Cancer-associated fibroblasts induce sorafenib resistance of hepatocellular carcinoma cells through CXCL12/FOLR1

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Abstract

Background: Due to the high drug resistance of hepatocellular carcinoma (HCC), Sorafenib has limited efficacy in the treatment of advanced HCC. Cancer-associated fibroblasts (CAFs) play an important regulatory role in the induction of chemo-resistance. This study aimed to clarify the mechanism underlying CAF-mediated resistance to sorafenib in HCC.

Methods: Immunohistochemistry and immunofluorescence showed that the activation of CAFs was increased in cancer tissues of HCC. CAFs and para-cancer normal fibroblasts (NFs) were isolated from the cancer and para-cancer tissues of HCC, respectively. Cell cloning assay, Elisa, and flow cytometry were used to detect that CAFs induced sorafenib resistance of HCC cells via CXCL12. Western blot and qPCR detected that CXCL12 induces sorafenib resistance of HCC cells by up-regulating FOLR1. We investigated that FOLR1 was the target molecule of CAFs regulating sorafenib resistance in HCC cells by querying the gene expression dataset platform of human HCC specimens from GEO genomic data platforms.

Results: CAFs were increasingly activated in cancer tissues of HCC, compared with pare-cancer tissues of HCC samples. CAFs inhibited the sensitivity of HCC cells to sorafenib. CAFs secreted CXCL12 to induce sorafenib resistance of HCC cells. CXCL12 upregulated the expression of FOLR1 in HCC cells to induce sorafenib resistance.

Conclusions: We found that CAFs induce sorafenib resistance of HCC cells through CXCL12/FOLR1.

Background

Hepatocellular carcinoma (HCC) is one of the most malignant tumors worldwide, and the incidence is increasing[1]. Studies have shown that more than 50% of HCC patients are initially diagnosed at an advanced stage[2]. Sorafenib is the first Food and Drug Administration (FDA) approved targeted therapy for advanced HCC[3]. However, since most advanced HCC patients acquired resistance to Sorafenib, only 10–30% of patients show objective responses to sorafenib[2]. Therefore, the prognosis of advanced HCC patients remains poor prognosis, even after sorafenib treatment[4, 5]. Therefore, we need to further investigate the mechanisms of sorafenib resistance in HCC. It is significant to develop novel therapeutic strategies to overcome sorafenib resistance in patients with advanced HCC.

Cancer-associated fibroblasts (CAFs) are the major cellular component of the tumor microenvironment (TME)[6]. CAFs could stimulate tumor cell drug resistance, proliferation, invasion, and metastasis in various cancers, including HCC[7, 8]. Recent studies have shown that CAFs play a crucial role in the induction of chemoresistance in a variety of cancers, including HCC[9–12]. However, the mechanism of CAFs-mediated sorafenib resistance in HCC remains to be fully elucidated. It is a crucial step towards identifying novel therapeutic targets to overcome chemotherapy resistance and predict treatment response.

Therefore, we aim to investigate the mechanism of CAF-induced sorafenib resistance in HCC. It can help us to identify novel therapeutic targets for overcoming sorafenib resistance. It will be significant to reduce the rate of sorafenib resistance and improve the prognosis of advanced HCC patients.

Methods

Isolation of fibroblasts from cancer tissues and para-cancer tissues of HCC samples

Human HCC tissues were obtained in post-surgical samples, from the department of hepatobiliary surgery, at the Fifth Affiliated Hospital of Sun Yat-sen University. All subjects signed an informed consent form, and the study was approved by the Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University.

Cancer-associated fibroblasts (CAFs) and para-cancer normal fibroblasts (NFs) were isolated from HCC tissues and non-tumor tissues adjacent to the HCC, respectively. Fresh cancer tissues and para-cancer tissues of HCC samples were washed with phosphate buffer (PBS; GenDEPOT, Barker, TX, USA) and mince into small pieces (< 1 mm^3). Five minuted small tissues were attached to the cell culture dishes and treated with Dulbecco's modified eagle medium (DMEM medium, Gibco, USA), containing 10% fetal bovine serum (FBS; Invitrogen, Waltham, MA, USA), 50 U/mol penicillin (Sigma-Aldrich, USA) and 50 mg/ml streptomycin (Sigma-Aldrich, USA). The DMEM medium was changed every two days. The fibroblasts extending from the HCC tissue were then trypsinised and transferred to the dish, followed by incubation in fresh medium to facilitate attachment of the isolated fibroblasts to the dish. Cells were maintained in complete medium at 37 ° C in a humidified incubator with 5% CO_2 and 21% O_2.

