AP-2α inhibits hepatocellular carcinoma cell growth and migration

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Abstract. Transcription factor AP-2α is involved in many types of human cancers, but its role in hepatocellular carcinogenesis is largely unknown. In this study, we found that expression of AP-2α was low in 40% of human hepatocellular cancers compared with adjacent normal tissues by immunohistochemical analysis. Moreover, AP-2α expression was low or absent in hepatocellular cancer cell lines (HepG2, Hep3B, SMMC-7721 and MHHC 97-H). Human liver cancer cell lines SMMC-7721 and Hep3B stably overexpressing AP-2α were established by lentiviral infection and puromycin screening, and the ectopic expression of AP-2α was able to inhibit hepatocellular cancer cell growth and proliferation by cell viability, MTT assay and liquid colony formation *in vitro* and *in vivo*. Furthermore, AP-2α overexpression decreased liver cancer cell migration and invasion as assessed by wound healing and Transwell assays, increasing the sensitivity of liver cancer cells to cisplatin analyzed by MTT assays. Also AP-2α overexpression suppressed the sphere formation and renewed the ability of cancer stem cells. Finally, we found that AP-2α is epigenetically modified and modulates the levels of phosphorylated extracellular signal-regulated protein kinase (ERK), β-catenin, p53, EMT, and CD133 expression in liver cancer cell lines. These results suggested that AP-2α expression is low in human hepatocellular cancers by regulating multiple signaling to affect hepatocellular cancer cell growth and migration. Therefore, AP-2α might represent a novel potential target in human hepatocellular cancer therapy.

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Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer-related death worldwide with extremely poor prognosis (1). Liver transplantation and surgical resection are potentially curative options for HCC patients at an early stage of cancer progression. However, when the disease develops to an advanced stage, these treatments are not useful. Moreover, the most challenging problem for HCC clinical treatment is a high incidence of tumor recurrence and metastasis (2). Currently, sorafenib, an oral multikinase inhibitor targeting RAF kinases, is the only approved targeted therapy to prolong the overall survival of patients with advanced HCC (3). Molecular mechanisms of malignant transformation of hepatocytes, and development and progression of HCC still remain largely elusive. Therefore, identification of key genes that serve as early diagnosis and novel therapeutic targets is urgently needed.

AP-2α extensively functions as a tumor suppressor in various types of human solid tumors, including melanoma, breast cancer, prostate cancer and colorectal cancer. Clinical studies demonstrated that loss of AP-2α expression associates with cancer progression and predicts poor prognosis (4-9). Exogenous AP-2α in cancer cell lines inhibited cell growth *in vitro* (10), and transfection of AP-2α reduced the tumorigenicity and metastatic potential in nude mice, whereas inactivation of AP-2α enhanced tumorigenicity (7,11,12). However, some data showed that low cytoplasmic and high nuclear AP-2α expression were related to increased tumor malignancy and poor survival (13-17), suggesting that AP-2α acts as an oncogene in certain histological subtypes, such as acute myeloid leukemia (18) squamous cell carcinomas (19) and head and neck squamous cell carcinoma (HNSCC) (20).

AP-2α was able to inhibit cancer cell growth and motility, by modulating many downstream pathways including the HIF-1α-mediated VEGF/PEDF signaling pathway, β-catenin/TCF/LEF signaling, bax/cytochrome c/Apaf1/caspase 9-dependent mitochondrial pathway, CdK-inhibitor p21WAF in p53-dependent and -independent pathways (7,21-24), thus suppressing angiogenesis, cell cycle progression, cell proliferation and invasion. AP-2α expression sensitized cancer cells to chemotherapy and loss of AP-2α led to chemoresistance (25,26). Adriamycin, cisplatin, taxol, etoposide and carboplatin could synergistically combine with

AP-2α expression to enhance tumor killing (26). Alteration or post-translational modification in AP-2α protein can affect molecular pathogenesis of human cancers (27,28). Although AP-2α is reported to function in hepatocellular carcinogenesis (10), the molecular mechanisms are still elusive. To further study the role of AP-2α in human carcinogenesis, we compared the expression of AP-2α between human hepatocellular carcinoma tissues and live cancer cell lines. Moreover, the effects of AP-2α on cellular proliferation, migration, invasion and stem cell sphere-forming were detected not only *in vitro* but also *in vivo* assays. Furthermore, whether AP-2α increased the sensitivity of hepatocellular cancer cells to cisplatin and the molecular mechanisms of AP-2α in hepatocellular cancer cells were investigated in this study.

Materials and methods

Immunohistochemistry. Ninety hepatocellular carcinoma samples and 18 adjacent normal tissues were examined. The experiment was approved by Hunan Normal University Human Ethics Committee and informed consent was obtained from all patients. The immunohistochemical staining was performed on paraffin-embedded samples. Sections (5-μm thick) were deparaffinized and rehydrated. Endogenous peroxidase was quenched with 3% hydrogen peroxide (H_2O_2) for 10 min. For antigen retrieval, sections were immersed in 0.01 M citrate buffer (pH 6.0) and boiled for 10 min in microwave oven. Non-specific binding was blocked by 5% normal goat serum in PBS for 30 min. The mouse monoclonal anti-AP-2α antibody (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added at 4˚C overnight in a moist chamber. The sections were then sequentially incubated with HRP-conjugated goat anti-mouse IgG (1:500 dilution, Sigma-Aldrich Corp., St. Louis, MO, USA) for 45 min and 3,3-diaminobenzidine (DAB)/H_2O_2 for 10 min at room temperature. The sections were counterstained with hematoxylin, mounted and photographed using an optical microscope (Olympus CX41, Tokyo, Japan). The percentage of tumor cells stained was scored as: 0 (no cell staining), 1 (≤30%), 2 (31-60%) and 3 (61-100%). The staining between two score values was given 0.5.

Cell culture. SMMC-7721, Hep3B, HepG2, MHHC 97-H and HeLa cells were cultured in DMEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FBS, Hyclone, Australia), 100 U/ml penicillin and 100 μg/ml streptomycin. All the cells were maintained in a humidified atmosphere with 5% CO_2 at 37˚C.

