Cancer-associated fibroblasts induce immunotherapy resistance in hepatocellular carcinoma animal model

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Abstract: Hepatocellular carcinoma is known to be a common predominant cancer in adults, especially in eastern countries. Immune response and cancer-associated fibroblasts (CAFs) have significant influences on tumor development. However, the interaction between CAFs and immunotherapy is unclear in hepatocellular carcinoma. We measured the number of activated fibroblasts in hepatocellular carcinoma samples and samples taken from normal liver tissues. A total of 20 patients’ fresh hepatocellular carcinoma and normal tissues which were surrounding the tumor were obtained from the surgery and used for evaluating alpha-SMA expression. We investigated the effects of CAFs in anti-tumor immunity in hepatocellular carcinoma animal model. The effects of CAFs in inducing anti-PD-1 treatment resistance were also measured in a preclinical animal model. Activated fibroblasts were highly accumulated in hepatocellular carcinoma tissues but not in surrounding normal tissues. CAFs showed a significant tumor-promoting effect in an immunocompetent model. The infiltration and function of some immune cells like myeloid-derived suppressive cells and T-cells were increased by CAFs. CAFs also reduced the number and activation of tumor-infiltrating cytotoxic T-cell in tumor tissue. In the treatment model, tumors with a higher amount of CAFs had been insensitive to therapy with anti-PD-1. CAFs are potent inducers of immunosuppression in hepatocellular carcinoma. Depleting CAFs rescued the antitumor immunity in the hepatocellular model and could be a novel treatment to combine with the existing immunotherapy.

Key words: Hepatocellular carcinoma; Cancer-associated fibroblasts; Immune response; Anti-PD-1 treatment; Preclinical model.

Introduction

Hepatocellular carcinoma is known to be one of the most common malignant tumors in Asian counties (1). The introduction of combinational chemotherapy and surgery for localized hepatocellular carcinoma may increase the survival rate of patients (2). However, the cure rate for patients with the metastatic or relapsed disease remains dismal with short long-term survival (2). Hence, understanding the mechanisms of hepatocellular development is urgent.

Tumors are communities of malignant cells as well as surrounding stromal cells, as well as fibroblasts and infiltrating immune cells (3). The significance of immune cells in determining the cancer patient’s survival and treatment has been widely studied (4, 5). Recently, the US FDA has approved the immunotherapies, such as immune checkpoint blockades, to treat several types of tumors, including melanoma, lung cancer, and renal cancer (6-8). However, immune checkpoint blockades’ efficiency is determined by the overall immune cell function, which can be regulated by non-immune cells, such as cancer-associated fibroblasts (CAFs) (9).

CAFs in cancerous tissues are as like as myofibroblasts in morphology (10). Functionally, CAFs are perpetually activated in cancer tissue and don’t undergo apoptosis-like non-cancerous fibroblasts (10). Designing efficient medications for cancers needs more knowledge about CAFs. Herein, we investigated the immunoregulatory roles of CAFs in a hepatocellular carcinoma model.

Materials and Methods

Cell culture and transfection

Murine hepatocellular carcinoma cell line H22 was received from the Chinese Academy of Sciences (Shanghai, China) Cell Bank. Cell lines were cultured in DMEM medium (Thermo Fisher Scientific, IL, USA) comprising 5% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U / ml penicillin in a 5% CO2 incubator at 37 ° C humidified. At a confluence of 70 percent of the growing cell, layer subculture took place. Main cancer-associated fibroblasts (CAFs) have been derived from hepatocellular fresh H22 murine tissue. Standard hepatic stellate cells (HSCs) were isolated.
Animal model
A syngeneic animal model was developed using 6-week old female BALB/c mice (21-23 g, Shanghai SLAC Laboratory Animal Center at Chinese Academy of Sciences, China) and H22 cells to examine the in vivo immunoregulatory function of CAFs. Each mouse was injected on the hind legs flank with 2 x 105 H22 cells with or without 8 x 10^5 CAFs or 8 x 10^5 HSCs. Each group included ten mice. The productivity of the tumors was tracked every five days. The size of the tumor was determined on the basis of the generally known formula: tumor volume = length \* width^2 \* \pi/6. For the orthotopic model, the same number of cells were injected. The detailed procedure was reported previously (11). The animal work was approved by the local Animal Care and Use Committee. Every mouse was kept in a specific pathogen-free area with free exposure to autoclaved water, regular food, and a day and night period of 12 hours. The treatment plan for each experiment was included in the corresponding Figure legend.

Flow cytometry
A study of the flow cytometry was used in an experimental model to analyze the immune infiltration. CD8^+ T cells (CD19^-, CD3^+, CD4^-, and CD8^+), regulatory T cells (Treg, CD19^-, CD3^-, CD4^+, CD8^-, CD25^+ and FOXP3^+), helper T-cells (Th1: CD19^-, CD3^+, CD4^+, CD8^-, and IFN-\gamma^+; Th2: CD19^-, CD3^+, CD4^+, CD8^-, and IL4^+), and myeloid-derived suppressive cells (MDSCs, CD45^-, CD11b^+, and Gr1^+) were classified and analyzed. We isolated single cells from animal tumor tissues and washed them with PBS once. To extract red blood cells the red blood cell lysis buffer was applied. Cells were then washed with PBS once and resuspended in blocking buffer for 10min. Cell membrane staining was then performed, and cells were incubated 15min at room temperature. After cell membrane staining, cells were fixed by fixation/permeabilization buffer for 30min at room temperature. The cytoplasm proteins were then stained at room temperature for 30min. We used the FACSCanto II equipment (Becton Dickinson and Company, San Jose, CA) for data acquiring. Flow Jo software was used to visualize the data.

