Renal tumor-derived exosomes inhibit hepaCAM expression of renal carcinoma cells in a p-AKT-dependent manner

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HepaCAM mediates cancer cell proliferation, migration and differentiation. Our previous studies showed the effects of hepaCAM on the inhibition of renal carcinoma cell proliferation. To further investigate the reason for the low expression of hepaCAM in renal carcinoma and the corresponding molecular mechanisms, we detected renal carcinoma OS-RC-2 cell lines containing high expression of hepaCAM; and hepaCAM and p-AKT were also detected in these cells. Exosomes were isolated and purified from the supernatant liquid of OS-RC-2 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and flow cytometry analysis were conducted to determine the effect of exosomes on the proliferation and cycle distribution of OS-RC-2 cells. OS-RC-2 cells (high expression of hepaCAM) were treated with exosomes or plus MK-2206 (AKT inhibitor); and hepaCAM, AKT and p-AKT were detected in these cells by western blot analysis. The correlation between hepaCAM and p-AKT was analysed by immunohistochemical method. Results showed that hepaCAM re-expression in OS-RC-2 cell lines resulted in significant weakening of proliferation ability and more prominent G0/G1 population as well as reduction of p-AKT protein. The increase in proliferation caused by exosomes was followed by hepaCAM downregulation and p-AKT upregulation in OS-RC-2 cells (high expression of hepaCAM). By comparison, the promotion of proliferation caused by exosomes was weakened and hepaCAM expression changed after MK-2206 treatment; however, this change was not significant. HepaCAM was negatively correlated with p-AKT protein in renal cell carcinoma tissues. Therefore, renal tumor-derived exosomes may be an important factor resulting in the low expression of hepaCAM by upregulating p-AKT in renal carcinoma.

Key words: renal cancer, exosomes, p-AKT, hepaCAM

Renal cell carcinoma (RCC) is a common malignant tumor of the urinary system, accounting for 2% to 3% of cancers worldwide [1]. Over the years, great progress has been achieved in the diagnosis and treatment of RCC, but prognosis remains unsatisfactory. The lack of sensitivity to conventional radiation and chemotherapy of late metastatic kidney cancer is one of the main reasons of poor prognosis. Gene targeting therapy can achieve satisfactory efficacy in some advanced or metastatic RCC [2]; thus, the potential mechanisms of renal cell cancer should be elucidated.

HepaCAM was first identified in 2005 as a cell adhesion molecule belonging to the immunoglobulin superfamily in [3]. The expression of hepaCAM gene is frequently lost in various human cancer cells and tissues [3,4]. The re-expression of hepaCAM in RCC and breast cancer cells inhibits cell proliferation [5]; as a result, cell cycle arrest occurs at the G2/M phase [4]. These aforementioned data show that hepaCAM may be a carcinoma suppressor gene. However, the reason for the inhibition of hepaCAM expression in renal carcinoma tissues and the relationship between hepaCAM and other oncogenes remain unclear.

Exosomes are small membrane vesicles that are usually released by most cell types, such as tumor cells. Increasing evidence shows that exosomes have an important function in cell-to-cell communication by transferring biologically active molecules into target cells. Tumor-derived exosomes are secreted constitutively and damage the immune system function. For instance, these exosomes can weaken lymphocyte responses to IL-2 [6], inhibit monocyte differentiation into dendritic cells [7], or induce apoptosis of activated T lymphocytes [8,9]. In addition, exosomes may promote the proliferation of adjacent tumor cells. The effect of gastric

cancer-derived exosomes on the proliferation of the released cells has been documented [10], but the exact mechanism of cell proliferation remains unclear. p-AKT, also called protein kinase B, mediates cell apoptosis and proliferation [11]; the overexpression of p-AKT may contribute to the development and progression of malignancies. Tumor-derived exosomes carry several biologically active molecules from precursor cells and are present in a tumor microenviroment. Therefore, studies should be conducted to elucidate whether or not exosomes can re-activate growth signals that lead to the deregulated growth of tumor cells after a tumor suppressor is re-expressed by gene therapy. This study aimed to demonstrate the relationship among exosomes, hepaCAM and p-AKT. We reported for the first time that renal cancer-derived exosomes promoted tumor cell proliferation by inhibiting hepaCAM expression, at least in part, by AKT re-activation.