AP-2α overexpression lentivirus generation. The full-length AP-2α cDNA was subcloned to pGC-FU-3xFlag-IRES-Puromycin vector, and pGCFU-GFP-3xFlag -IRES-Puromycin vector served as a negative control (NC). Lentiviral particles were prepared as described in our previous work (18). Briefly, the lentivirus expression plasmid and packaging plasmids (pHelper 1.0 and pHelper 2.0) were cotransfected into 293T cells, supernatants were harvested 48 h after transfection and filtered through a 0.45-μm pore size filter (Millipore, Billerica, MA, USA) and concentrated by ultracentrifugation. The infectious titer was determined using hole-by-dilution titer assay. SMMC-7721 and Hep3B cells were infected with AP-2α-Flag-lentivirus or GFP-Flag-lentivirus at the multiplicity of infection (MOI) of 10 in the presence of 5 μg/ml polybrene (Sigma) and detected on the 4th day by the invert fluorescence microscope followed by the screening of 1.5 μg/ml of puromycine for stable cell lines.

Cell survival assay. 100,000 cells stably expressing AP-2α-Flag or GFP-Flag and parental cells were plated in triplicate in 6-well plates in complete medium. After 1-4 days, cell numbers were counted with a hemocytometer after trypan blue staining of viable cells.

MTT assay. To detect the cell growth rate, 3,000 cells per well were plated in 96-well plates untreated or treated with 30 μM of cisplatin (DDP) or 0.9% NaCl (NS) for 48 h. On day 1-7, cells were analyzed with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) at 37˚C for 4 h. Then 100 μl dimethylsulfoxide (DMSO) per well was added to dissolve the formazan crystals. The absorbency at 490 nm was measured with a spectrophotometer (UV-2102C, Changsha, China). Triplicate independent assays were performed in octuplicate.

Liquid colony formation was performed as previously described (29). Briefly, 2,000 cells were seeded in duplicate in 6-well plates and grown for over 10 days. Colonies were fixed with methanol, stained with Giemsa (BBI International, Cardiff, UK) and photographed with the digital camera (Olympus). Only colonies containing over 30 cells were counted. All experiments were carried out for at least three times.

Tumor xenograft mouse model. Approximately $2x10^7$ of lentivirus-infected AP-2α-SMMC-7721 or GFP-SMMC-7721 cells in 0.2 ml of sterile PBS were injected subcutaneously into the left and right dorsal regions of 4- to 5-week-old female nude mice (n=6 mice), respectively. Mice were checked every 2 days and the formed tumors were measured as previously described (30). After 41 days, mice were sacrificed, and tumors were excised, weighed and photographed. The mouse experiments were carried out according to the ethical guidelines for laboratory animal use and approved by the Ethics Committee of Hunan Normal University.

Wound-healing assay. SMMC-7721 cells were cultured in 6-well plates until over 95% confluence. A 100-μl pipette tip was used to generate wounds. After wound creation, the medium was changed to remove cellular debris. Three wounded areas in each well were marked on the bottom of plates and photographed at 1 and 3 days with an invert microscope (Zeiss Axioskop 2, LLC, Thornwood, NY, USA).

Cell migration and invasion assay. The Transwell cell migration and invasion assays were performed in polyethylene terephthalate (PET)-based migration chambers and BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA, USA) with 8 μm porosity according to the manufacturer's instructions. Tumor cells ($4x10^4$) in serum-free DMEM/F12 were seeded onto uncoated or Matrigel-coated filters in the upper chambers. DMEM/F12 containing

15% FBS was added to the lower chambers. After 48-72 h of incubation, cells on the upper surface of the filters were removed with a cotton swab, and the filters were fixed with 100% methanol and stained with Giemsa. The migration and invasive ability of the tumor cells was expressed as the mean number of cells in all fields. The experiments were performed three times independently.

Sphere-forming and self-renewal assay. Tumor cells were seeded at a density of 10,000 cells/ml in low-attachment culture bottles and grown in serum-free DMEM/F12 supplemented with 20 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor (PeproTech, Inc., Rocky Hill, NJ, USA), 0.4 μg/ml insulin and B27 (1:50; Gibco, Karlsruhe, Germany). The number of spheres was counted after 5 or 10 days, respectively. After sphere-forming culture, photos were observed with the invert microscope every 2 days to observe cell size and morphology. The sphere-forming cells were separated into single cells using 1X Trypsin-EDTA and analyzed by cell survival and MTT assays.

To investigate self-renewal capacity of liver cancer spheres, single cell suspension from the sphere forming cells was prepared and plated in 96-well ultra-low plates containing 200 μl serum-free medium per well. Single cell in these wells was monitored daily under the invert microscope for 9 days.

To analyze the effects of cisplatin on sphere forming of AP-2α-overexpressing SMMC-7721 cells, suspension cells from the sphere forming cells were plated at a density of 10,000 cells/well in 24-well ultra-low plate. Cisplatin (DDP) (30 μM) or 0.9% NaCl (NS) was added to medium on day 2. After further culturing for 3 days, the spheres were analyzed by optical microscopy, MTT assays and cell counting.

Western blotting. Cells were lysed in RIPA buffer as previously described (31). Briefly, the lysates were denatured and heated to 95˚C for 5 min. Samples were then separated on 10-15% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were detected with rabbit polyclonal antibodies against GFP and MMP9, mouse monoclonal antibodies against ERK and phosphorylated ERK, cyclin D1 (CCND1), Flag, c-Myc, p53, CD133, E-cadherin, Vimentin, β-catenin and GAPDH (Santa Cruz Biotechnology). HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Sigma) were used. The signal was detected with SuperSignal West Pico chemiluminescent Substrate (Thermo Scientific Pierce, Rockford, IL, USA) and visualized with tanon-5200 system (Bio-Tanon, Shanghai, China). The data are presented as mean ± SD (n=3).

Luciferase reporter assays. Hep3B cells were cultured in 12-well plates and transfected with reporter plasmid pTOP-Flash and the indicated plasmids using Lipofectamine 2000 as previously described (32).

Statistical analysis. All statistical analyses were performed using the SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Data are shown as mean ± SD from at least 3 independent experiments. Statistical significance was determined using Student's t-test at P-value <0.05.

Table I. Patient clinicopathological characteristics in 90 cases of hepatocellular carcinoma.

