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Original Article

Loss of caveolin-1 in cancer associated fibroblasts promotes hepatocellular carcinoma development

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Abstract: Background: Caveolin-1 (Cav-1) expressed by cancer associated fibroblasts (CAFs) has been shown to be an inhibitory factor in many tumors. However, its functions in hepatocellular carcinoma (HCC), a top killing disease, were not clear. Materials and Methods: In this study, we explored the roles of Cav-1 expressed by CAFs in HCC development and its clinical significance. We collected human HCC tumor tissues to evaluate the Cav-1 expression and correlated it with the overall survival. We also established in-vitro experimental systems to investigate the roles of Cav-1 low expression CAFs in HCC invasion and chemotherapy resistance. Results: We found that loss of Cav-1 in CAFs was an adverse prognosticator for the HCC patients. Biologically, loss of Cav-1 in CAFs promoted the HCC tumor cell invasion and induced multiple drug resistance. In HCC tumor model, tumors with Cav-1 low expression CAFs were not sensitive to chemotherapy. Conclusion: We concluded that the loss of Cav-1 in CAFs might be a promoting factor of HCC development and a biomarker of HCC.

Keywords: Hepatocellular carcinoma, caveolin-1, cancer associate fibroblast, drug resistance, survival

Introduction

Caveolins, consisting of caveolin-1 (Cav-1), Cav-2 and Cav-3, are the major structural proteins of caveolae, a special type of lipid raft of the plasma membrane in many vertebrate cell types, especially in endothelial cells and adipocytes [1, 2]. Among the tree caveolins, Cav-1 has shown controversial roles in tumor development [1, 2]. In breast cancer, prostate cancer, and esophageal squamous cell carcinoma, Cav-1 expressed in tumor cells has shown oncogenic roles, primarily through increasing tumor cell survival and drug resistance [3-5]. However, cancers are integrations of cancer cells and the tumor microenvironment [6]. In the tumor microenvironment, specifically in cancer associated fibroblasts (CAFs), high expression of Cav-1 was deemed as an inhibitory mechanism of tumor development. Loss of Cav-1 expression induced autophagy in CAFs, resulting in high catabolism in CAFs and secretion of metabolic nutrient to the tumor microenvironment [7-9]. The nutrient derived from Cav-1 low expression CAFs was then taken by the surrounding tumor cells to accelerate tumor growth and drug resistance [7-9].

Hepatocellular carcinoma (HCC) is a common malignant worldwide and causes more than 422,000 deaths each year in China [10]. Treatment of HCC, especially the late stage HCC, relies on surgical based comprehensive treatments [11]. However, drug resistance occurs and the overall survival of late-stage patients is still dismal. It is worth to note that the molecular mechanisms driving HCC progression and drug resistance remain unclear, causing difficulties in developing novel treatments. In this study, we aimed to investigate the roles of CAFs expressed Cav-1 on HCC progression. Specifically, we hypothesized that loss of Cav-1 in CAFs promotes HCC development by inducing tumor cell invasion and drug resistance.

Materials and methods

Cell culture, conditioned media, and CAFs isolation

Human HCC cells lines, HepG2 and Hep3B (both from ATCC) were cultured in EMEM medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2 Mm glu-

tamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. CAFs were isolated from fresh human HCC tumor tissues following the methods reported preciously [12]. CAFs were cultured with DMEM medium with 10% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin. All the cells were cultured within the humidified incubator with 5% CO_2 at 37°C. The cells were passaged every 3-4 days. CAFs' conditional medium was produced by adding 5 ml fresh medium to 10 cm cell culture dish with 70% confluent CAFs. After 24 h, the supernatant without CAFs was collected and used as conditional medium.