Materials and methods

Cell lines and transfection. Human renal cancer OS-RC-2 (RC-2) cell lines were provided by the Institute of Cell Biology (Shanghai, China). These cell lines were cultured in RPMI 1640 medium (Gibco Life Technologies) with 10% foetal bovine serum (Sijiqing, China) at 37 °C in a humidified atmosphere containing 5% CO_2 incubator. Cells were transfected as described in a previous study [12] and divided into three groups: RC-2/GFP-hepaCAM; RC-2/GFP; and RC-2.

Determination of relative hepaCAM and p-AKT expression levels after transfection. The total RNA of RC-2 cells was extracted by Trizol (Invitrogen, Carlsbad, CA, USA), and reverse transcription polymerase chain reaction (RT-PCR) was performed using a two-step RT-PCR kit (Takara, Japan). All of the experimental procedures were performed according to the manufacturer's protocol. β-Actin was used as an internal control standard. hepaCAM and β-actin primers were used as described previously [13]. Gel image analysis (BIO-RAD) was conducted to analyse the grey values of hepaCAM and β-actin. AKT and p-AKT protein expression levels were detected by western blot analysis, and a detailed experimental procedure was described according to the following experiment.

Preparation and identification of exosomes. The RC-2 cell-derived exosomes were isolated and purified as described previously [14]. Bradford assay (Pierce Biotechnology, Rockford, USA) was performed to measure the protein concentrations of exosomes. The morphological characteristics of cells were observed under a transmission electron microscope. Molecular markers were analysed by western blot analysis; anti-HSP70 and anti-ICAM-1 antibodies were used.

MTT assay. MTT assay was performed to determine cell proliferation. In brief, RC-2 cell lines were seeded into 96-well plates and treated with exosomes at the indicated concentrations for 0, 24, 48 and 72 h. The cells were transfected by hepaCAM and seeded at 1.5×10^3 cells/well onto 96-well plates. The cells were co-cultured at 37 °C with 200 μg/ml

exosomes for 0, 24, 48 and 72 h. Approximately 1 μM MK-2206 (Shanghai Science Peptide Biological Technology Company, China) was used or unused at the indicated time for 1 h. The medium was removed. Subsequently, the MTT solution was added, incubated for 1 h, and replaced with 100 μl of DMSO (Sigma, USA). An ELX-800 spectrometer reader (Bio-Tek Instruments Inc., USA) was used to measure the optical density (OD) at 570 nm. The following equation was used to measure cell proliferation: Cell proliferation = (OD of the experimental group – OD of the blank group) / (OD of the control group – OD of the blank group) × 100%. The blank group contained PBS and exosomes without cells. The control group contained cells treated with PBS but without exosomes.

Cell cycle analysis. RC-2/GFP-hepaCAM cells were treated with exosomes (200 μg/ml) for 72 h and then treated or untreated with MK-2206 (1 μM) for 1 h or DMSO as a control for 72 h. The cells were collected and processed for FACS analysis as previously described [15].

Determination of hepaCAM and p-AKT expression levels by western blot analysis after exosome treatment. RC-2 cells (low or high expression of hepaCAM) were cultured, treated or untreated with exosomes and collected at the indicated time. The detailed experimental procedures of western blot analysis were performed according to a previously described method [16]. Anti-AKT (Bio-Rad, Hercules, CA, USA), anti-p-AKT (Bio-Rad, Hercules, CA, USA), anti-hepaCAM (Santa Cruz, CA, USA) and anti-GAPDH (Santa Cruz, CA, USA) were used to detect protein expression. The intensity of each protein was read and analysed with the AlphaEaseFC software.

Immunohistochemistry and staining evaluation. A total of 6 normal renal tissues (normal control) and 85 paired (RCC and its adjacent non-tumorous renal tissue) renal specimens were surgically collected at the Department of Urology in the First Affiliated Hospital of Chongqing Medical University from 2010 to 2012. The hospital's ethics committee approved the collection and use of human renal tissues for investigational purposes. Renal tissues were used for routine histopathological diagnosis. Tumor stage was evaluated according to the UICC/TNM system, and tumor grades were assessed according to the WHO guidelines. Table 1 shows the clinicopathological parameters. Primary antibodies against hepaCAM and p-AKT (DAKO, Glostrup, Denmark) were applied according to the manufacturer's instructions. HepaCAM and p-AKT expression were semi-quantitatively evaluated in each tissue in terms of extent (absent, 0; <10%, 1; 10% to 50%, 2; >50%, 3) and intensity (negative, 0; weak, 1; moderate, 2; and strong, 3). The total score of each patient was determined by calculating the sum of two parameters. Total scores 0–3 and 4–6 indicated low expression and high expression, respectively.

