Carboxypeptidase A6 Promotes the Proliferation and Migration of Hepatocellular Carcinoma by Up-regulating AKT Signaling Pathway*

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Summary: Hepatocellular carcinoma (HCC) has a poor treatment prognosis and high mortality worldwide. Understanding the molecular mechanism underlying HCC development would benefit the identification of diagnostic biomarkers and the improvement of the treatment strategies. The expression of carboxypeptidase A6 (CPA6) has been reported in epilepsy and febrile seizures rather than in any type of cancers. However, the function of CPA6 expression in HCC is not yet understood. In this study, we aimed to investigate the clinicopathological significance of the expression of CPA6 in HCC and the underlying mechanisms. We observed that the expression of the CPA6 protein was increased significantly in HCC tissues than in paracancerous tissues. To explore its function in HCC, both gain- and loss-of-function studies demonstrated that CPA6 played a vital role in promoting HCC growth and metastasis. When knocking down CPA6 with shRNA, HCC cell proliferation and migration could be suppressed. Meanwhile, CPA6 overexpression could promote proliferation and migration of HLF cells. Moreover, CPA6 could activate AKT serine/threonine kinase (AKT) signaling pathway as confirmed by Western blotting. In conclusion, our study revealed that CPA6 could promote HCC cell proliferation and migration via AKT-mediated signaling pathway. These findings suggest that CPA6 is a promising diagnostic biomarker and therapeutic target to improve the prognosis of HCC.
Key words: carboxypeptidase A6; hepatocellular carcinoma; proliferation; migration; AKT

Hepatocellular carcinoma (HCC) is one of the most common malignancies with high mortality worldwide[1]. Less than 30% of HCCs are diagnosed at their early stage and are suitable for liver transplantation, resection, or local ablation[2]. In many cases, the only effective systemic therapy depends on the chemotherapeutic drugs, i.e., sorafenib. However, none of these treatment strategies showed any positive results on the prognosis of HCC[3]. Clearly, it is urgent to clarify the molecular mechanism of HCC development to identify better diagnostic biomarkers and to improve treatment strategies.

It has already been reported that carboxypeptidases are composed of three groups: the A/B group, the N/E group, and the Nna1/CCP group[4, 5]. All metallo-CPs have structurally similar CP domains. It is known that the majority of A/B and N/E enzymes are secreted and function outside of the cell[5]. Carboxypeptidase A6 (CPA6) is a member of the M14 metallocarboxypeptidase family, which is highly expressed in the adult mouse olfactory bulb and broadly expressed in embryonic brain and other tissues. A disorder in the human CPA6 gene is linked to Duane syndrome, a defect in the abducens nerve/lateral rectus muscle connection[6, 7]. However, its expression and function in HCC remain unclear.

Previous studies also demonstrated that AKT plays an important role in various cellular processes and its aberrant activation is often identified in various cancer types[8, 9]. AKT is located at the central position of a notable signaling pathway–phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, which participates in HCC progression and is related with poor prognosis of HCC patients[10]. Therefore, deeply understanding of the mechanisms of the AKT signaling pathway could provide new insight into the carcinogenesis of HCC.

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In the present study, we provide for the first time to our knowledge that CPA6 is up-regulated in HCC and its expression is related with proliferation and migration of HCC by activating AKT signaling pathway. Our results revealed the role of CPA6 in HCC progression.

1 MATERIALS AND METHODS

1.1 Patients and Tissue Specimens

A total of 60 pairs of randomly selected snap-frozen tissues from consecutive patients received hepatectomy for HCC at the Hepatic Surgery Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). They were enrolled into this study from January 2012 to December 2014. The diagnosis of HCC was confirmed by two independent histopathologists. The surgical indication was an HCC patient with liver tumor of enough residual liver volume, but without distant metastasis, decompensated cirrhosis, or organic dysfunction. All researches on human materials were approved by the Ethic Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The study was conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from each patient.

1.2 Cell Lines and Cell Culture

97H and LM3 cell lines were obtained from the Liver Cancer Institute of Fudan University (China). Human fetal liver cell line HL-7702, hepatoma cell line HepG2, and HCC cell lines Hep3B and SK-Hep1 were purchased from China Center for Type Culture Collection (CCTCC, China). BEL7402 cells were deposited in the Hepatic Surgery Center, Tongji Hospital. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, USA) and incubated in 5% CO_2 at 37°C.