shRNA transfection and western blotting

The shRNA lenti-virus vector targeting CAV-1 gene was purchased from Origene (Catalog number: TG314183). The manufacturer recommended standard procedure was followed for cell transfection and infection. After the shRNA was stably expressed in CAFs, we measured the Cav-1 expression in CAFs with Western blotting analysis. Briefly, cells were collected and then lysed by RIPA buffer with proteinase inhibitor and phosphorylase inhibitor to extract total protein. The total protein concentration was tested by BCA protein assay and normalized between different groups. The protein was then separated by electrophoresis and was transferred to a PVDF membrane by a semi-dry transferring system. Anti-Cav-1 antibody (1:2000 dilution, Novus, USA) was then added and incubated overnight at 4°C. β-Actin (1:1000 dilution, Novus, USA) was used as internal control. HRP-conjugated secondary antibody (1:4000 dilution, Novus, USA) and ECL Western blotting detection reagents (ThermoFisher Scientific, USA) were used for imagining. Expression of multidrug resistance protein 1 (MRP1) (1:1500 dilution, Novus, USA) was also measured by Western blotting analysis with a similar procedure.

Patient samples and immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tumor tissues of 105 HCC patients' samples were collected before chemotherapy or radiotherapy. All HCC patients were diagnosed from February 2008 to February 2010, at the Sichuan Provincial People's Hospital, China. Written or oral informed consent was obtained from each patient. Using of human tissues in this study was approved by the medical ethics committee of the Sichuan Provincial People's Hospital.

A standard immunohistochemistry (IHC) procedure was conducted to measure the expression of Cav-1 in HCC patients' tissues. In short, tissue sections were first deparaffinized and rehydrated following by submerging in 3% H_2O_2 in methanol for 20 minutes. Then slides were subjected to antigen retrieval by streaming with Reveal Decloaker (Biocare Medical, CA, USA) for 10 minutes. 5% bovine serum albumin buffer was applied on slides and incubated for 30 minutes at room temperature. Primary antibody (1:100 dilution) was overnight incubated at 4°C. HRP conjugated secondary antibodies were incubated for 1 h at room temperature. DAB was used for color development. Counterstain was performed with hematoxylin. The primary antibody was omitted in the negative controls. All cases were observed under a light microscope at × 200 magnification. Expression of Cav-1 in the tumor stromal part was evaluated. We defined low expression as 1%-30% positive staining, high expression as more than 30% positive staining. The Activated-Caspase 3 expression in mouse model tumors was studied by immunofluorescence with a similar procedure.

Cell viability assay

The viabilities of HCC cell lines (HepG2 and Hep3B) after 5-fluorouracil (5-FU) or gemcitabine (Gem) treatments were evaluated using CCK-8 kit following the manufacturer's recommendation. 1×10^5 cells (per well) were seeded to 96-well plates with 100 μl culture medium or CAFs' conditional medium. Then, various concentrations of 5-FU or Gem were added to incubate for 24 h, followed by adding 10 μl CCK-8 solution to each well. After 30 min incubation at 37°C, the 450 nm absorbance of each well was measured using the MRX microplate reader (Dynex Technologies). Every treatment of these HCC cells were repeated three times and the average values were used for further analysis.

Flow cytometry

To prepare the single cell suspensions, fresh human HCC tumor tissues were minced to be small pieces and disassociated with collagenase I (10 mg/ml) for 60 min on the shaker at