Statistics. SPSS 17.0 software was used for statistical analyses. Data were presented as means ± SD. The correlation between hepaCAM and p-AKT was determined by Pearson analysis. Statistical significance was determined by Student's

Table 1 Clinicopathological characteristics of 85 patients with renal cell carcinoma (RCC) and their correlations with hepaCAM expression

Characteristics	Number of patients (%)	HepaCAM Low (n = 57)	Expression High (n = 28)	P*
Age (years)				
<65	38 (44.7)	22	16	0.106
>65	47 (55.2)	35	12	
Gender				
Male	78(91.7)	54	24	0.155
Female	7(8.2)	3	4	
Tumor size [23]				
<7	71(83.5)	46	25	0.316
>7	14(16.4)	11	3	
TNM stage				
I–II	57(67.0)	43	14	0.019
III–IV	28 (32.9)	14	14	
Fuhrman grade				
1–2	63 (74.1)	38	25	0.025
3–4	22 (25.9)	19	3	

A

B

Figure 1. HepaCAM inhibited the expression of p-AKT in renal cancer cell lines. (A) HepaCAM mRNA levels and (B) p-AKT protein levels in renal cell carcinoma RC-2 cells after completion of transfection with different vectors. mRNA levels were quantified using semi-quantitative reverse transcription polymerase chain reaction. Data were normalised using β-actin as an internal control, and protein levels were quantified using western blot analysis. Data were normalised using GAPDH as an internal control. *P < 0.05 vs. control group (RC-2 cells).

t-test in some experiments. P < 0.05 was considered statistically significant.
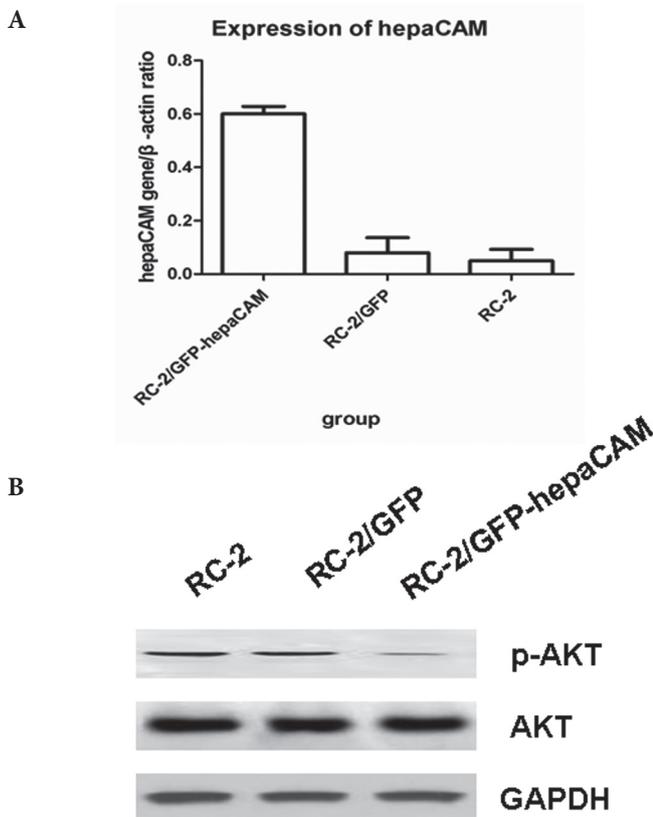
Results