Cell lines

HCC cell lines (HepG2, Huh7) were obtained from the Cancer Center, Sun Yat-sen University, Guangzhou, Guangdong, People's Republic of China. Cancer-associated fibroblasts (CAFs) and normal fibroblasts (NFs) were cultured from post-surgical HCC tissues in our center. All cells were cultured in Dulbecco's modified eagle medium (DMEM medium, Gibco, USA), which was supplemented with 10% fetal bovine serum (FBS), 50 U/mol penicillin (Sigma-Aldrich, USA) and 50 mg/ml streptomycin (Sigma-Aldrich, USA). AMD3100, an inhibitor of CXCL12, was purchased from Selleck (S8030). The CXCL12 protein was purchased from MedChemExpress (HY-P7287). The anti-CXCR4 was purchased from Proteintech (60042-1-Ig). The cultured cells were kept in a humidified incubator at the temperature of 37°C, under the concentration of 5% CO_2, and 21% O_2.

HCC cells were co-cultured with CAFs and NFs

CAFs (1×10^6 cells /ml) or NFs (1×10^6 cells /ml) were cultured in Dulbecco's modified eagle medium (DMEM medium, Gibco, USA), which were supplemented with 10% fetal bovine serum (FBS) for 48 hours. Then, their supernatant was collected and used to treat the HCC cells (HepG2 and Huh7) for 48 hours.

Western blotting analyses

After all sample proteins were separated by SDS-PAGE gel, they were transferred to the PVDF membrane and then blocked with 5% skimmed milk. Then, they were subsequently incubated with the primary antibody and secondary antibody. The primary antibodies used to detect the target protein were β-actin (abclonal, AC026, 1:1000), FOLR1 (Proteintech, 23355-1-AP, 1:1000), CXCR4 (Proteintech, 60042-1-Ig, 1:1000), Cleaved Caspase-3 (Cell Signaling Technology, 5A1E, 1:500). The targeted bands were analyzed by ImageJ software (v1.8.0; National Institutes of Health, USA). The β-actin was used as the internal control. The relative protein levels were quantified through comparison to β-actin.

Cell Viability Assays

Cell proliferation was analyzed by the cell counting kit-8 (CCK-8, MedChemExpress, Cat. No. HY-K0301). Cells were seeded at a density of 1×10^4 /well into 96-well microplates. Then, the cells were treated with various concentrations of Sorafenib (0.25, 0.5, 1, 2, 4, 8, 16, and 32 μM). The CCK-8 assay was performed after 48 hours of treatment. Treated cells were incubated for 4 hours with a culture medium containing the CCK-8 reagent, and absorbance was recorded at 450 nm using the iMark™ Microplate Absorbance Reader (Bio-Rad, iMark, United States). All experiments were repeated three times. The inhibition of cell proliferation was expressed by the absorbance.

Flow cytometry apoptosis assay

Cell apoptosis analysis: cells were implanted into a 6-well plate for apoptosis analysis. Then, the medium was replaced with a fresh medium supplemented with 3 μM Sorafenib. After treatment of 48 hours, the cell apoptosis was detected by Apoptosis Detection Kit (eBioscience™ Annexin V Apoptosis Detection Kits, Thermo Fisher Scientific, Lot No. 2106736). All cells were detected by Polychromatic analytical flow cytometry (Beckman, Cytoflex LX, United States).

Colony Formation Assay

HCC cells (800 cells/well) were implanted into a 6-well plate. Then, they were treated with the supernatant of CAFs or NFs, CXCL12 protein, AMD3100 (20 μM), and sorafenib (3 μM). After treatment of 15 days, cells were fixed with 4% paraformaldehyde and stained with crystal violet (5%). Each experiment was done thrice. Cells colonies formation rate = Number of colonies formed in each treatment group / Number of implanted cells (800 cells) × 100%.