Clinical features	Number (%)
Gender	
Female	13 (14.3)
Male	77 (85.7)
Age (median, 48 years)	
<48 years	33 (36.7)
≥48 years	57 (63.3)
Tumor size, cm	
≤5	61 (67.8)
>5	29 (32.2)
Cell differentiation	
Well (I-II)	47 (52.2)
Moderately (III)	43 (47.8)
Tumor stage	
Stage III	22 (24.5)
Stage II	56 (62.2)
Stage I	12 (13.3)
Primary tumor	
Tx	2 (2.2)
T1	5 (5.6)
T2	56 (62.2)
T3	24 (26.7)
T4	3 (3.3)
Normal tissue	18

Results

AP-2α is lowly expressed in high-grade hepatocellular cancer tissues and high-invasive cell lines. The expression level of AP-2α was examined in 12 WHO grade I, 56 WHO grade II, 22 WHO grade III hepatocellular cancers and 18 adjacent normal tissues by immunohistochemistry analysis using mouse monoclonal anti-AP-2α antibody. We found that AP-2α was completely localized in the nucleus (Fig. 1A). AP-2α expression was detected in 34 (37.8%) of the 90 hepatocellular cancers with strong staining (3+), 20 (22.2%) of the 90 hepatocellular cancers were moderately stained (2+), and 36 (40%) were weakly stained or negative for AP-2α expression (+/0) (Fig. 1B), which indicated AP-2α was lowly expressed in hepatocellular cancers (P<0.05) according to Student's t-test. A high expression of AP-2α was observed in human normal liver and hepatocellular cancers (I) (Fig. 1A and B). Therefore, AP-2α expression was significantly decreased in human high-grade hepatocellular cancer tissues (II and III) than normal liver and hepatocellular cancer tissues (I). Patient characteristics are summarized in Table I.

We next analyzed the expression of AP-2α proteins in human hepatocellular cancer cell lines. Low expression or loss of AP-2α proteins was evident in Hep3B, HepG2, SMMC-7721 and MHHC 97-H cells compared to HeLa cells

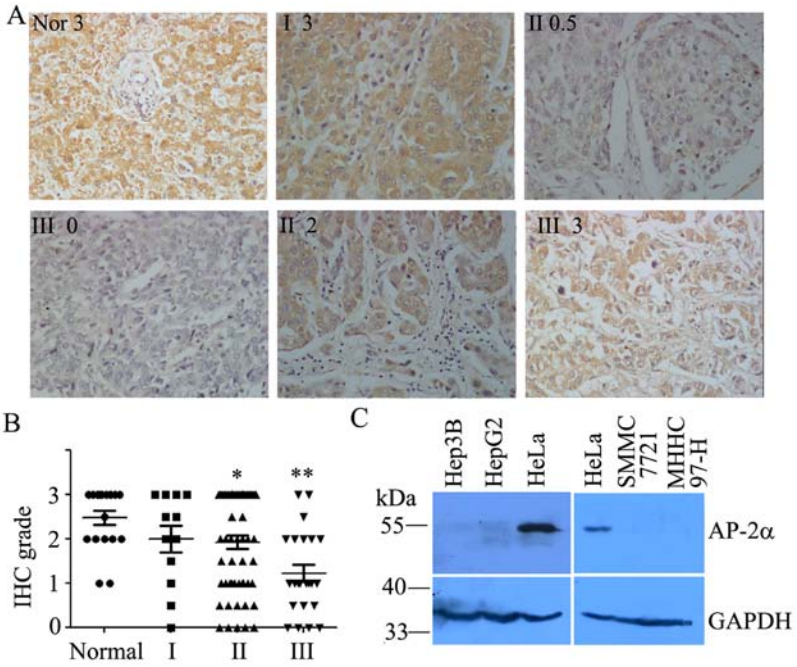

Figure 1. The expression levels of AP-2α in hepatocellular cancer tissues and cell lines. (A) AP-2α expression was examined by immunohistochemical analysis in 90 hepatocellular tumors and 18 adjacent normal tissues. Strong nucleic expression of AP-2α (brown staining) was detected in normal tissues and stage I hepatocellular cancer cells while the nucleus was stained blue with hematoxylin. (B) Immunohistochemical stages of hepatocellular cancers and normal tissues stained with monoclonal anti-AP-2α antibody. The staining intensity was scored with grades 0-3. Each symbol represents an individual sample. Statistical comparisons between hepatocellular cancers and normal tissues were performed according to Student's t-test. *P<0.05; **P<0.01. (C) AP-2α protein levels in different hepatocellular cancer cell lines were detected by western blotting. GAPDH was used as an internal control.

