incubated with HUVECs cells had increased PI3K/AKT and kept no difference in the recipient cells, potentially indicating that HCC-exosomes transfer HIF-1α via exosomes to HUVECs by activating the PI3K/AKT signaling pathway (*Figure 3B*).

HCC serum-derived exosomes accelerate xenograft tumor proliferation and angiogenesis

The present study performed an HCC xenograft model by injecting different types of exosomes into nude mice. Consistent with the present aforementioned results, exosomes from HCC patients appeared to accelerate tumor proliferation compared to healthy individuals or control group (P=0.007; *Figure 4A,B,C*; 2,278±589.8 *vs.* 812.4±147.9; P=0.0329; 2,278±589.8 *vs.* 438.7±119.5; P=0.0164). In addition, mice injected with high levels of exosomal HIF-1α from HCC patients had promoted vascularization (P=0.0042; *Figure 4D,E*). Overall, these results may indicate that HIF-1α accelerates the growth of HUVECs via exosomes, and enhanced tumor cell proliferation and angiogenesis.

Discussion

Proteins, RNAs and other types of molecules involved in the pathology of cancer were carried by exosomes (13,20). Colon cancer cells contain microRNAs (miRNAs) secreted exosomes involved in cancer progression and mediated by the major vault proteins that sort miRNAs (21). Tumor-associated macrophage-derived exosomes