Enzyme-Linked Immunosorbent Assay (Elisa)

The supernatants of CAFs and NFs were carried out with ELISA analyses. The OD value was detected with the enzyme plate analyzer. Meanwhile, the amount of target protein secreted per 100,000 cells was calculated. The Elisa kit was a Human stromal cell-derived factor 1β (CXCL12β/SDF1B) ELISA kit (Cusabio, B04011121).

Immunohistochemistry staining (IHC)

Human HCC tissues and the xenograft tumors of mice were sectioned into 5μm slices. Hematoxylin and eosin staining was applied to confirm the status of cancer or cancer-free. All tissue sections were baked, dehydrated, hydrated, and antigen-retrieved. Then, they were incubated with primary and secondary antibodies. An N-ACHROPLAN microscope (ZEISS, Germany) was used to photograph the representative areas. Image-Pro Plus v6.0 software (Media Cybernetics Inc., Bethesda, MD, USA) was used to analyze the Information Object Definition (IOD) values of all images. We calculated the relative IOD values based on three parameters (sum of area, average density, and IOD), which were used for further analysis.

IHC was performed according to the kits (Boster, SA1028, SA1027). Primary antibodies were used for IHC staining: α-SMA (Abcam, ab119952, 1:100), Cleaved Caspase-3 (Cell Signaling Technology, 5A1E, 1:100).

Immunohistofluorescence staining (IF)

Human HCC tissue samples were sectioned into 5μm slices. Hematoxylin and eosin staining was applied to confirm the status of cancer or cancer-free. IF was performed according to the kits (Boster, SA1028, SA1027). Primary antibodies were used for IHC staining: CXCL12 (Boster, BA1389, 1:100), and α-SMA (Abcam, ab119952, 1:100).

Animal Xenograft Models

The growth of the tumor was observed in vivo. A total of 9 BALB/c mice (4 weeks old; 9 males) were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China) and housed with a 12 hrs light/dark cycle and fed standard laboratory food and water. We randomly divided the 9 mice into the NC group, CAFs group, and CAFs + AMD3100 group equally, with 3 mice in each group.

1×10^6 HepG2 cells (50 μL) combined with 1×10^6 NFs cells (50 μL) were mixed, and then injected subcutaneously into the left and right flanks of nude mice in the NC group. Meanwhile, 1×10^6 HepG2 cells (50 μL) and 1×10^6 CAFs (50 μL) were mixed and then injected subcutaneously into the left and right flanks of nude mice in the CAFs group, and CAFs + AMD3100 group. Therefore, we would get 6 tumors in each group. The tumor volume (mm^3) = (length of tumor × width of $tumor^2$)/2. When tumor volume reached 100–150 mm^3, the mouse of the NC group and CAFs group were began to receive tail vein injections of an equal volume of normal saline + Sorafenib (30 mg/kg) for 3 weeks. Meanwhile, the mouse of the CAFs + AMD3100 group was began to receive tail vein injections of an equal volume of AMD3100 (2.5 mg/kg) + Sorafenib (30 mg/kg) for 3 weeks.

Statistical analysis

Statistical analysis was performed using GraphPad Prism V.8 software. All data were repeated at least three times. Data were presented as mean +/- SEM and analyzed by Student's t-test and Pearson correlation. One-way analysis of variance (ANOVA) and Brown-Forsythe tests were carried out for multiple group comparisons. Kaplan-Miere analysis and Log-rank test as used to analyze the survival of HCC patients. For each test, values of $p < 0.05$ were considered statistically significant. *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$; ****$p < 0.00001$. N.S, not significance.

Results

The CAFs were increasely activated in cancer tissues of HCC, compared with para-cancer tissues

α-SMA is a common marker of CAFs activation[13, 14]. The results of IHC and IF revealed that the activation of CAFs was significantly increased in ccancer tissues of HCC, compared with para-cancer tissues of HCC (Fig. 1a-b).

CAFs induced sorafenib resistance of HCC cells

Then, we sorted CAFs from caner tissues in HCC (Fig. 2a). Meanwhile, we sorted NFs from para-cancer tissues in HCC (Fig. 2a). To explore whether CAFs can induce sorafenib resistance in HCC cells, we co-cultured CAFs, NFs and HCC cells. The result showed that CAFs significantly enhance the resistance of HCC cells to sorafenib (Fig. 2b-d). Therefore, CAFs were significantly activated in caner tissues of HCC, which suppressed the sensitivity of HCC cells to sorafenib.