1.3 Lentivirus Production, Transfection and Establishment of Stable Cell Clones

The pLKO.1-TRC cloning vector (cat. No. 10878; from Addgene, Inc., Cambridge, USA), pMD2.G and psPAX2 (cat. Nos. 12259 and 12260; Addgene, Inc., USA), cDNA encoding full-length human CPA6 (CH816589, Vigenebio) were obtained commercially. cDNA encoding full-length human CPA6 was subcloned into the *Xba*I/*Bam*HI sites of the pLenti vector (cat. No. 39481; Addgene, Inc., USA). Short hairpin (sh)RNAs against human CPA6 were subcloned into the *Age*I/*Eco*RI sites of pLKO.1 vector. The sense sequences (#1, TRCN0000047006; sense 5′ CCGGGTTTGGATAGACTGTGGTATTCTCGAGAATACCACAGTCTATCCAAACTTTTTG-3′ and #2, TRCN0000047007: sense 5′-CCGGCAGATGTTACTGTATCCCTATCTCGAGATAGGGATACAGTAACATCTGTTTTTG-3′) as well as control vectors (cat. No. SHC005) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). For plasmid transfection, 293T cells (China Center for Type Culture Collection, China) were plated into 6-cm dishes (2×10^6 cells) and were co-transfected with target plasmids (1 μg) and virus packaging plasmids (pMD2.G, 0.25 μg; psPAX2, 0.75 μg) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., USA) at 37°C. Eight h after transfection, the cells were cultured with fresh medium containing 10% FBS and were incubated for 48–72 h at 37°C. Lentiviral supernatants were filtered through a 0.45-μm filter (Pall Life Sciences, Port Washington, USA), concentrated with Centricon Plus 70 (Merck KGaA) according to the manufacturer's protocol, and used to infect ALEX cells (1×10^6 cells/well in 6-well-plates). Lentiviruses were infected into HCC cells in the presence of polybrene (8 μg/mL). After a total of 48 h post-infection, 5 μg/mL puromycin was added to the growth medium to select cells. Knockdown or overexpression efficiency was verified by Western blotting.

1.4 Cell Proliferation Assay

To determine the effects of CPA6 on the proliferation of HCC cells, 1000 cells/well were cultured in 96-well plates; each well contained 200 μL medium. Cell proliferation was detected using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Japan), according to the manufacturer's protocol. After 24, 48, 72 and 96 h, 10 μL CCK-8 assay reagent was added to each well mixed with 90 μL serum-free medium. The absorbance (*A*) was measured 1.5 h later using a microplate reader at a test wavelength of 450 nm.

1.5 Wound Scratch Assay

A confluent monolayer of HCC cells (confluence, 95%) was cultured overnight and a scratch was introduced using a pipette tip. Cell migration was recorded under a phase contrast microscope (Nikon Digital Eclipse C1 system; magnification, ×100; Nikon Corporation, Japan) with white light 0 and 24 h after scratch generation. Images of five random fields across three replicate wells were captured for semi-quantification using Image-Pro Plus 6.0 (Media Cybernetics, Inc., USA).

1.6 Transwell Cell Migration and Invasion Assays

Cell migration assays were performed using a 24-well Transwell plate (pore size, 8 μm; Corning Incorporation, Corning, USA), according to the manufacturer's protocol. For the matrigel invasion assay, filters were precoated with 50 μL 1:4 mixture of Matrigel (BD Biosciences, Franklin Lakes, USA) and DMEM for 4 h at room temperature. For invasion and migration assays, culture medium containing 10% FBS was quickly added to the lower chambers

and aliquots of 5×10^4 cells in 100 μL serum-free medium were seeded into the upper chambers. After 24 h incubation at 37°C, non-migrated or non-invaded cells were removed by scraping the upper surface of the membranes with a cotton swab. Cells on the lower surface of the membranes were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with 0.1% crystal violet at room temperature for 20 min. Cell number was counted under an optical microscope. Each experiment was repeated at least three times.