Expressions of hepaCAM and p-AKT in renal carcinoma RC-2 cells after transfection. RT-PCR analysis results showed that the mRNA level of hepaCAM in RC-2/GFP-hepaCAM group cells was much higher than that in RC-2/GFP and RC-2 cells (P < 0.05; Figure 1A). This result is consistent with that in a previous study [12]. Western blot analysis results showed that p-AKT protein expression was significantly decreased in the RC-2/GFP-hepaCAM group than in the RC-2/GFP and RC-2 groups after transfection (Figure 1B). Total AKT level remained unchanged.
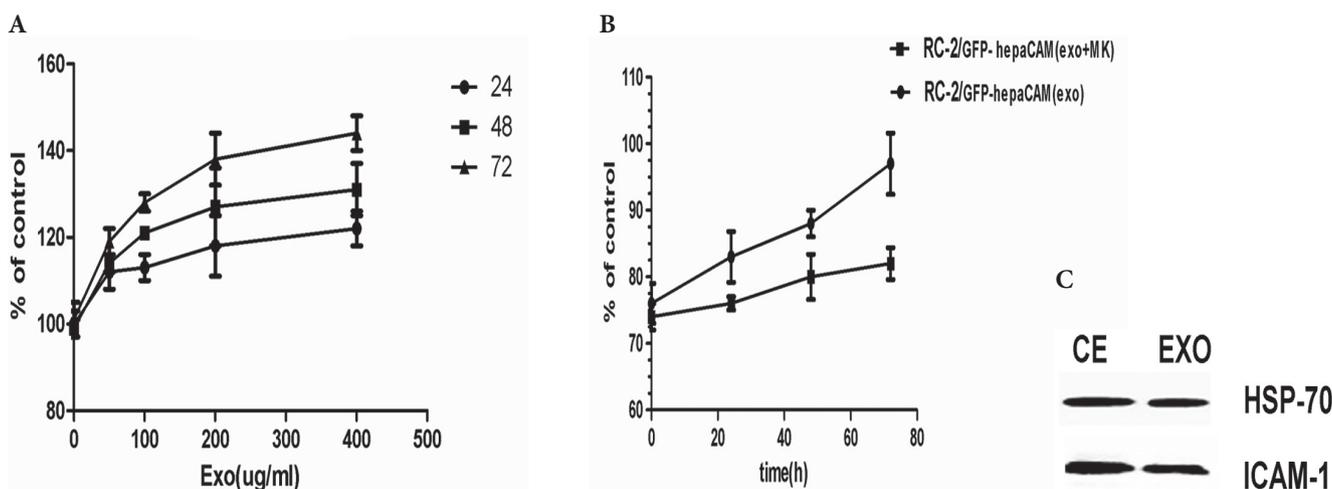
Identification of tumor-derived exosomes. Exosomes were isolated and purified from RC-2 cells and observed under a transmission electron microscope. Exosomes with diameters ranging from 40 nm to 100 nm (57.35 ± 26.59 nm) exhibited a saucer-like shape. Western blot analysis results showed that the multivesicular body marker HSP70 and ICAM-1 antigens were rich in exosomes (Figure 2C). All of these results are consistent with those in our previous study [14].

Effects of tumor-derived exosomes on renal cancer cell proliferation. To further identify the effect of tumor-derived exosomes on cell proliferation, we used different concentrations of exosome-treated RC-2 cells at indicated times. MTT assay was performed to assess cell proliferation. The results revealed that renal cancer exosomes significantly increased RC-2 cell proliferation in a classical time- and dose-dependent manner. At >200 μg/ml exosomes, the cell proliferation of RC-2 was promoted at 72 h (Figure 2A). Therefore, a concentration of 200 μg/ml exosomes was selected for the subsequent experiments. Prior to exosome treatment, the inhibition of RC-2/GFP-hepaCAM cell proliferation was observed and compared with that of RC-2 and RC-2/GFP cells. To further observe the effects of RC-2 cell-derived exosomes on the proliferation of RC-2/GFP-hepaCAM cells, we used 200 μg/ml exosomes and stimulated viable cells for 0 h to 72 h. MTT assay results revealed that renal cancer exosomes significantly increased RC-2/GFP-hepaCAM (untreated with MK-2206) cell proliferation in a classical time-dependent manner. The cell proliferation of RC-2/hepaCAM (untreated with MK-2206) increased sharply at 72 h. Moreover, the proliferation of RC-2/hepaCAM cells after exosome treatment for 72 h was almost restored to the level of the cells in the control group (Figure 2B). However, no significant growth-promoting activity of exosomes was observed in RC-2/GFP-hepaCAM (treated with MK-2206) cells compared with RC-2/GFP-hepaCAM (untreated wih MK-2206) cells (Figure 2B).