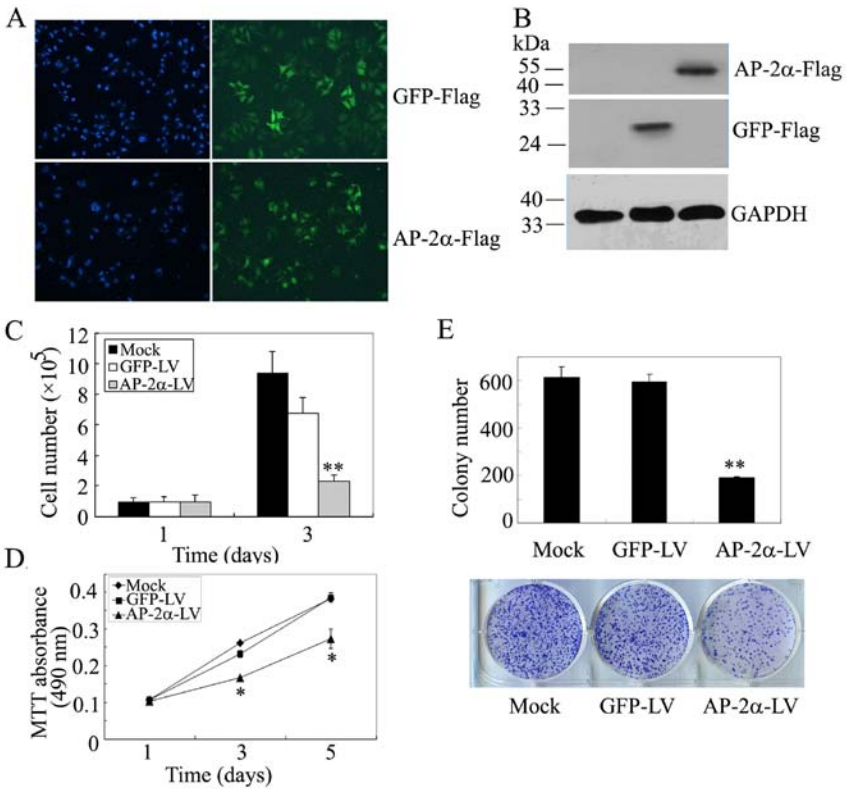

Figure 2. Effects of AP-2α overexpression on SMMC-7721 hepatocellular cancer cell proliferation. (A) Immunofluorescence staining of AP-2α and GFP expression in SMMC-7721 cells stably expressing AP-2α and GFP. (B) Western blot of GFP/Flag and AP-2α/Flag expression in SMMC-7721 cells using anti-Flag antibodies. GAPDH served as a loading control. (C) Cell survival assays of AP-2α-infected SMMC-7721 cells, GFP-infected SMMC-7721 cells and parental SMMC-7721 cells. Cells (100,000) were plated into 6-well plates in triplicate, grown in DMEM with 10% FBS for 3 days and stained with trypan blue in PBS, viable cells were counted. (D) MTT assays of mock or infected SMMC-7721 cells. Cells (3,000) were plated in octuplicate in 96-well plates and grown in DMEM with 10% FBS. The absorbance was analyzed for 1, 3 and 5 days. (E) Liquid colony formation analysis of mock or infected SMMC-7721 cells. Cells (1,000) were seeded in triplicate in 6-well plates, and grown for 10 days. Colonies were fixed with methanol, stained with Giemsa, images were taken (lower panel) and counted (upper panel). The data represent at least three independent experiments with similar results. **P<0.01, compared with parental and control cells.

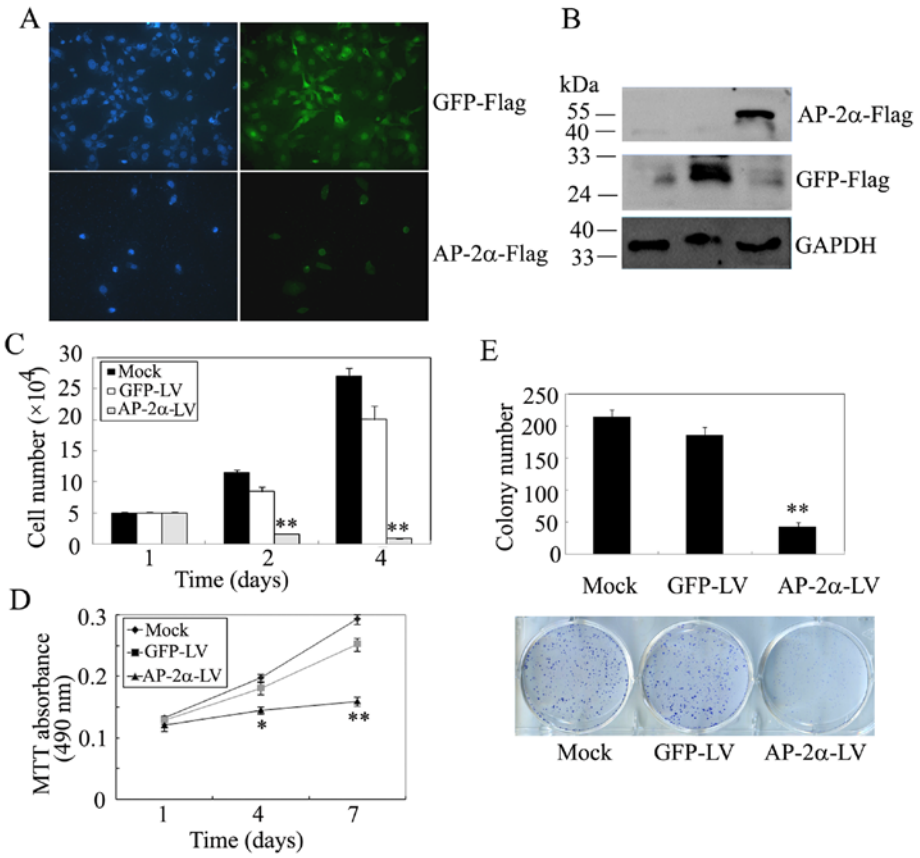

Figure 3. Effects of AP-2α overexpression on Hep3B hepatocellular cancer cell growth. (A) Immunofluorescence assays for the measurement of the transduction efficiency of Hep3B cells infected with AP-2α overexpression lentivirus. (B) Western blot of GFP/Flag and AP-2α/Flag expression in Hep3B cells using anti-Flag antibodies. GAPDH served as a loading control. (C) Cell survival assays of AP-2α overexpression-infected Hep3B cells, GFP-infected Hep3B cells and parental Hep3B cells on 1st, 2nd and 4th day. (D) MTT assays of mock or infected Hep3B cells. Cells (3,000) were plated and grown. The absorbance was analyzed for 1, 4 and 7 days. (E) Liquid colony formation analysis of mock or infected Hep3B cells. Hep3B cells (2,000) were seeded and grown for 2 weeks. Colonies were fixed with methanol, stained with Giemsa, images were taken (lower panel) and counted (upper panel). These data represent at least three independent experiments with similar results. $^{**}P<0.01$, compared with parental and control cells.

with high expression of AP-2α (Fig. 1C). Thus, the level of AP-2α expression appears low in hepatocellular cancer cell lines. These cell lines were further selected to overexpress AP-2α proteins, respectively.

AP-2α overexpression inhibits the proliferation of human hepatocellular cancer cell lines in vitro and in vivo. To further investigate the role of AP-2α in human hepatocellular cancer cells, we overexpressed AP-2α and asked whether AP-2α inhibits the proliferation of hepatocellular cancer cells. Then, lentivirus-based vector Ubi-gene-3xFlag-IRES-puromycin-AP-2α containing the full-length AP-2α and packaging plasmids pHelper 1.0 and pHelper 2.0 were cotransfected to 293T cells. pGCFU-GFP-3xFlag-IRES-Puromycin served as negative control. Lentiviral particles were prepared to infect SMMC-7721 cells. The fluorescence intensity was markedly increased 4 days after infection and the infection efficiency was ~95% in SMMC-7721 followed by the screening of 1.5 μg/ml of puromycin (Fig. 2A). Western blot analysis demonstrated overexpression of AP-2α and GFP (Fig. 2B).