1.7 Western Blot Analysis

Cells were lysed with RIPA (Beyotime, China) containing a protease inhibitor mixture on ice for 30 min. Cell lysates were quantitated by BCA protein assay before being subjected to SDS-PAGE and then transferred to the PVDF membrane (Roche Life Sciences, Switzerland). The membranes were blocked with 5% non-fat milk and incubated with the primary antibodies at 4°C overnight. After that, the membranes were incubated with HRP goat anti-rabbit or goat anti-mouse IgG (1:4000, Jackson ImmunoResearch Laboratories, Inc., USA) at 37°C for 1 h. Finally, the ECL detection system was used for visualization. Sources of antibodies and concentrations used were as follows: rabbit anti-CPA6 (1:1000, abs138857, Absin, China), rabbit anti-AKT (1:1000, #4685, CST, USA), rabbit anti-p-AKT (Ser473) (1:1000, #4060, CST, USA), mouse anti-Flag (1:1000, F1804, Sigma, Germany), mouse anti-GAPDH (1:10000, KC-5G4, KangCheng, China).

1.8 Animal Study

A total of 10 male BALB/c nude mice (age, 4 weeks; weight, 18–19 g) were purchased from the Animal Center of East China Normal University, Shanghai, China, after signing the animal-raising agreement. Mice were randomly divided into two groups (5 in each group). After anesthesia, the control group was injected with 2×10^6 HLF cells transduced with vector virus in 100 μL DMEM, and the treatment group with 2×10^6 HLF cells transduced with CPA6 virus in 100 μL DMEM. The length and width of tumor were manually monitored by using a vernier caliper in nude mice twice a week. The tumor volume was calculated according to the equation of V (volume, mm^3) = 0.5×L (length, mm) × W^2 (width, mm^2). Six weeks after injection, all the mice were sacrificed after euthanasia, tumor tissue was removed and tumor weight was measured. All of the animal experiments met the National Institutes of Health guidelines and were approved by the Committee on the Ethics of Animal Experiments of the Tongji Medical College, HUST.

1.9 Statistical Analyses

All experiments were repeated at least twice with consistent results. Quantitative results are presented as $\bar{x}\pm s$. The significance of differences, unless otherwise indicated, was determined by an unpaired two-tailed Student's *t*-test (with Welch's correction for unequal variances where necessary). All the statistical analyses were performed with SPSS Statistics version 20 (Armonk, USA) and GraphPad Prism 6 (San Diego, USA). Differences were considered statistically significant at $P<0.05$.

2 RESULTS

2.1 Up-regulation of CPA6 in Human HCC

To investigate the potential role of CPA6 in HCC, HCC tissues and paired paracancerous tissues were collected from 60 HCC patients for comparison of CPA6 protein levels by Western blotting (fig. 1A and 1B). Results showed that, as compared with paracancerous tissues, up-regulation of CPA6 protein was observed in 14 paired HCC tissues. Thus, the data indicated that the expression of CPA6 was significantly increased in HCCs.

2.2 Expression of CPA6 in HCC Cell Lines

To explore CPA6 expression in different HCC cell lines, the expression levels of CPA6 protein were detected in 9 different types of HCC cell lines by Western blotting (fig. 2A). We found significantly increased CPA6 levels in MHCC-97H, HCC-LM3, and ALEX cell lines, which have higher invasive and metastatic abilities. In cell lines with lower metastatic potentials, i.e., Bel-7402, Hep3B, and HepG2, CPA6 protein levels were significantly lower and there were no significant differences compared with normal liver cell line 7702 (fig. 2A). We then generated two CPA6-specific shRNAs to silence the endogenous CPA6 expression (shCPA6) of ALEX cells (fig. 2B). We also transfected HLF cell line with lentiviral construct CPA6-FLAG to up-regulate CPA6 protein level (fig. 2C). These data showed that CPA6 expression was drastically decreased in ALEX cells treated by shCPA6 and CPA6 expression was highly increased in HLF cells treated by CPA6-FLAG.