Effects of tumor-derived exosomes on the cell cycle of renal cancer cells. Cell cycle analysis results showed that hepaCAM transfection produced a more prominent G0/G1 population in RC-2/GFP-hepaCAM cells than in untransfected cells. G0/G1 population was almost restored to that of RC-2

Figure 2. Identification of RC-2 cell line-derived exosomes and its effect on renal cancer cell growth. (A) RC-2 cell lines were seeded into 96-well plates and treated with exosomes (Exo) with the indicated concentrations for 0, 24, 48 and 72 h. **(B)** After hepaCAM transfection, renal cancer cell lines were seeded on 96-well plates and treated with 200 μg/ml exosomes for 0 h to 72 h. The cells treated with PBS but not with exosomes (exo) were used as the control group. MK indicates that RC-2/GFP-hepaCAM cells were treated with 1 μM MK-2206 at the indicated time for 1 h. MTT experiment was used to assess cell proliferation. Cell proliferation was expressed as the mean ± S.D. for the three independent wells of three different experiments. **(C)** Western blot analysis was used to detect the protein composition of RC-2 cell line-derived exosomes (EXO) and RC-2 cell lines (CE). Anti-HSP70 and anti-ICAM-1 antibodies were used.

cells after exosome treatment. However, this change in the cell cycle caused by exosomes was not observed after MK-2206 treatment (Figure 3A). All of these results showed that the pro-proliferation effect of exosomes could be blocked by the AKT inhibitor MK-2206.

Upregulated p-AKT and downregulated hepaCAM after exosome treatment in renal carcinoma cells. After exosome treatment for 0 h to 72 h, hepaCAM protein expression decreased in a time-dependent manner (Figure 3B). p-AKT level increased in RC-2/GFP-hepaCAM cells (Figure 4A). The p-AKT levels were almost restored to the level of the cells in the control group, but no significant change in the hepaCAM protein expression of RC-2/GFP-hepaCAM cells was observed (treated with MK-2206; Figure 4B). To further analyse the relationship of exosomes, hepaCAM and p-AKT, we compared p-AKT protein expressions after the cells were treated with exosomes for 72 h. The RC-2 cell-derived exosomes could significantly increase the p-AKT expression in RC-2 cell lines. The p-AKT expression in RC-2/GFP-hepaCAM cells was almost restored to the same level as the cells in the control group after exosome treatment. However, the exosomes that promoted p-AKT expression was not detected in RC-2/GFP-hepaCAM cells (treated with MK-2206; Figure 4C).