Next, we examined whether AP-2α is a critical regulator of hepatocellular cancer cell proliferation and detected the effect of AP-2α overexpression on SMMC-7721 cell growth. The same number of cells was plated in triplicate in 6-well plates, and cell number was counted on day 1 and 3. We found that the overexpression of AP-2α in SMMC-7721 cells showed a decreased cellular growth compared with controls (Fig. 2C). Likely, we examined cell viability using MTT assays, AP-2α overexpression resulted in a remarkable decrease in viable cells (Fig. 2D). Further, the liquid colony formation assay indicated a great decrease in colony number and size (Fig. 2E). These results indicated that AP-2α overexpression inhibits hepatocellular cancer cell SMMC-7721 proliferation *in vitro*.

To further demonstrate the effects of AP-2α on cell proliferation, we performed the above experiments in Hep3B cells. From Fig. 3A-D, we observed that AP-2α overexpression significantly inhibits the survival and proliferation of Hep3B cells, whereas GFP has no effect compared with parental cells. The liquid colony formation assays showed that exogenous AP-2α displays considerably fewer and smaller colonies in Hep3B cells, while control GFP had no effect compared with uninfected parental cells (Fig. 3E). Therefore, AP-2α overexpression suppresses the proliferation of human hepatocellular cancer Hep3B cells *in vitro*.

The inhibitory effect of AP-2α on hepatocellular cancer cell proliferation *in vitro* suggested that AP-2α might suppress tumor growth *in vivo*. Tumorigenicity assay was performed by subcutaneous injection of AP-2α cells into nude mice, whereas GFP was used as controls. Within 41 days, solid tumors were

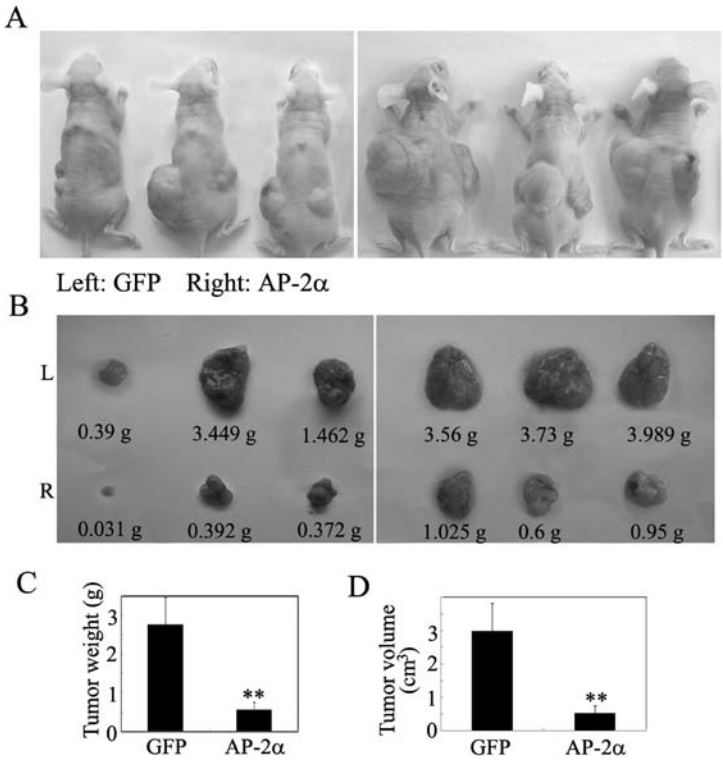

Figure 4. AP-2α overexpression results in decreased cell proliferation of SMMC-7721 cells *in vivo*. Approximately $2x10^7$ of lentivirus-infected cells were injected s.c. into the left and right back of female nude mice (BALb/c) (n=6 per group). After 41 days, tumors were excised, photographed (A and B), and weighed (C and D). The weight and volume of the tumors excised are mean ± SD in three independent experiments. $^{**}P<0.01$ compared with controls.

readily visible in left and right dorsal regions of all mice (Fig. 4A and B), but the average tumor weight and volume of the AP-2α group was reduced by >70% compared with the negative controls (Fig. 4C and D).

AP-2α overexpression suppresses hepatocellular cancer cell migration and increases the sensitivity of hepatocellular cancer cells to cisplatin. Since cell motility is an important factor regulating cancer invasion and metastasis, the effect of AP-2α on cell motility was characterized by woundhealing, Transwell migration, and Matrigel invasion assays. As shown in Fig. 5A, the wound-healing assay showed that AP-2α overexpression produces 62% inhibition of cell migration at the edge of exposed regions in SMMC-7721 cells. In contrast, control groups significantly increased cell migration. Subsequently, the Transwell migration assay showed that overexpression of AP-2α led to a marked decrease in cell motility, whereas more cells were observed to migrate through the 8-μm pores in control GFP compared with AP-2α (Fig. 5B). Similarly, the invasion assay showed that AP-2α cells obtained a significantly slower rate of cell invasion than that of control cells (Fig. 5C). Thus, these data demonstrated that AP-2α is able to significantly decrease cell motility.

We next investigated the implication of AP-2α in chemosensitivity of hepatocellular cancer cells. SMMC-7721 cells were treated with cisplatin, chemotherapeutic agents. Reduction of MTT absorbance (63%) was detected in AP-2α overexpressing cells compared with 48% inhibition in their control cells (Fig. 5D). Therefore, AP-2α could sensitize SMMC-7721 cells to cytotoxicity of cisplatin as reported in endometrial cancer cells (25).

AP-2α overexpression attenuates the self-renewal ability of liver TICs. We next decided to examine whether AP-2α can regulate the self-renewal ability of liver TICs. As shown in Fig. 6A and C, AP-2α overexpression significantly suppressed the spheroid formation ability of these cells when grown in non-adherent serum-free conditions *in vitro*. Moreover, we evaluated whether AP-2α could suppress the self-renewal of TICs *in vitro*, single cell suspension from the primary tumorspheres was used to form the secondary sphere. AP-2α overexpression significantly decreased the size of the secondary tumorsphere, suggesting a decreased self-renewal ability of these TICs (Fig. 6B). Furthermore, we also investigated the effects of AP-2α overexpression combined with cisplatin on TICs. As shown in Fig. 6C-E, AP-2α overexpression displayed a markedly lower growth and proliferation, when cells were treated with cisplatin for 3 days, cisplatin synergistically inhibited the survival and growth of TICs.