2.3 CPA6 Promotes HCC Growth and Metastasis

In order to explore the functions of CPA6 in HCC cells, CCK-8 assay was performed to detect the effect of CPA6 on cell growth in HCC cells (fig. 3A). By using cell lines which had been constructed above, we found that CPA6 knockdown significantly attenuated the proliferation of ALEX compared with control group. When CPA6 was overexpressed, the proliferation ability of HLF was highly increased (fig. 3B). *In vitro* scratch wound healing assay suggested that CPA6 could increase cellular migration, while the inhibition of CPA6 by shCPA6 decreased cellular migration (fig. 4A and 4B). Consistent with the results from the scratch wound healing assay, *in vitro* Transwell migration assay also confirmed that shCPA6

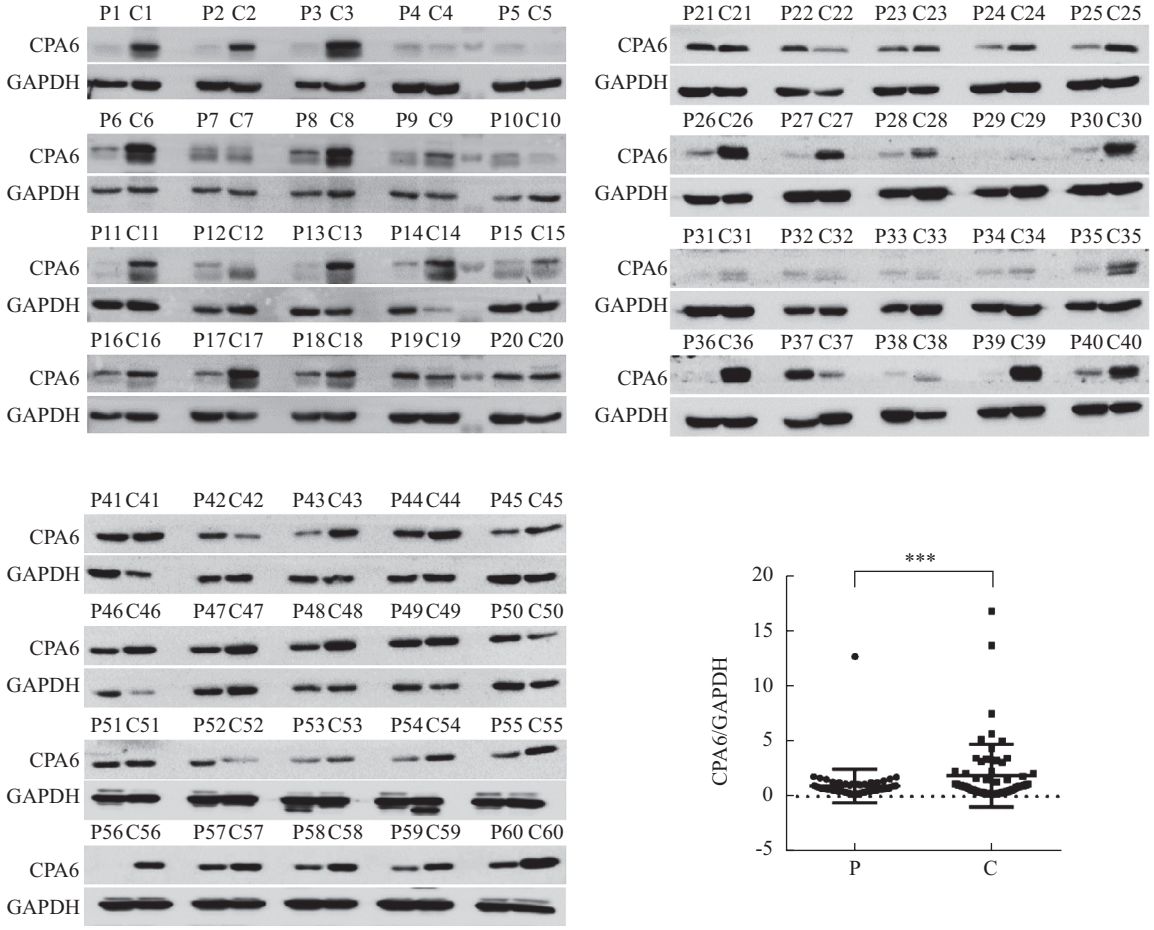

Fig. 1 Detection of expression of CPA6 in HCC by Western blotting
CPA6 protein expression in 60 pairs of HCC tissues was analyzed, and statistically significant difference was found in relative expression level of CPA6 between HCC tissues and normal tissues. ***P<0.001; P: para-carcinoma tissue; C: HCC tissue