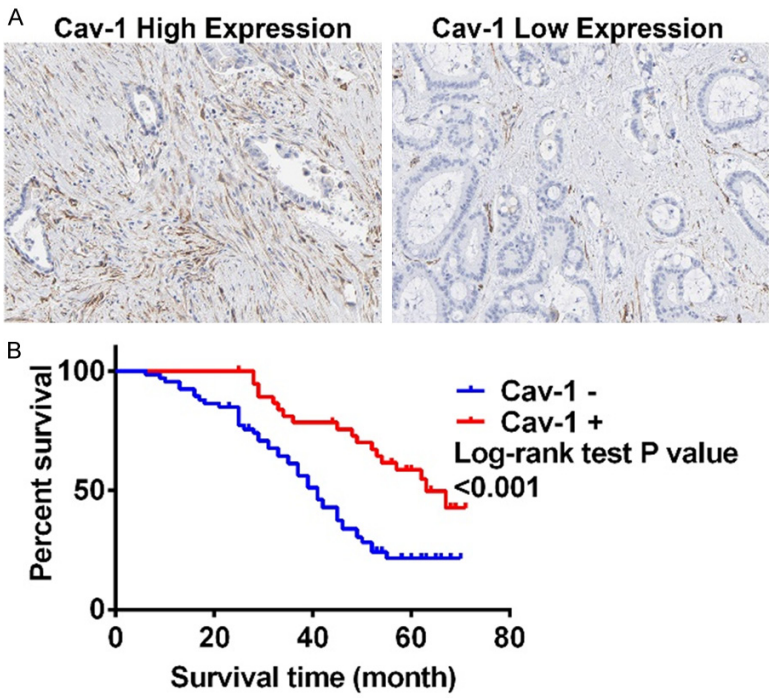

Figure 1. Tumor stromal Cav-1 expression in HCC patients. A. Various expression levels of stromal Cav-1 were observed in the tumor tissues of HCC patients. B. The patients with high Cav-1 expression (Cav-1+) had higher overall survival than the patients with low Cav-1 expression (Cav-1-).

37°C, and then filtered with 40 μm cell strainers. The single cells were thenwashed by cold FBS. Subsequently, primary antibodies (all from Abcam, USA), including E-cadherin, vimentin, and Cav-1 antibodies, were added to the cell pellets and incubated for 15 min. Fluorescence conjugated secondary antibodies were then incubated at room temperature for 10 min. E-cadherin and vimentin were used as epithelia and fibroblasts markers respectively to isolate the tumor cells and CAFs from the fresh human HCC tissues. The final data was analyzed by FlowJo software, and the mean fluorescence intensity (MFI) were calculated to quantify the expression of proteins.

Cell invasion assay

The Boyden chambers (Costar Transwell 24-well plate) were used to perform the cell invasion assay following the manufacturers' instruction. Briefly, the HCC cells were preconditioned for 48 h by the conditioned medium from different CAFs before loading to the transwell inserts. DMEM medium with 1% FBS was added in the inserts while DMEM medium with 10% FBS was add on the bottom. The cells were the cultured for 12 h before stained by crystal violet stain solution. The number of invaded cells in each well were quantified under bright light microscopy.

ELISA

The amount of MMP-2 and MMP-9 expressed by the HCC cell lines (HepG2 and Hep3B) were measured by ELISA kits (Thermo Fisher Scientific, USA). The manufacturer's instructions were carefully followed. BAC assay was conducted to measure the total protein concentrations which were normalized ahead of loading the plates.

Animal model

HCC subcutaneous mouse model was created using 6-week old BALB/c nude mice (male, 20-22 g, Shanghai Experimental Animal Center, China), HepG2 cell line and CAFs. For each mouse, a total number of 4×10^5 cells were injected into its flank (n=8 in each group). The ratio of tumor cells to CAFs was 1:3. The CAFs were either wild type, transfected with control vector or Cav-1 shRNA vector. To understand drug response, mice were treated with 5-FU (150 mg/kg) and Gem (150 mg/kg). Treatments were performed on the 10th day after tumor inoculation. The tumor volume (calculated by $\text{width}^2 \times \text{length} \times \pi/6$) was recorded every 7 days. This animal study was approved by the Ethics Committee of Sichuan Provincial People's Hospital.

Statistical analysis

All the statistical analyses were performed using GraphPad Prism 6.0 software. The difference of means among experimental groups were analyzed by t test or one-way ANOVA. Survival analysis was conducted by the Kaplan-Meier method. Log-rank test was performed to compare the difference of survival curves of different experiment groups. Two-tail *P* values of the statistical tests less than 0.05 were recognized to be significant.