AP-2α affects the expression of phosphorylated ERK, β-catenin, p53 and EMT markers. Since AP-2α influences many genes involved in the development and progression of human cancers, we decided to investigate whether AP-2α regulates these target genes in hepatocellular cancer cells. As shown in Fig. 7A, AP-2α overexpression had no effect on ERK protein, but it decreased the expression of β-catenin and the phosphorylated level of ERK expression, and downregulated the expression of c-Myc and cyclin D1 as a downstream target of ERK signaling and Wnt signaling. Moreover, AP-2α markedly suppressed the TOPFlash reporter activity and gave an inhibitory effect on Wnt signaling pathway (Fig. 7A and B). AP-2α overexpression increased the expression of tumor

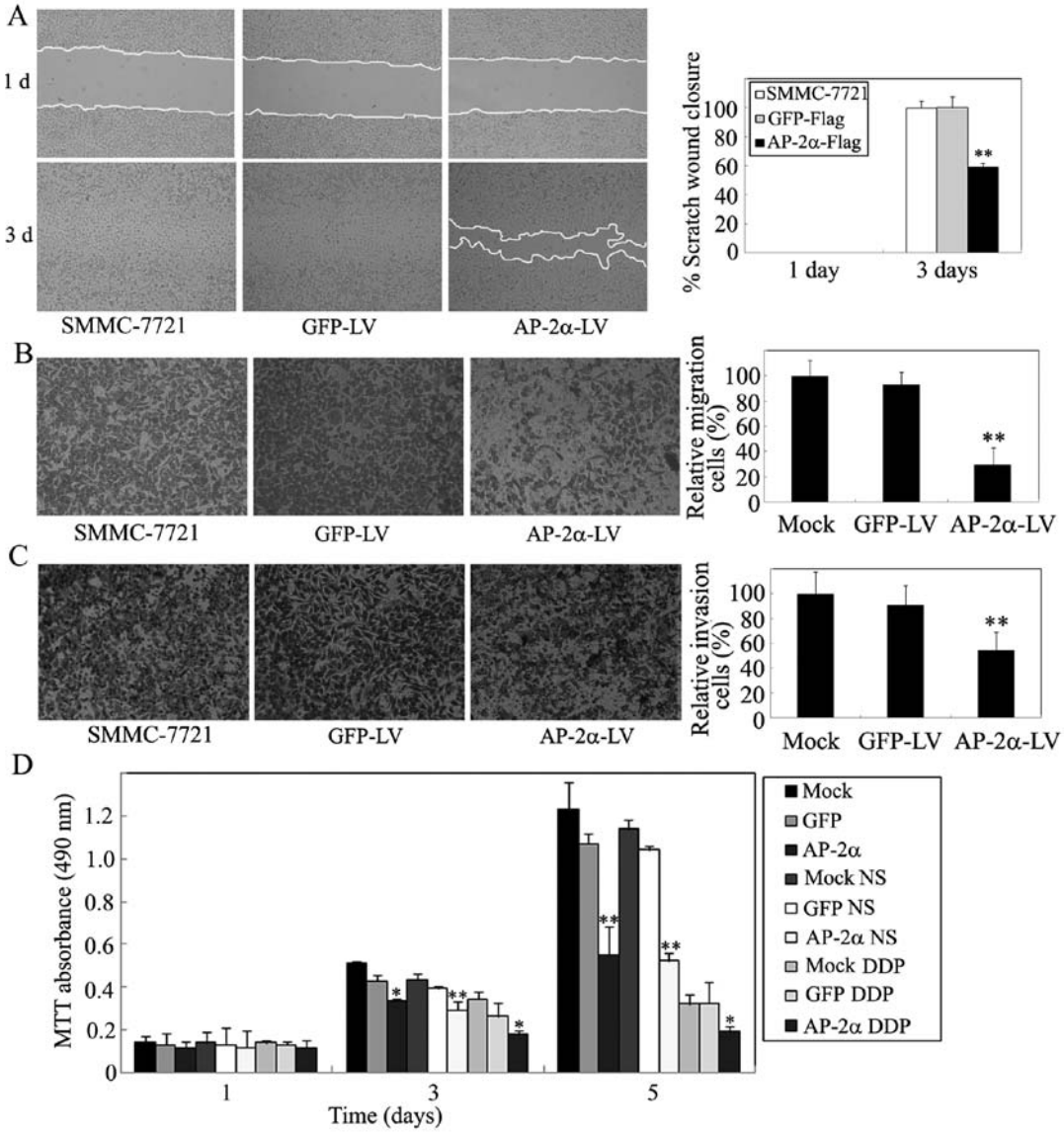

Figure 5. AP-2α enhances SMMC-7721 cell migration and invasion *in vitro* and increases the sensitivity of hepatocellular cancer cells to cisplatin. (A) Representative images of wound-healing assays were obtained at indicated time following initiation of the scratch in lentivirus-infected and parental SMMC-7721 cells. These areas were calculated by AutoCAD software. (B) Effect of AP-2α overexpression on cell migration in Transwell assays. Examples of cells migrated through the PET-membrane (pore size: 8 μm) are shown in the left panel. Relative migration proportion of cells is shown in the right panel. (C) Effect of AP-2α overexpression on cell invasion through Matrigel. Examples of cells migrated through Matrigel-coated Transwell are shown in the left panel. Relative invasion proportion of cells is shown in the right panel. (D) Lentivirus-infected and parental SMMC-7721 cells were treated with 30 μM of cisplatin and control agent 0.9% NaCl (NS) for 48 h, and cell viability was measured by MTT assays as described in Materials and methods. All results indicate the mean of triplicate experiments. *P<0.05, **P<0.01.

suppressor p53. Furthermore, the level of EMT epithelial marker E-cadherin was concomitantly increased in AP-2α-infected cells while AP-2α overexpression decreased the levels of mysenchymal marker vimentin and MMP9 (Fig. 7A). Taken together, these data showed that AP-2α regulates cell proliferation and migration, at least in part, by affecting ERK signaling and EMT-related cell movement.

We wished to clarify whether the epigenetic modification might regulate low expression of AP-2α in hepatocellular carcinoma, thus, we determined the effect of the histone deacetylase inhibitor, trichostatin A (TSA) or sodium butyrate (NaBu) and/or the DNA methyltransferase inhibitor, 5-aza-2'deoxycytidine (5-aza-dC) on AP-2α expression in SMMC-7721 cells. We found that TSA, NaBu and 5-aza-dC significantly increased the expression of AP-2α protein (Fig. 7C), suggesting the possible modification influences of AP-2α function in hepatocellular carcinoma.