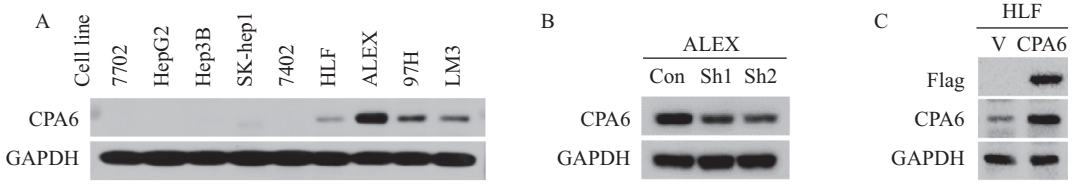

Fig. 2 Expression of CPA6 in HCC cell lines
A: Protein expression of CPA6 in 9 HCC cell lines. GAPDH was used as an internal control. B: ALEX cells were infected with CPA6 shRNA lentivirus (sh1 and sh2) or control shRNA lentivirus (Con). CPA6 protein expression was analyzed by Western blotting. C: Overexpressed CPA6 in HLF cells with lentivirus and CPA6 protein expression was also analyzed. V: vector

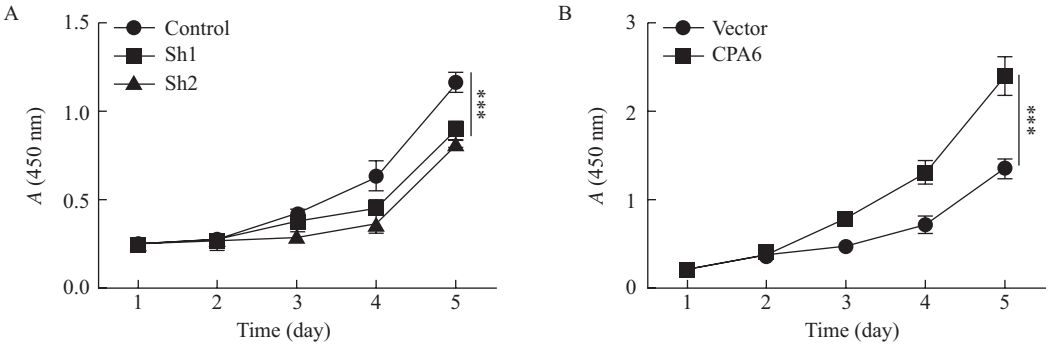

Fig. 3 CPA6 promoted proliferation in HCC cells
A: Effects of ALEX CPA6 knockdown on proliferation was evaluated by CCK-8 assays and clone formation assay; B: Effects of HLF CPA6 overexpression on proliferation was evaluated by CCK-8 assays. ***P<0.001