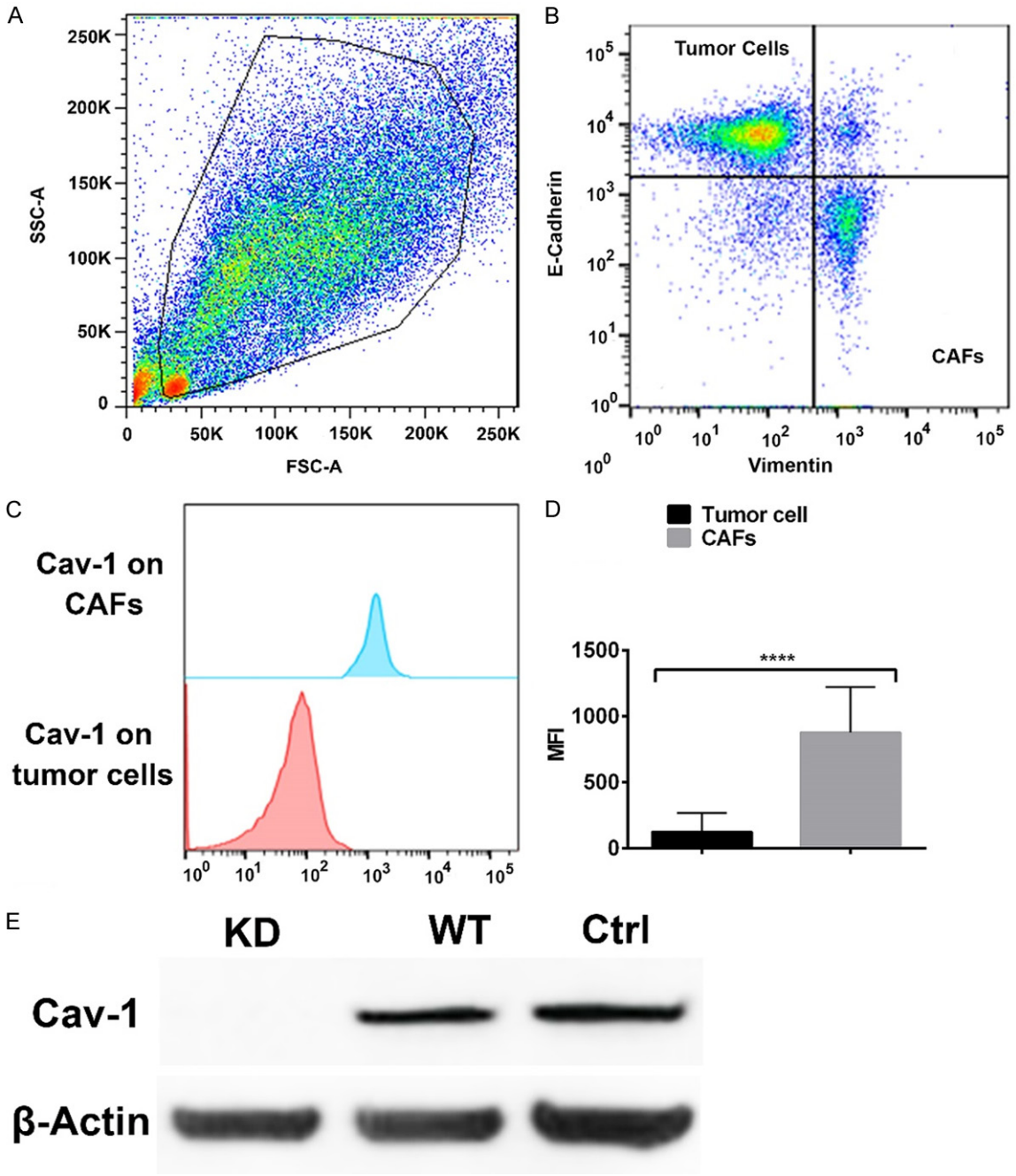

Figure 2. Cav-1 expression in human HCC tumor cells and CAFs. A. Single cells of fresh human HCC tumor tissues were gated in FACS analysis. B. Gating strategy of isolating HCC tumor cells (E-Cadherin) and CAFs (vimentin). C. CAFs expressed a high level of Cav-1, but the tumor cells almost didn't express Cav-1. D. Mean fluorescence intensity (MFI) was calculated to quantify the Cav-1 expression. The CAFs expressed a much higher level of Cav-1 than the tumor cells. E. The Cav-1 expression of shRNA transfected CAFs wasmeasured by Western blotting analysis. The Cav-1 shRNA dramatically decreased Cav-1 expression. (*****P* value less than 0.0001; KD: Cav-1 knockdown CAFs; WT: wide type CAFs; Ctrl: transfection control CAFs).