Discussion

Hepatocellular carcinoma (HCC) is a common malignant tumor. Carcinogenesis of HCC is a multi-factor, multi-step and complex process, which is associated with genetic alterations that involve the gain-of-function mutation, amplification, and/or overexpression of key oncogenes together with the loss-of-function mutation, deletion, and/or epigenetic silencing of critical tumor suppressors. Previously, loss of AP-2α has been associated with tumor malignancy in various cancers, including melanoma (12), breast cancer (27), and glioma (14). It has been well reported that DNA methylation of CpG

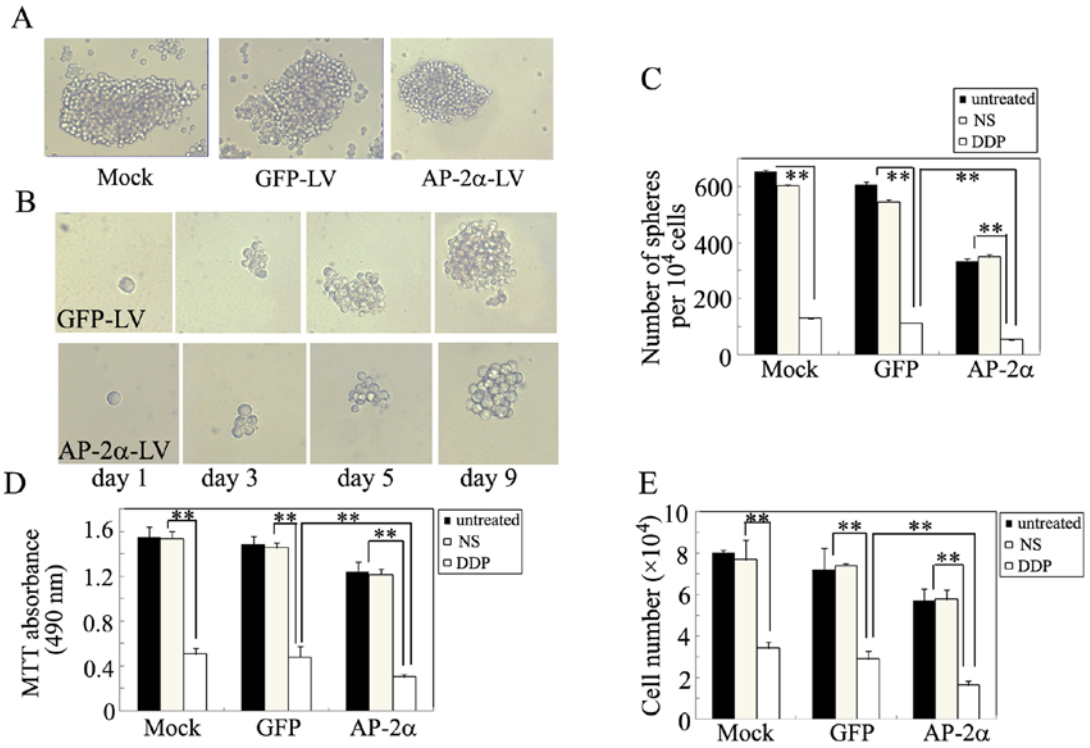

Figure 6. AP-2α is critical for growth and self-renewal of liver TICs. (A) SMMC-7721 cells were plated in serum-free stem cell medium and allowed to grow as spheres for 13 days. Images of the spheres were taken using inverted microscopy. (B) Single cell suspension from the primary tumorspheres was used to form the secondary sphere. The process of single cell to form sphere was defined at 1, 3, 5 and 9 days, respectively. (C) The SMMC-7721 spheres were treated with 30 μM of cisplatin or 0.9% NaCl for 3 days and then counted by optical microscopy on the 5th day. (D) The SMMC-7721 sphere-forming cells were treated, trypsinised and analyzed by MTT assays on the 5th day. (E) The SMMC-7721 sphere-forming cells were treated, trypsinised and detected by cell survival assays on the 5th day.