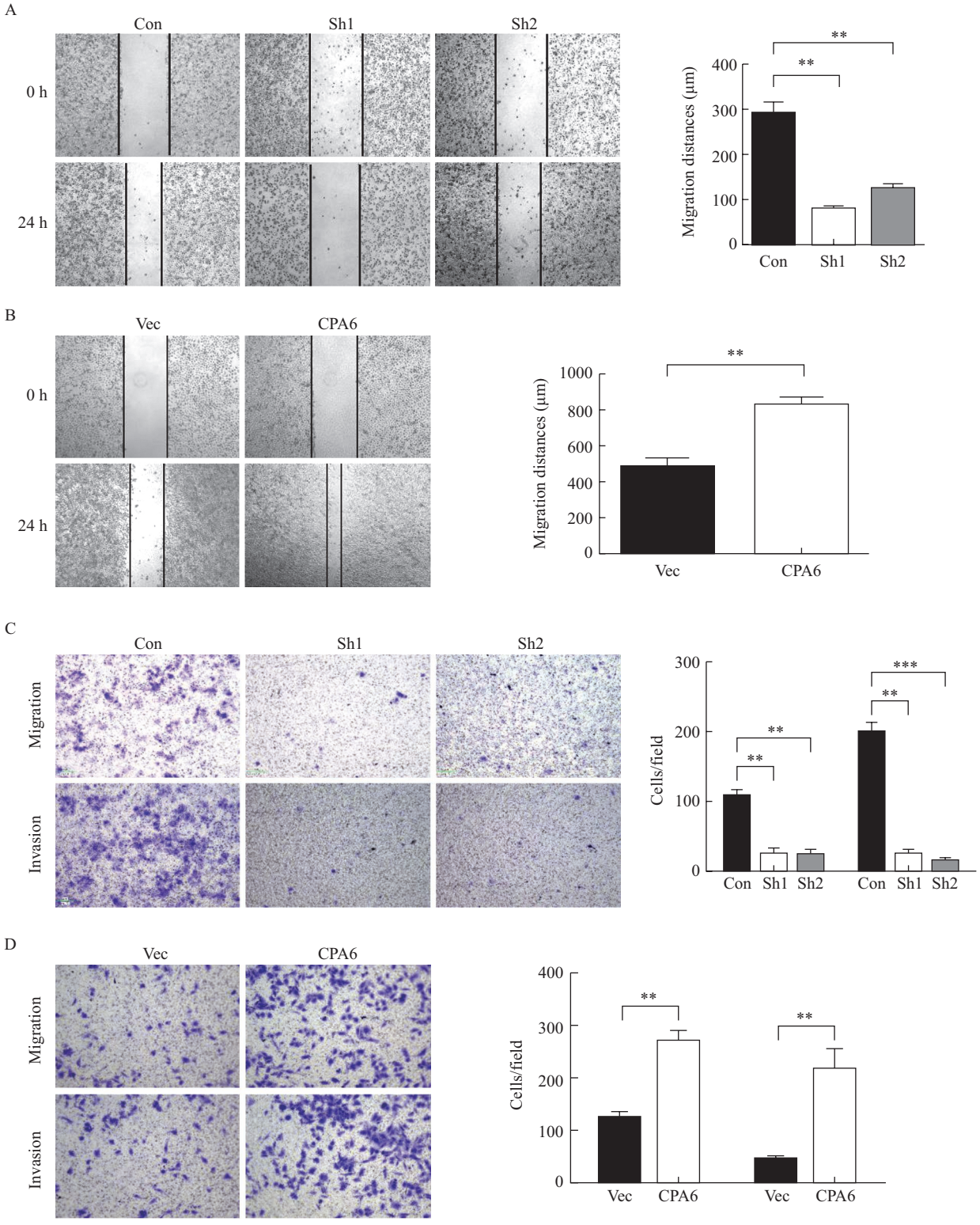

Fig. 4 CPA6 promoted migration and invasion in HCC cells

A, C: Effects of CPA6 knockdown on migration and invasion were measured by transwell assays; B, D: Effects of CPA6 overexpression on migration and invasion were measured by transwell assay. Results are presented as mean±SEM ($^{***}P<0.001$; $^{**}P<0.01$) of triplicate determination from three independent experiments. Vec: vector; Con: control

inhibited the migration and invasion of ALEX cell line (fig. 4C). Similarly, migration and invasion ability were significantly increased in cells with endogenous up-regulation of CPA6 by transfection of CPA6-FLAG than in cells with low expression of CPA6 (fig. 4D). Taken together, these *in vitro* studies of CPA6 with enhancement and functional loss identified that CPA6 plays significant roles in facilitating HCC growth and

metastasis.

2.4 CPA6 Activates AKT Signaling Pathway

To elucidate the molecular mechanisms of CPA6 in promoting HCC metastasis, we checked the expression of several key signaling pathways. Notably, the level of p-AKT (Ser473) was increased in CPA6 overexpression cells but decreased in CPA6 low expression cells (fig. 5). As activation of AKT signaling pathway is closely correlated with oncogenesis and metastasis of many cancers, CPA6 may promote the development and metastasis of HCC through regulation of the AKT signaling pathway.

2.5 CPA6 Promotes HCC Growth *In Vivo*

To further analyze the effect of CPA6 on HCC growth *in vivo*, we exploited the xenograft mouse model. HLF cells transduced with a CPA6 overexpression virus or vector virus were transplanted into nude mice. We found tumor growth significantly increased in CPA6 groups as compared with the vector group (fig. 6A). At 6 weeks after inoculation, tumor volume (fig. 6B) and weight (fig. 6C) was noticeably higher in the CPA6 groups than in the vector group.