Results

Low stromal Cav-1 expression was associated with worse prognosis of HCC patients

Aiming to understand the role of stromal Cav-1 in the development of HCC, we evaluated the Cav-1 expression in 105 HCC patients' tumor tissues. Interestingly, we found that Cav-1 was dominantly expressed in the tumor stromal part, rather than the tumor cells. The HCC patients showed various levels of tumor stromal Cav-1 expression (**Figure 1A**). Further survival analysis indicated that the patients with

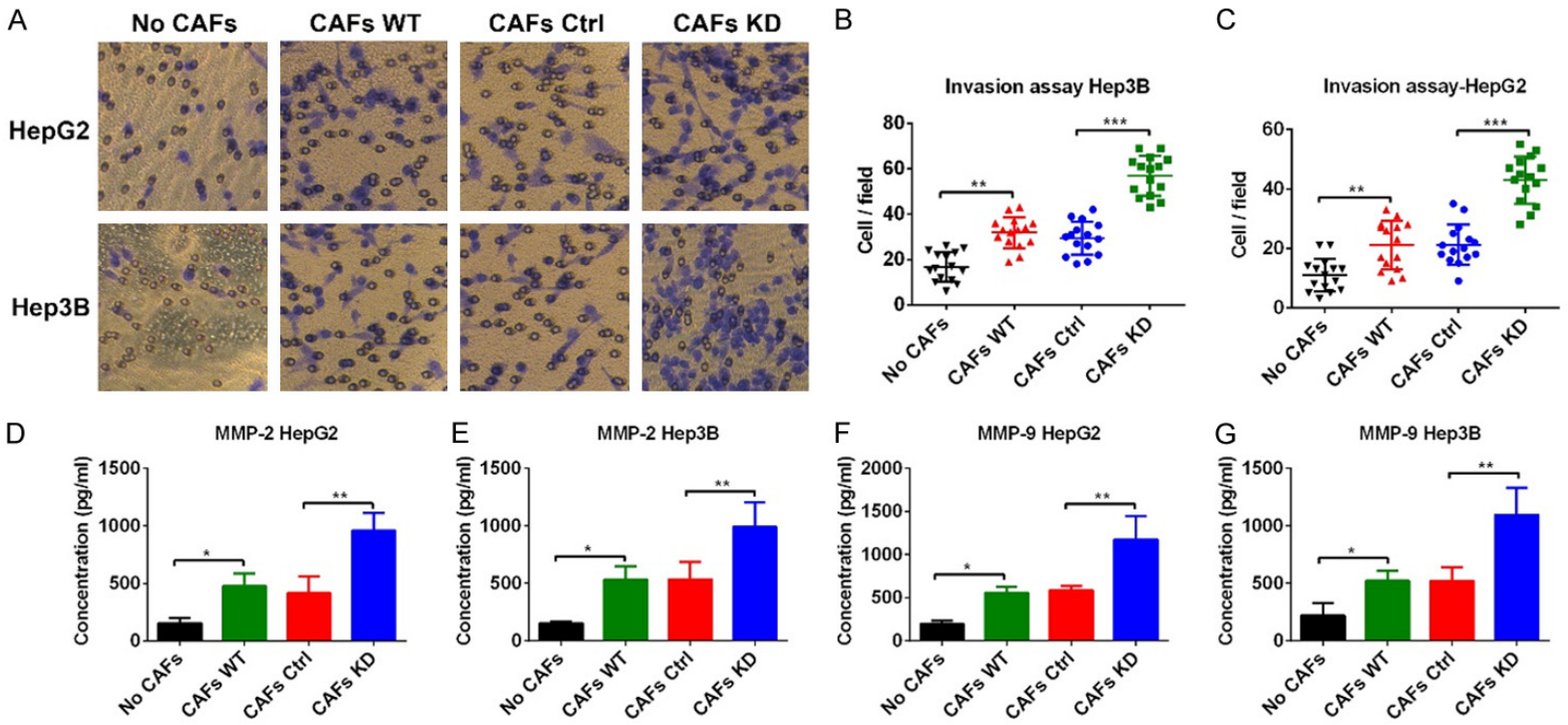

Figure 3. Effects of Cav-1-knockdown CAFs on HCC invasion. A. Representative pictures of the invasion assay of HCC cell lines, HepG2 and Hep3B, which were treated by CAF conditional medium. B, C. The quantified data showed that HepG2 and Hep3B cells had higher invasion ability after treated by the conditional medium of Cav-1 knockdown CAFs. D-G. The amount of MMP-2 and MMP-9 expressed by HCC cells were significantly increased after treated by the conditional medium of Cav-1 knockdown CAFs. (**P* value less than 0.05; ***P* value less than 0.01; ****P* value less than 0.001; CAFs KD: Cav-1 knockdown CAFs; CAFs WT: Cav-1 wide type CAFs; CAFs Ctrl: transfection control CAFs).

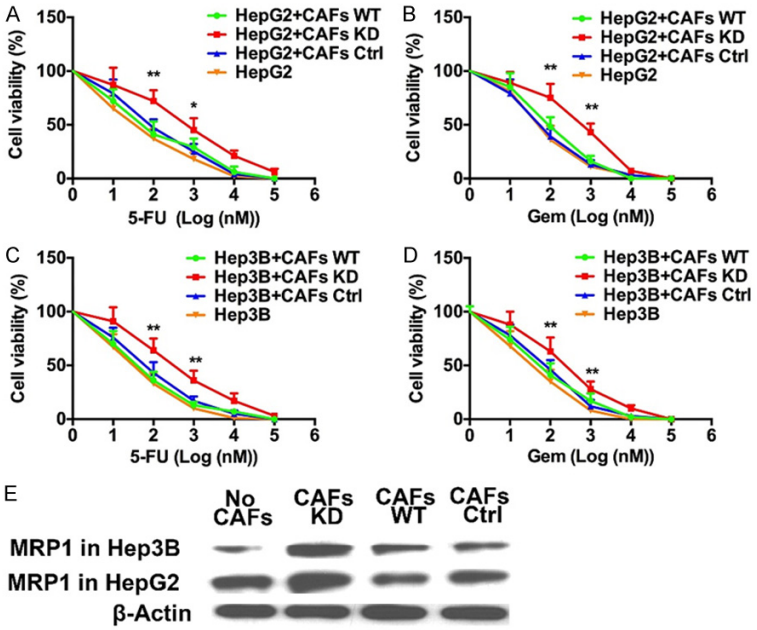

Figure 4. CAFs with Cav-1 knockdown induced drug resistance of HCC cell lines. A, B. Under the treatment of 5-FU and Gem, the response of HepG2 cells cultured with Cav-1 knockdown CAFs' conditional medium was not as sensitive as the cells cultured with normal Cav-1 expression CAFs' conditional medium. C, D. Hep3B cells also showed increased drug resistance to the 5-FU and Gem treatments when cultured with the Cav-1 knockdown CAFs' conditional medium. E. Expression of multidrug resistance protein 1 (MRP1) in each group after treated by 1,000 nM 5-Fu and 1,000 Gem for 24 h. (Difference of the cell viability among the experimental groups was analyzed at the 2nd and 3rd treatment concentrations. **P* value less than 0.05; ***P* value less than 0.01; CAFs KD: Cav-1 knockdown CAFs' conditional medium; CAFs WT: Cav-1 wide type CAFs' conditional medium; CAFs Ctrl: transfection control CAFs' conditional medium).

low stromal Cav-1 expression had lower overall survival rate than the cases with high stromal Cav-1 expression (**Figure 1B**). The log-rank test indicated this difference was statistically significant (*P* value < 0.001). Based on these observations, we hypothesized that the loss of stromal Cav-1 might promote the development of HCC.