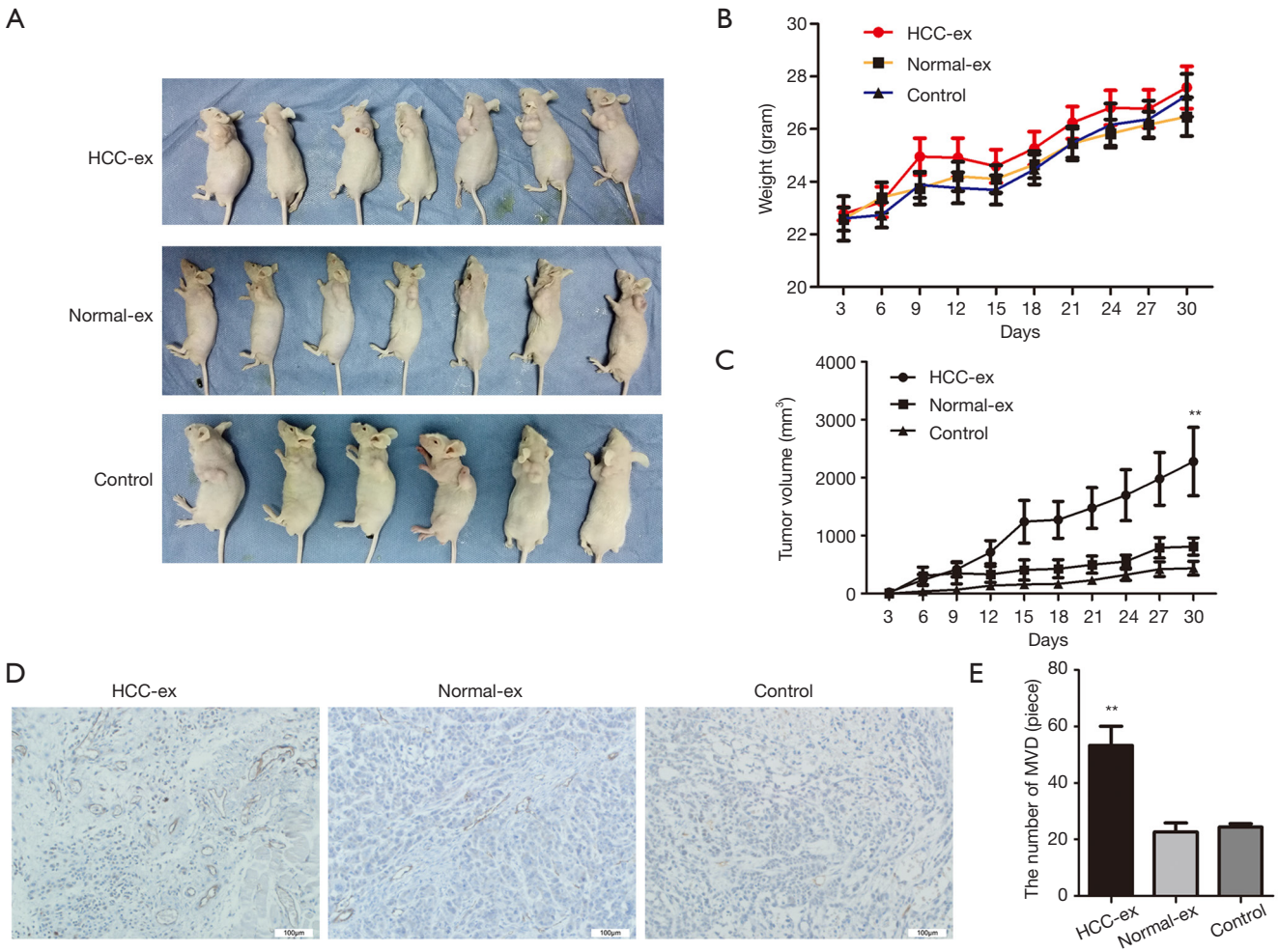

Figure 4 HCC exosomes promote xenograft tumor growth and angiogenesis. (A) Representative examples of mice treated with different types of exosomes. (B) The mice weight overtime. (C) Tumor growth over time. (D and E) Tumors were analyzed by immunohistochemistry analysis for CD31 expression and vascular density (scale bar, 100 μm). **P<0.01. HCC, hepatocellular carcinoma; HCC-ex, exosomes derived from HCC patients; normal-ex, exosomes derived from healthy individuals; CD, cluster of differentiation.

transferred functional Apolipoprotein E to promote the migration of gastric cancer cells (22). Recently, many studies were conducted to reveal the role of exosomes in HCC angiogenesis and proliferation (15,23,24). In the present study, the exosomal concentration in HCC patients and healthy donors was analyzed. We found the HCC patients exhibited a greater capacity to convert HIF-1α in serum exosomes than healthy individuals, and high serum exosomal HIF-1α levels were correlated with the PI3K/AKT signaling pathway, which promoted xenograft tumor proliferation and angiogenesis. Liu *et al.* (25) reported that HIF-1α induced a phenotypic switch in human vascular smooth muscle cells through PI3K/AKT/astrocyte elevated gene-1 signaling. Furthermore, we indicated that high HIF-1α expression in serum exosomes is correlated with the BCLC stage of HCC, which has important implications for efficient prevention and therapeutic strategies. This was consistent with the results of the Li *et al.* (26) study, which demonstrated that co-expression of HIF-1α and chemokine (C-X-C motif) ligand 8 was a prognostic factor in HCC.

HCC is considered to be a dynamic model of the associations between the tumor microenvironment and tumor intercellular communications (27). In addition, there is emerging evidence that has indicated that hypoxia in the

tumor microenvironment promotes tumor angiogenesis and proliferation (28). Tumor-derived exosomes hold important role in the intercellular communications and angiogenesis associated with the tumor environment (29). Therefore, study of the interactions between the tumor microenvironment and angiogenesis, and proliferation mediated by exosomes is required. Exosomes identified from the serum of HCC patients may be a vital component of HCC microenvironment.

The HIF-1α/PI3K/AKT signaling pathway in malignant tumors is not a novel concept. Many studies have indicated that HIF-1α in the exosomal pathway under hypoxic conditions secretes several growth factors to stimulate PI3K signaling activation (30). The HIF-1α/PI3K/AKT signaling pathway of oral squamous cell carcinoma may contribute to the anti-carcinogenic activity of Sal-B (31). In the present study, the HIF-1α/PI3K/AKT signaling pathway mainly contributed to the angiogenesis and proliferation in HCC.

In conclusion, the present results indicated that HCC serum derived exosomes activated HUVECs by increasing HIF-1α/PI3K/AKT signaling in HCC. In addition, HCC patients exhibited a greater capacity to convert HIF-1α in serum exosomes than healthy individuals, which could promote xenograft tumor proliferation and angiogenesis. Notably, high expression of HIF-1α in serum exosomes exhibited a positive correlation with BCLC stage in HCC patients. The present study may elucidate a molecular mechanism underlying the crosstalk between HCC cells and exosomes to promote angiogenesis and proliferation.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.08.07). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Research procedures were approved by the Ethical Committee of Jiang Su Cancer Hospital, for clinical research and all patients signed written informed consent for biomedical research.