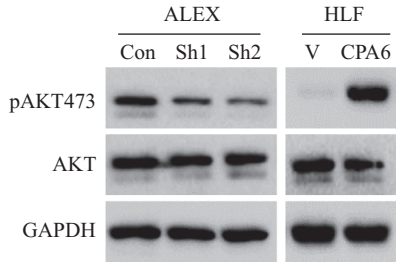

Fig. 5 CPA6 activates AKT signaling pathway in HCC cells
Expression levels of p-AKT (Ser473) and AKT protein were analyzed in ALEX and HLF cells. Con: control; V: vector

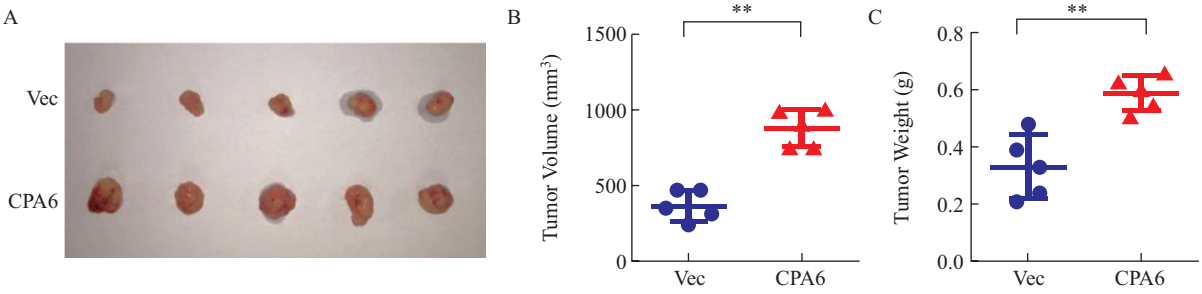

Fig. 6 CPA6 promotes HCC growth in vivo
A: Representative images of isolated tumors in the control group (first row), CPA6 group (second row). B: Xenograft tumor volume in CPA6 nude mice was larger than that in the control group. C: Xenograft tumor was heavier in the CPA6 nude mice than in the control group. $^{***}P<0.001$. Vec: vector

3 DISCUSSION

CPA6, a vital subtype of metallocarboxypeptidase family, plays an important role in the process of epilepsy and febrile seizures[4, 5]. Previous studies have shown four types of rare pseudomutations in patients with focal epilepsy, of which CPA6 is one of the most common and significantly associated with pathogenesis[5]. Another study also demonstrated that CPA6 protein cleaves angiotensin Ⅰ into angiotensin Ⅱ and may play a role in brain development and neuronal migration[6]. CPA6 also suppresses neurotensin, which plays a role in thermal regulation, stress, and depression[11]. To the best of our knowledge, no reports have identified CPA6 expression and its relationship with cancers. The goal of this study is to investigate the effect of CPA6 on the development of HCC and the action mechanism. By comparing 60 HCC tissues and paired paracancerous tissues, we found that CPA6 expression was significantly higher in cancer tissues than in paracancerous tissues. To validate the Western blotting result, we also examined its expression in HCC cell lines. Our results demonstrated that CPA6 might play an important role in HCC proliferation and migration.

According to the Western blotting result observed above, CPA6 expression was extremely weak in cell lines with lower metastatic potentials. Reversely, CPA6 expression was significantly higher in cell lines with higher metastatic potentials. Next, we selected two different cell lines to construct knockdown cell lines and overexpression cell lines, namely ALEX and HLF. The results identified the knockdown and overexpression of CPA6 protein levels in the HCC cell lines. Another vital phenomenon we observed was that CPA6 deficiency could ameliorate HCC cell proliferation by CCK-8 assay. In addition, through Transwell assay and wound healing assay, it was identified that CPA6 could facilitate HCC migration and proliferation. Therefore, our findings indicate that CPA6 could facilitate the migration and proliferation of HCC.

AKT is an oncogene and its activation could be observed in nearly every type of tumors including HCC[12, 13]. It is well known that PI3K/AKT is involved

in cell survival, growth, proliferation, repair, migration and angiogenesis[14–17]. CPA6, according to our result, could activate AKT, which supports its function of promoting invasion and migration[18–20].

In conclusion, our study is the first to report the potential role of CPA6 in HCC invasion and migration. More importantly, our study illustrates the connection of CPA6 with AKT signaling pathway and provides new insights of CPA6 in HCC migration. CAP6 might be a promising diagnostic biomarker and treatment target for HCC.

Conflict of Interest Statement

The authors declare no potential conflicts of interest.

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