Cav-1 was mainly expressed by CAFs in HCC tissues

Given that in the HCC sections, Cav-1 was seen in the tumor stromal part, we further validated the Cav-1 expression location by flow cytometry. We evaluated the Cav-1 expression on both the tumor cells and CAFs (**Figure 2A**, **2B**) from fresh HCC tumor tissues. In line with the results from tissue staining, we found that Cav-1 was mainly expressed by the CAFs, but not the tumor cells (**Figure 2C**, **2D**). To understand the roles of Cav-1 low expression CAFs on tumor development, we created the Cav-1 knockdown CAFs with shRNA transfection for further study (**Figure 2E**).

Loss of Cav-1 in CAFs promoted the HCC cell invasion

Human HCC cell lines, HepG2 and Hep3B, were cultured with the conditional medium of different CAFs, including Cav-1 knockdown CAFs, transfection control CAFs, and Cav-1 wild type CAFs. Then these HCC cells were subjected to invasion assay. As shown in **Figure 3A-C**, the HCC cell lines cultured with Cav-1 knockdown CAFs' conditional medium had increased invasion ability than the control groups and baseline group (HCC tumor cells with no conditioned medium treatment). We further measured the two key invasion-related proteins MMP-2 and MMP9 levels in tumor cells. As expected, the MMP-2 and MMP-9 amounts expressed by the HCC cell lines cultured with Cav-1 knockdown CAFs' conditional medium were significantly increased (**Figure 3D-G**).

Loss of Cav-1 in CAFs induced multidrug resistance in HCC cell lines and tumor model

The HCC cell lines, HepG2 and Hep3B, were treated with two kinds of chemotherapeutic drugs (5-FU and Gem). As shown in **Figure 4**, the tumor cells cultured with Cav-1 knockdown CAFs' conditional medium showed obvious resistance to 5-FU treatment and Gem treatment compared to the tumor cells cultured with Cav-1 wild type CAFs' conditional medium. The estimated half maximal inhibitory concentration of each drug showed more than ten times' difference in these two groups. Expression of MRP1 in HCC cells was also upregulated by conditioned medium from Cav-1 low expression CAFs (**Figure 4E**).

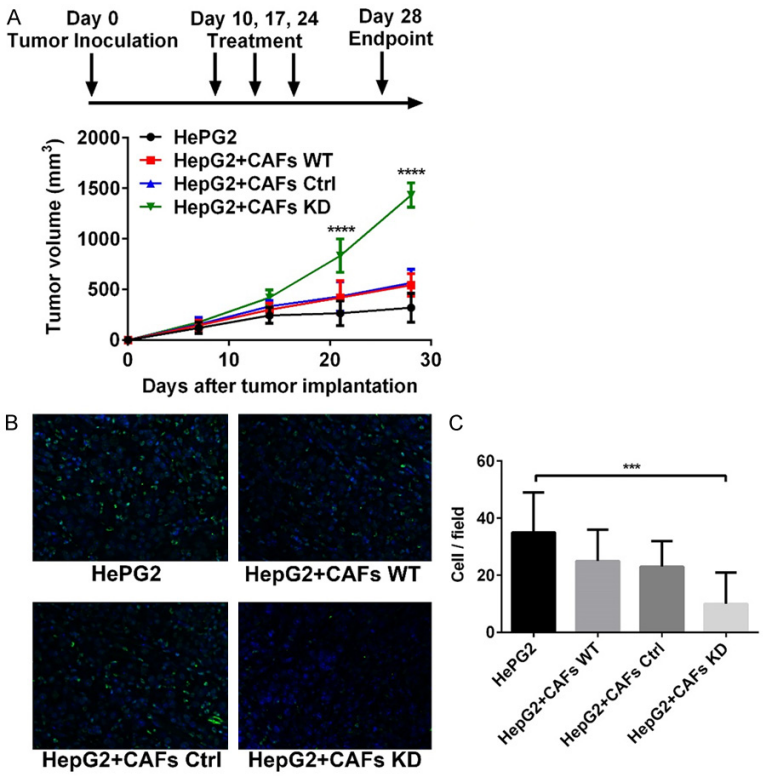

Figure 5. CAFs with Cav-1 knockdown induced drug resistance in HCC tumor model. A. Cancer model treatment plan and tumor volume in each group. The mice were treated with 5-FU (150 mg/kg) and Gem (150 mg/kg) for three times. B. Staining of Activated-Caspase 3 in the tumor tissue of each group. C. Number of Activated-Caspase 3 positive staining cells per field in each group. (****P* value less than 0.001; *****P* value less than 0.0001; CAFs KD: Cav-1 knockdown CAFs; CAFs WT: Cav-1 wide type CAFs; CAFs Ctrl: transfection control CAFs).