HepaCAM expression is correlated with clinicopathological characteristics and p-AKT in RCC samples. To further determine relevant evidence between hepaCAM and p-AKT in vivo, we analysed hepaCAM and p-AKT expressions in tissues obtained from 85 RCC tissues and 6 normal renal tissues (Table 1). HepaCAM staining was present mainly on the cell surface (Figure 5), and 57 (67%) patients revealed low expression. Furthermore, hepaCAM expression was positively
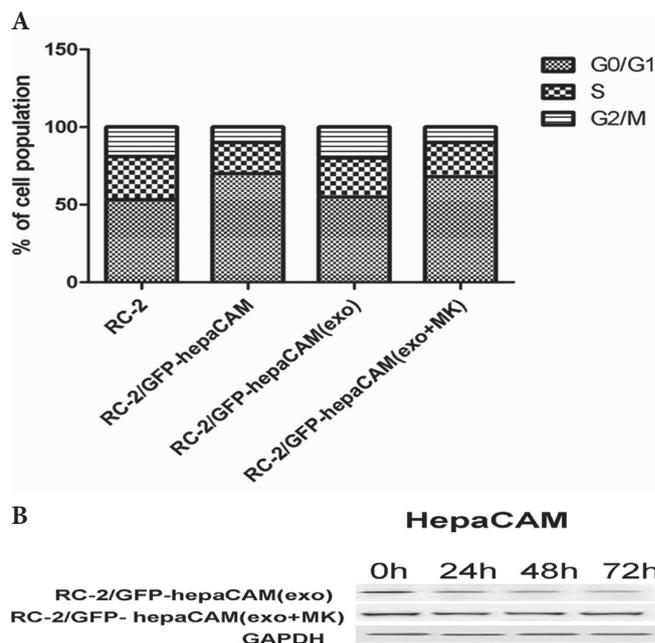
Figure 3. Effect of RC-2 cell-derived exosomes (exo) on its cycle and expression of hepaCAM. (A) After RC-2 cells (transfected by hepaCAM) were treated with exo for 72 h, MK-2206 (MK) was either added or not added to cells for 1 h. RC-2 cells (transfected or untransfected by hepaCAM) were treated with the same amount of DMSO. These cells were harvested and then processed for cell cycle analysis. One of the three similar experiments is shown. **(B)** After the cells were treated with 200 μg/ml RC-2-derived exosomes for 0 h to 72 h. HepaCAM protein levels were quantified using western blot analysis. Data were normalised using GAPDH as an internal control. The data shown are representative of three independent experiments. exo (treated with 200 μg/ml exosomes for the indicated time); MK (treated with 1 μM MK-2206 at the indicated time for 1 h).
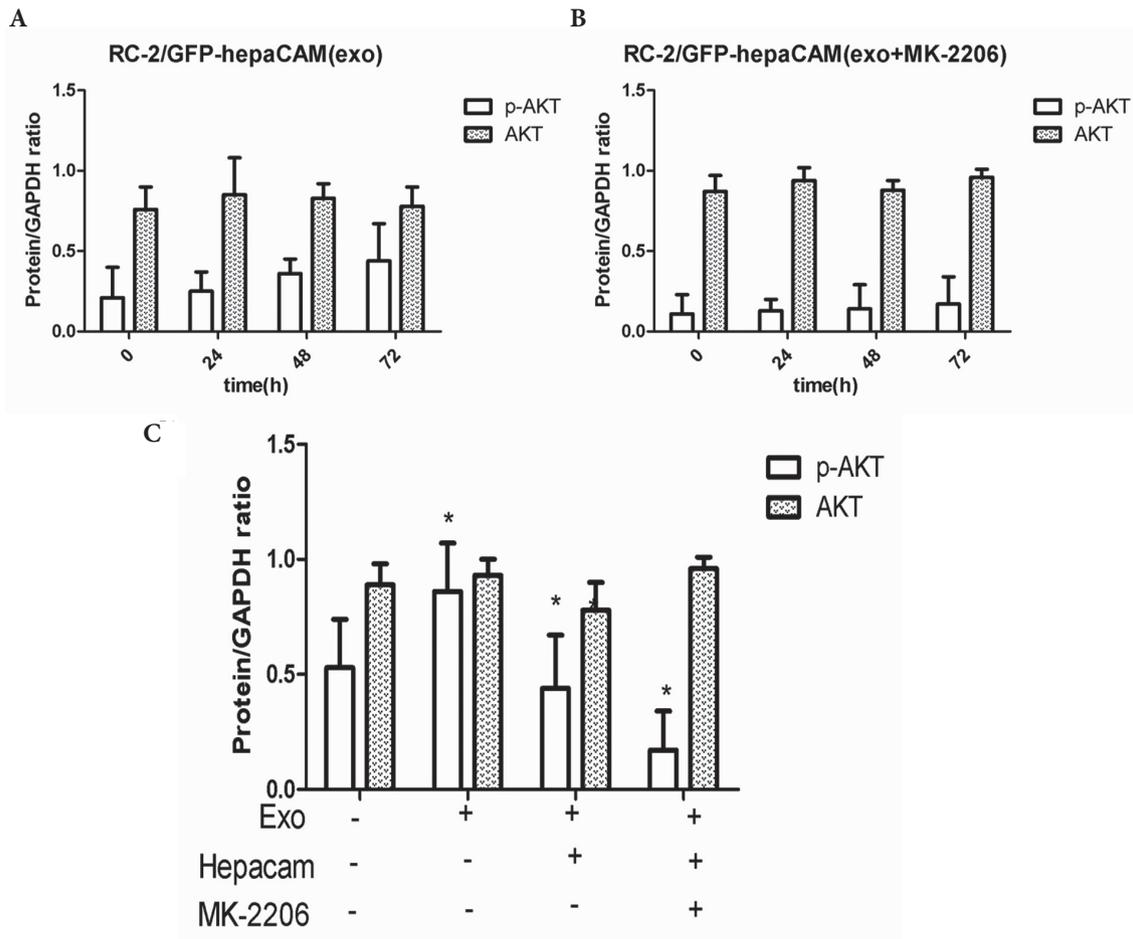
Figure 4. Effect of hepaCAM on RC-2 cell line-derived exosomes (exo)-mediated activation of AKT. (A, B) RC-2 cell lines (transfected by hepaCAM were pretreated with 200 μg/ml RC-2 cell line-derived exosomes for indicated time and then treated with AKT inhibitor MK-2206 (1 μM) at the indicated time for 1 h. (C) Cells (transfected or notransfected by hepaCAM) were pretreated or not with exosomes for 72 h and then treated or untreated with AKT inhibitor MK-2206 (1 μM) for 1 h. p-AKT and AKT were determined using western blot analysis. Data are expressed as mean ± SD of each group of cells from three independent experiments.*$P < 0.05$ vs. control group (RC-2 cells).