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Supplementary

Table S1 Demographic and clinical characteristics of the 24 healthy individuals enrolled in the study

Variable	Sample	Age (y)	Sex (0: male; 1: female)	Serum HIF-1α levels in exosomes (pg/mL)	Serum exosome concentration (μg/μL)
Cohort of serum exosome concentration	1	32	0	270.071	1.61574
	2	42	0	250.244	2.904544
	3	45	0	223.031	1.05877
	4	48	0	287.376	4.070296
	5	39	0	268.144	4.627266
	6	34	1	302.856	2.885114
	7	37	0	270.071	0.9098124
	8	41	0	305.027	4.102678
	9	50	1	186.007	3.565136
Cohort of serum HIF-1α levels in exosomes	10	47	0	302.931	–
	11	40	1	195.701	–
	12	36	1	279.541	–
	13	36	0	295.836	–
	14	38	1	258.989	–
	15	42	1	290.365	–
	16	44	0	188.644	–
	17	31	1	243.608	–
	18	35	0	235.875	–
	19	51	0	258.683	–
	20	47	1	204.702	–
	21	55	1	283.817	–
	22	37	0	179.351	–
	23	49	1	302.593	–
	24	32	1	210.876	–

HIF, hypoxia-inducible factor.

Table S2 Demographic and clinical characteristics of the 48 HCC patients enrolled in the study

Variable	Sample	Age (y)	Sex (0: male; 1: female)	Tumor size (0: ≤5 cm; 1: >5 cm)	BCLC stage (1: A; 2: B; 3: C stage)	Venous invasion (0: none; 1: presence)	Serum exosome concentration (μg/μL)	Serum HIF-1α levels in exosomes (pg/mL)
Cohort of serum exosome concentration	01	63	0	0	3	1	4.43945	350.184
	02	47	1	0	2	0	9.516948	265.127
	03	42	1	0	2	0	5.372052	264.266
	04	59	0	1	2	0	2.846256	218.626
	05	87	0	0	2	0	4.931657	298.5
	06	59	0	0	2	0	2.308715	271.378
	07	57	0	0	2	0	11.49225	294.68
	08	69	0	1	3	1	2.949878	255.244
	09	55	1	0	3	0	5.410911	452.049
	10	61	0	1	3	1	3.183029	362.828
	11	36	0	1	3	1	3.732407	389.292
	12	58	0	0	3	0	2.682166	444.908
	13	30	0	1	3	0	2.871905	328.89
	14	41	0	1	2	0	2.475503	354.431
	15	31	0	0	2	0	2.184215	254.555
	16	51	0	1	3	1	2.868341	397.25
	17	84	0	1	2	0	2.442544	377.628
	18	79	0	0	2	0	4.343488	416.774
	19	42	0	1	2	0	7.406912	400.933
	20	33	0	1	2	0	4.88687	422.935
	21	47	1	0	3	1	4.193836	233.529
	22	84	1	1	3	1	3.177445	234.68
	23	32	1	1	3	1	5.155888	500.594
	24	53	0	1	3	0	3.727062	495.199
	25	33	0	0	2	0	4.233921	541.786
	26	61	0	0	2	0	6.370034	212.567
	27	29	0	1	2	0	4.714056	497.536
	28	48	1	0	2	0	3.639765	188.795
	29	80	0	1	2	0	3.140923	208.941
	30	82	0	0	2	0	2.013184	329.68
	31	50	0	1	3	1	5.939783	239.554
	32	76	0	1	2	0	4.689115	213.839
	33	69	1	0	2	0	2.796187	251.566
Cohort of serum HIF-1α levels in exosomes	34	38	0	0	2	0	3.734189	–
	35	65	1	1	2	0	7.545876	–
	36	60	1	0	3	1	1.634598	–
	37	61	0	1	2	0	3.268306	–
	38	44	0	1	2	0	3.062533	–
	39	51	1	0	3	0	7.126314	–
	40	58	0	1	3	1	8.434883	–
	41	60	0	1	2	0	4.258863	–
	42	58	0	1	2	0	4.032603	–
	43	47	0	0	2	0	5.198646	–
	44	41	0	1	3	1	11.68894	–
	45	64	1	0	2	0	3.748441	–
	46	59	0	0	3	0	2.839836	–
	47	49	0	0	3	1	2.637627	–
	48	52	0	1	2	0	5.447176	–

HCC, hepatocellular carcinoma; HIF, hypoxia-inducible factor; BCLC, Barcelona Clinic Liver Cancer.