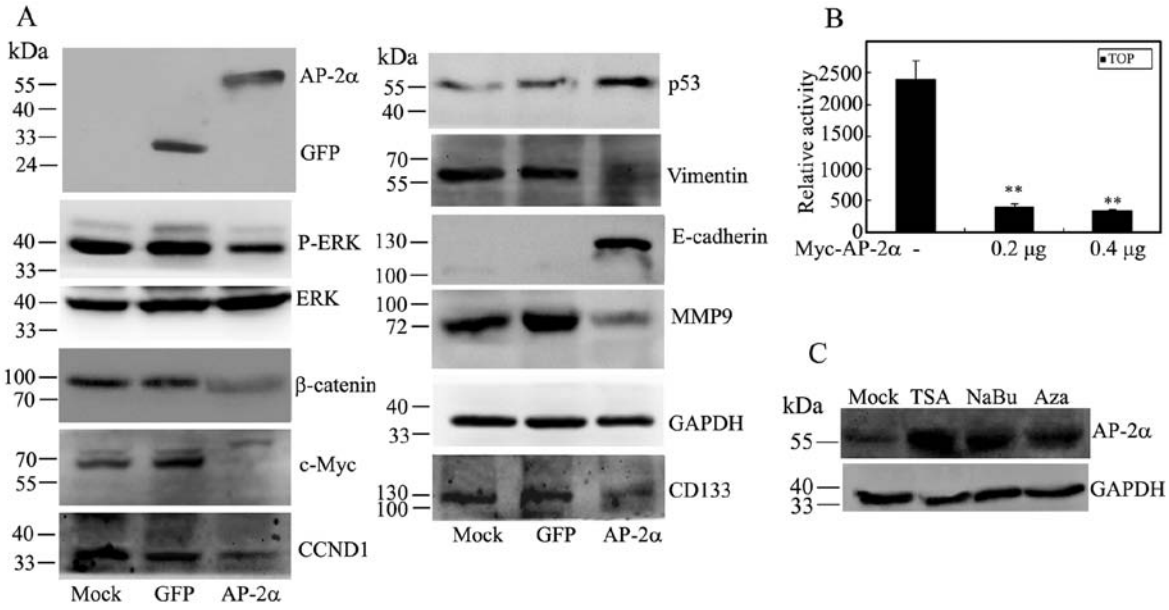

Figure 7. AP-2α modulates the expression of multiple downstream target genes. (A) Western blotting was carried out to detect the effect of AP-2α overexpression on the expression of target genes ERK, p53 and β-catenin. GAPDH was used as a loading control for total lysate samples. (B) Effects of AP-2α overexpression on the TOPFlash reporter activity. Hep3B cells were transfected with a TOPFlash reporter plasmid, and pCMV-Myc-AP-2α plasmids. The amount of DNA in each transfection was kept constant by the addition of control empty vectors. Luciferase and β-galactosidase activities were measured 24 h after transfection. Relative reporter activity was presented as mean ± SD from three independent transfection experiments performed in triplicate. **P<0.01 compared with controls. (C) Effects of epigenetic modification on AP-2α expression. SMMC-7721 cells were treated with the demethylating agent 5-Aza-dC or the histone deacetylase inhibitor, TSA or NaBu for 48 h followed by western blots. GAPDH served as an internal control for sample loading.

islands near the promoter and exon 1 affects the transcription of AP-2α in breast cancer (27), and acetylation of AP-2α upregulates Toll-like receptor 2 gene expression in THP-1 cells, a human monocytic cell line (28). In the present study,

that loss or low expression of AP-2α was detected in 40% of HCC samples, indicating a tumor suppressive role of AP-2α in HCC. Moreover, the levels of demethylated and acetylated AP-2α were increased in SMMC-7721 cells after 5-aza-dC or TSA treatment. Therefore, in a future study we will investigate whether the downregulation of AP-2α might associate with DNA hypermethylation and deacetylation in hepatocellular carcinoma.

The *in vitro* and *in vivo* functional assays were performed to characterize the role of AP-2α in regulating cell proliferation, migration and invasion of hepatocellular cancer, and the results showed that AP-2α has strong tumor-suppressive ability. We found that AP-2α-infected cells were able to form smaller and fewer colonies in liquid colony formation and inhibited tumor formation in nude mice. Furthermore, AP-2α decreased HCC cell migration and invasion by wound healing and a serial of Transwell assays. More intriguingly, we showed that AP-2α overexpression significantly enhanced cisplatin-induced death of HCC cells, supporting that AP-2α increased the sensitivity of liver cancer cells to cisplatin. Consistent with our previous data that miR-200b/200c/429 induced cisplatin resistance by repressing AP-2α expression in endometrial cancer cells (25), all these findings strongly supported that AP-2α plays an important role in HCC growth and motility.

Recent studies in a variety of solid tumors and leukemia show that there is significant heterogeneity with respect to tumor-forming ability within a given population of tumor cells, suggesting that the capability to maintain tumorigenesis is found only in a small population of cells called cancer stem cells (CSCs) or tumor-initiating cells (TI Cs) (33). CSCs have been shown to be resistant to conventional therapies and are thus thought to drive disease relapse (34). Hepatic stem/progenitor cell markers including CD44 and EpCAM with the transmembrane cell-surface glycoprotein CD133 can mark liver TICs (35). Our results showed that AP-2α overexpression significantly suppressed the growth and self-renewal of liver CSCs by downregulating CD133 expression.

Many pathways including Ras/MAPK, PI3K/AKT, and Wnt/β-catenin signaling, have been shown to be of significance in HCC. The Ras-Raf-MEK-ERK signaling cascade plays a crucial role in regulating cellular processes including differentiation, proliferation, survival and apoptosis (36). We demonstrated that AP-2α inhibits the expression of phosphorylated ERK and downstream genes c-Myc and CCND1 in cellular growth of liver cancer. Moreover, c-myc is a major driver of tumor angiogenesis and its expression has been associated with HCC recurrence (37). In addition, c-Myc and CCND1 also serve as canonical Wnt pathway downstream target genes. β-catenin is the central effector of the canonical Wnt signaling, which is a highly conserved pathway regulating critical cellular processes such as proliferation, differentiation, survival and self-renewal (38,39). The Wnt/β-catenin pathway has been implicated in a subset of HCC where activating mutations in the β-catenin gene have been reported in 20-40% of patients (40). Knockdown of β-catenin in hepatoma cells leads to decreased growth and survival, and oncogene β-catenin is considered a valuable therapeutic target (41). AP-2α has been identified to associate with adenomatous polyposis coli/β-catenin and inhibits β-catenin/T-cell factor transcriptional activity in colorectal cancer cells (23). However, no evidence indicates that AP-2α regulates Wnt/β-catenin pathway in liver cancer. Here, we found that AP-2α overexpression can inhibit the Wnt/β-catenin pathway in HCC cells through downregulating β-catenin expression. In addition, the tumor suppressor gene p53 inhibits the development and growth of the majority of human tumors (42). Moreover, the tumor suppressor activity of AP-2α is mediated through a direct interaction with p53 (43), we also found that AP-2α augments p53 activation in HCC cells.

Epithelial-mesenchymal transition (EMT) is a critical process for tumor invasion and metastasis in which epithelial cells lose their junctions and apical-basal polarity, remodel their cytoskeleton, undergo a change in cell shape and reprogramme gene expression to increase cell motility and develop an invasive phenotype, while MET describes the reverse process (44,45). In this study we found that AP-2α overexpression gives a significant effect on EMT by increasing the expression of epithelial markers (E-cadherin) and decreasing mesenchymal markers (vimentin), and might achieve lower motility and invasiveness. Thus, these data suggested that AP-2α might partly inhibit EMT-like transition and invasion in HCC. Moreover, the invasiveness in tumor cells was enhanced by upregulation of MMPs such as MMP9 for extracellular matrix remodeling (46). We provided evidence that the levels of MMP9 are decreased in AP-2α overexpressed HCC cells.

In conclusion, our data suggest the pivotal role of AP-2α in hepatocellular cancer progression. Epigenetically modified AP-2α is lowly expressed in hepatocellular cancer tissues and cell lines. AP-2α regulates the proliferation, migration, invasion and self-renewal ability of hepatocellular cancer cells, at least in part, by affecting the expression of phosphorylated ERK, β-catenin, p53 and EMT markers. Preliminary data showed that AP-2α increases the cytotoxicity of chemotherapeutic drugs in hepatocellular cancer cells. Taken together, the tumor suppressor AP-2α might have an important role as molecular therapeutic and/or prognostic markers in human hepatocellular cancers.

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