correlated with nuclear grade ($P = 0.025$). The same trend between hepaCAM levels and tumor-node-metastasis (TNM) stage ($P = 0.019$) was also observed (Table 1). Six (100%) normal renal tissues revealed a high expression of hepaCAM. P-AKT staining was mainly located in the cytoplasm (Figure 5). A total of 44 (51.8%) samples displayed high expression,

but no significant p-AKT expression was found in six normal renal tissues. The results of hepaCAM immunostaining of the RCC specimens showed a significantly negative correlation with p-AKT ($P = 0.017$; Table 2).

Discussion

Given that hepaCAM is a cell adhesion molecule, the gene expression level of the patients' carcinoma tissue was repeatedly low, and the same result [5,12] was observed in patients suffering from renal cancers. HepaCAM can inhibit cell proliferation, and our previous study revealed that methylation may be an important factor resulting in the low expression of hepaCAM in bladder carcinoma [17]. However, no report has been presented yet regarding the exact mechanism of low hepaCAM expression and the relationship between hepaCAM and tumor microenvironment in renal carcinoma.

Table 2 HepaCAM immunostaining correlates with p-AKT in RCC samples

HepaCAM	P-AKT	
	Low	High
Low	29	40
High	12	4
R	-0.258	
P	0.017	
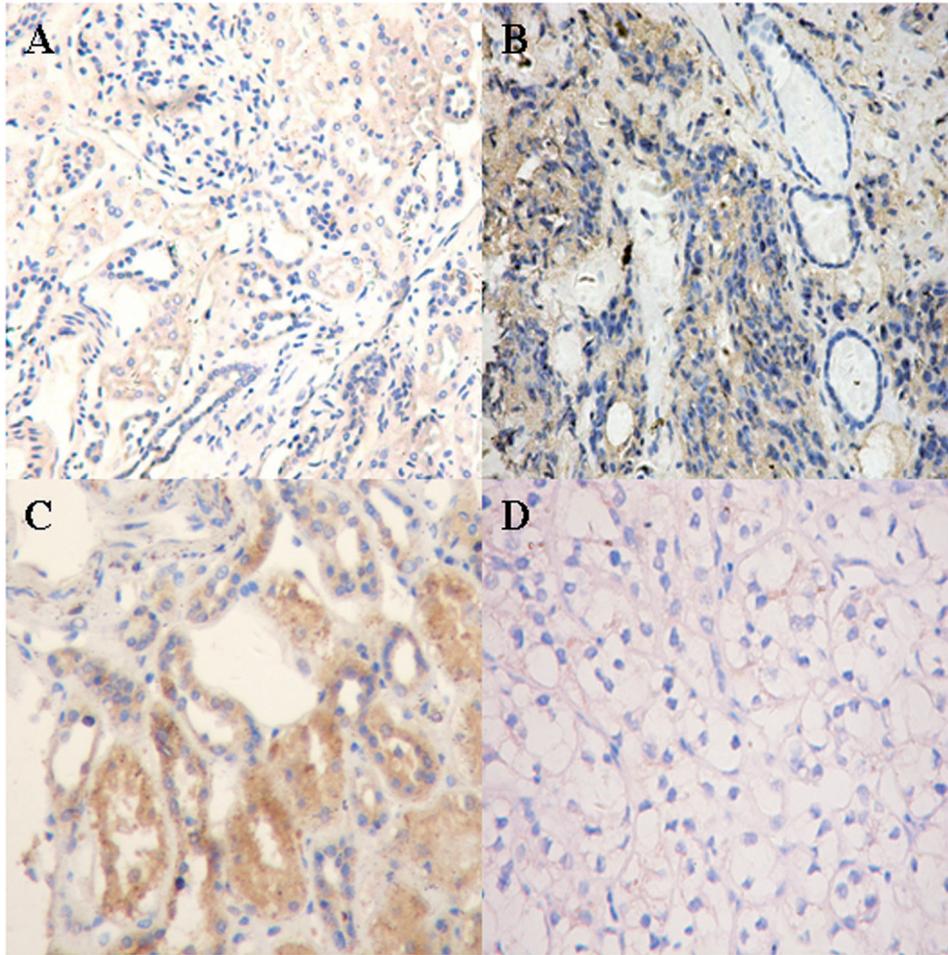
Figure 5. Immunolocalisation of hepaCAM and p-AKT in renal carcinoma and tumor-negative renal tissue. Immunohistochemistry was carried out on the paraffin sections of renal carcinoma and tumor-negative renal tissues by using hepaCAM and p-AKT antibodies. Low p-AKT expression in renal tumor-negative tissue (A), high p-AKT expression in renal carcinoma (B), hepaCAM overexpression in renal tumor-negative tissue (C) and low expression of hepaCAM in renal carcinoma. Magnification 400×.