Patient sample
A total number of 20 hepatocellular carcinoma tissues and 20 tumor-adjacent normal liver tissues were included in this study. These patients were diagnosed from January 2016 to December 2016. All the tissues were obtained during the surgery before chemotherapy or radiotherapy. This study was approved by the local ethics committee. All patients assigned written informed consent.

Western blotting
The protein content of fresh human tissue and cell lysates was calculated utilizing the BCA (Thermo Scientific) protein measurement test. Anti-smooth muscle actin antibody (1:1000 dilution, Abcam) was used for Western blotting to test the rates of various proteins in the lysates. Beta-actin (Abcam, 1:2000 dilution) has been used as a charge buffer. The general western blotting technique was implemented. Pierce ECL Western Blotting Substrate (Thermo Scientific) was also used to generate the signals.

Statistical analysis
Graph Pad software (CA, USA) was used for statistical analyses and data visualization. The data were shown as mean ± SEM. One-way ANOVA was used to analyze the difference of means between more than two different groups. T-test was performed for two-group comparison. Differences with a two-tailed P-value<0.05 were considered as statistically significant.

Results
Activated fibroblasts are accumulated in hepatocellular carcinoma
A total of 20 patients’ fresh hepatocellular carcinoma and surrounding normal tissues were obtained from the surgery and used for evaluating alpha-SMA expression. The hepatocellular carcinoma tissues showed higher alpha-SMA expression than tumor-adjacent normal tissues (Figure 1A and B). Alpha-SMA is expressed in activated fibroblasts, but not in quiescent fibroblasts. Thus, our data suggested that CAFs, a subtype of activated fibroblasts were accumulated in hepatocellular carcinoma.

CAFs induced immunosuppressive cells accumulation in tumor tissue
Flow cytometry quantified the major innate immune cell types in tumor tissues. The gating strategy of myeloid-derived suppressive cells (MDSCs) has been shown in Figure 2A. The number of tumor-infiltrating MDSCs was highest in tumors containing exogenous CAFs (Figure 2B). However, the frequency of tumor-infiltrating macrophages and dendritic cells (DCs) were not changed by the exogenous CAFs (Figure 2E and F). The immunosuppressive cytokines, IL-10 and PD-L1, were also accumulated in tumor-infiltrating MDSCs from the tumors with a high amount of exogenous CAFs (Figure 2C and D). When dasatinib, the functional inhibitor of

![Figure 1](https://example.com/figure1.png)

**Figure 1.** CAFs in hepatocellular carcinoma. (A)alpha-SMA expression in the representative case of hepatocellular carcinoma and surrounding normal liver tissues. (B) Quantification of alpha-SMA expression in 20 hepatocellular carcinomas and surrounding normal liver tissues. The data were normalized to \(\beta\)-actin expression. (**** P-value < 0.0001).
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we further investigated the therapeutic role of dasatinib in hepatocellular carcinoma. As shown in Figure 5A, the administration of dasatinib delayed tumor growth in tumors with a high number of CAFs. In an orthotopic model, we found that anti-PD-1 treatment alone didn’t dramatically increase mice survival time. However, when dasatinib was combined, the survival time was dramatically increased (Figure 5B).

Discussion

There is abundant evidence indicating that natural
The activation of CAFs was inhibited by dasatinib, the functional inhibitor of activated fibroblasts (16). When the number of Treg in tumor tissue was enlarged by exogenous CAFs. This data suggested that CAFs induced immunosuppression via excluding cytotoxic T-cell and accumulating Treg in tumor tissue. This is in line with the previous study in gastric cancer that activated CAFs can shift the ratio of cytotoxic T-cell to Treg (18). We also checked the functional markers of T-cell, IFN-γ and granzyme B, which are downregulated by functional CAFs as well. These data strongly supported that functional CAFs are immunosuppressive in hepatocellular carcinoma and reversing CAFs activation may release the suppression.

The immune checkpoint blockades have achieved impressive effects in melanoma patients (19, 20). However, when tested in solid tumors, the immune checkpoint blockades alone showed limited efficacy. This is partly due to the immunosuppressive microenvironment in solid tumors, such as hepatocellular carcinoma (21). Combinatory immunotherapy has been widely tested in clinical trials (22). Here, we showed that inhibiting the activation of CAFs could reduce exogenous CAFs mediated hepatocellular carcinoma growth. More importantly, the efficacy of anti-PD-1 treatment was enhanced by dasatinib treatment. These data highlighted the clinical value of targeting CAFs in hepatocellular carcinoma.

In conclusion, our study indicated that activated CAFs promoted hepatocellular development via inducing strong immunosuppression. Inhibition of activated CAFs released the immunosuppression in the tumor microenvironment and thus might be a promising target for combining with immunotherapies.

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Conflicts of interests
None.

References