To further validate the drug resistance induced by Cav-1 low expression in HCC, we performed an animal model experiment. As shown in **Figure 5A**, the tumors with CAFs grew faster than tumors without CAFs. When treated with 5-FU and Gem, the tumors with Cav-1 low expression CAFs were not as sensitive as other groups indicated by the tumor growth rate (**Figure 5A**). In the tumor tissue sections, we evaluated the expression of Activated-Caspase 3, a marker of cell apoptosis (**Figure 5B**). As expected, the tumors with Cav-1 low expression CAFs showed lowest number of apoptotic cells (**Figure 5C**). These data suggested that the loss of Cav-1 in CAFs could induce multidrug resistance in HCC in *vitro* and in *vivo*, thereby promoting HCC development.

Discussion

In the past decades, the significance of tumor stromal cells in promoting cancer progression has arisen extensive interests in cancer studies [6]. Fibroblasts are the most abundant "non-cancerous" cells in tumors. It is now clear that the CAFs in tumor microenvironment facility tumor development via enhancing tumor invasion, metastasis, drug resistance, and immunosuppression [6]. However, the specific molecular mechanisms in the CAFs mediated cancer development are not clear yet. Herein, we focused on Cav-1 expressed in CAFs, investigating its effects on HCC invasion and drug resistance.

Cav-1 is expressed in both tumor cells and stromal cells in many cancers [13-15]. In HCC samples, we analyzed the Cav-1 expression by IHC and flow cytometry. Interestingly, by these two methods, we localized high Cav-1 expression in CAFs, rather than in tumor cells. In HCC patients, the Cav-1 level in CAFs has significant prognostic values: high expression of Cav-1 in CAFs predicts better overall survival than low expression. This result is in line with the findings from breast cancer, prostate cancer, as well as gastric cancer, where high Cav-1 in CAFs is a favorable prognostic marker [8, 13, 15].

Loss of Cav-1 in CAFs promoted cancer development by multiple mechanisms. In previous breast cancer experimental systems, loss of Cav-1 could stimulate autophagy is CAFs [16, 17]. Activation of the catabolic pathways of autophagy in CAFs secreted a large amount of nutrients into the tumor microenvironment [16, 17]. Studies reveal that a metabolic coupling occurs between catabolic fibroblasts and anabolic cancer cells, thereby promoting cancer cell growth and survival in stress conditions [16, 17]. Cav-1 lost CAFs also promote tumor development by secreting cytokines and regulating biological features of cancer cells [18]. In our study, we focused on the invasion ability and drug response of cancer cells, two major malignant features of cancers. Interestingly,

the conditioned media from CAFs with low Cav-1 expression is very efficient in accelerating tumor cell invasion by upregulating MMP-2 and MMP-9 in cancer cells. Gemcitabine and 5-FU are among baseline therapies of advanced cancers. CAFs have shown critical roles in inducing drug resistance [19-21]. The resistance to multiple drugs is a hallmark of cancer progression and linked with poor prognosis in several cancers. Here, we indicated that loss of Cav-1 in CAFs could induce resistance to these drugs, implying the second mechanism by which Cav-1 low expression CAFs facilitates HCC progression.

In summary, our investigation indicated the HCC tumor-promoting roles of low Cav-1 expressing CAFs by inducing tumor cell invasion and multidrug resistance. Clinically, our study found that loss of Cav-1 expression in CAFs could serve as a prognostic marker in HCC patients. Further studies investigating the regulatory mechanisms of Cav-1 expression in CAFs and identifying potential targets to upregulate Cav-1 expression are highly desired.

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Disclosure of conflict of interest

None.

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