Tumor-derived exosomes, or membrane-bound nano-particles present in a tumor microenvironment, significantly influence extracellular signal transmission. Gastric cancer exosomes also promote tumor cell proliferation [10]. Our previous studies also showed that hepaCAM re-expression in renal cancer cells can significantly inhibit cell proliferation [12]. To identify the essential factors triggering the downregulation of hepaCAM expression in renal carcinoma, we should investigate the effect of exosomes on hepaCAM expression. Therefore, this study isolated and purified the RC-2 cell-derived exosomes and constructed RC-2 cell lines, which exhibited a stable hepaCAM gene expression. MTT analysis results showed that the proliferation rate of RC-2 cell lines transfected by hepaCAM gene was downregulated and inhibited the p-AKT level. P-AKT is the activated form of AKT that regulates proteins involved in cell proliferation and apoptosis. p-AKT suppresses the pro-apoptotic activity of caspase-9 and BAD but activates several anti-apoptotic substrates [11,18]. p-AKT overexpression is

conducive to the development of malignancies and negatively affects prognosis [19]. p-AKT expression is also increased in RCCs [20]. Thus far, studies have been conducted to investigate the correlation between the AKT activity and its expression of cell adhesion molecules. Some of these studies have focused on the regulation of PI3-kinase/Akt/MAPK/NF-kappaB in the expression of vascular cell adhesion molecule-1 (VCAM-1) in human intestinal microvascular endothelial cells [21]; these studies have also observed withaferin A inhibition on VCAM-1 expression by blocking Akt and downregulating NF-kappaB activity [22]. In contrast to conventional cell adhesion molecules, hepaCAM largely functions as a tumor suppressor gene, but the correlation between AKT activity and hepaCAM expression of the primary tumor has not been evaluated. The results of this study showed that hepaCAM expression was deficient in RC-2 cells. As such, the RC-2 cells transfected by the hepaCAM gene exhibited a lower p-AKT expression than the untransfected cells. After exosome treat-

ment, the proliferation rate of RC-2 cell lines (transfected or untransfected by hepaCAM) increased, and the expression of p-AKT was upregulated. The level of hepaCAM expression in RC-2/GFP-hepaCAM cells decreased after exosome treatment. The population of RC-2/GFP-hepaCAM cells at G0/G1 phase was almost restored to the same population as RC-2 cells after exosome treatment. However, this change was not observed after MK-2206 treatment, and MK-2206 was a highly selective non-ATP competitive allosteric inhibitor of Akt. To further understand the mechanism that results in the downregulation of hepaCAM expression, we detected p-AKT expression in all of the tissues. The correlation between hepaCAM and p-AKT levels was analysed. Renal cancer exhibited a lower hepaCAM expression than adjacent and normal tissues, whereas p-AKT expression was higher than hepaCAM expression. This study demonstrated that hepaCAM downregulation was partially mediated by upregulating p-AKT in tumor tissues. Moreover, p-AKT upregulation was likely caused by tumor-derived exosomes present in a tumor microenvironment. Some of the limitations of this study were listed as follows: (1) the use of only one kind of renal carcinoma cell line and (2) the lack of information to determine the specific component of exosomes that caused the downregulation of hepaCAM expression. Such limitations should be further studied.

In conclusion, this study found that renal tumor-derived exosomes inhibited the hepaCAM expression of renal carcinoma cells in a p-AKT-dependent manner. These findings also provided the basis for future gene therapy of RCC.

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