CAFs secreted CXCL12 to induce sorafenib resistance of HCC cells

CXCL12 is a common secreted factor in CAFs[15]. Studies have shown that CXCL12 could induce durg resistance in cancer[16–18]. However, there is no report on whether CXCL12 can induce sorafenib resistance in HCC cells. To explore whether CAFs secret CXCL12 to induce sorafenib resistance in HCC cells, the following experiments were performed. Immunofluorescence assay showed that compared with para-cancer tissues, CAFs expressed higher level of CXCL12 in cancer tissues of HCC (Fig. 3a). The result of Elisa showed that CAFs secreted higher levels of CXCL12 than NFs (Fig. 3b). Meanwhile, the results of cell cloning assays, and flow cytometry apoptosis analysis showed that CAFs significantly reduce the sensitivity of HCC cells to sorafenib (Fig. 3c-f). When CXCL12 was inhibited, the sensitivity of HCC cells to sorafenib was increased (Fig. 3c-h). These results suggested that CAFs induce sorafenib resistance in HCC cells through CXCL12.

CXCL12 induced sorafenib resistance of HCC cells by upregulating FOLR1

To explore the mechanism of CXCL12, secreted by CAFs, induces sorafenib resistance in HCC cells. We found two datasets of cancer cells treated with CXCL12 protein (GSE15893 and GSE40017) in the GEO database. We took the intersection of these two datasets and combined them with the reported drug-resistant genes of HCC[2, 19, 20]. The result showed that FOLR1 was the most significantly upregulated drug-resistant gene (Fig. 4a). Subsequently, we used the CXCL12 protein to treat HCC cells. The result of qPCR showed that CXCL12 protein significantly upregulated FOLR1 mRNA levels in HCC cells (Fig. 4b).

Furthermore, both CXCL12 protein and CAFs not only significantly upregulate FOLR1, but also induce sorafenib resistance in HCC cells (Fig. 4c-h). Meanwhile, When CXCL2 was inhibited by AMD3100, those effect were disappeared (Fig. 4c-h).

Studies have revealed that the upregulation of FOLR1 was associated with sorafenib resistance in cancer cells[19, 21]. Additionally, CXCR4 has been identified as the major cognate receptor for CXCL12. The CXCL12/CXCR4 biological axis played an important role in the malignant progression of cancer[17, 22, 23]. To investigate whether CXCL12 upregulates FOLR1 to induce sorafenib resistance in HCC cells through CXCR4. We inhibited CXCR4 of HCC cells and subsequently treated them with CXCL12 protein or CAFs supernatants. The results demonstrated that while the CXCR4 was inhibited, the ability of CXCL12 protein or CAFs supernatant upregulates FOLR1 in HCC cells was inhibited, and the sensitivity of HCC cells to sorafenib was increased (Fig. 4i-j). These findings suggested that CAFs induced sorafenib resistance in HCC cells through the CXCL12/CXCR4/FOLR1 pathway.

CAFs enhanced sorafenib resistance of HCC cells through CXCL12 in vivo

Next, to further verify the effects of CAFs induced sorafenib resistance in HCC cells in vivo, the xenograft model of nude mice was established (Fig. 5a). The results showed that CAFs significantly enhanced the resistance of HCC cells to sorafenib, while CXCL12 inhibition reversed this phenomenon (Fig. 5b-c). Furthermore, the result of IHC showed that during the treatment of sorafenib, CAFs reduced the sensitivity of HCC cells to sorafenib, while CXCL12 inhibition could increase the apoptosis of HCC cells (Fig. 5d-e). In conclusion, CAFs could induce sorafenib resistance in HCC cells by CXCL12 in vivo.

Discussion

Sorafenib is a first-line targeted drug for the treatment of advanced HCC, but more and more evidence showed that the high drug resistance of HCC cells leads to poor efficacy of sorafenib[2]. Various studies have attempted to investigate the unnderlying mechanisms of sorafenib resistance in HCC and try to identify new therapeutic targets to overcome drug resistance[24, 25]. In this study, we determined that the activation of CAFs is significantly increased in cancer tissues of HCC, which could induce sorafenib resistance in HCC cells. Meanwhile, CAF secreted C-X-C motif chemokine 12 (CXCL12) to upregulate FOLR1 expression in HCC cells via the CXCL12/CXCR4 axis. Furthermore, the overexpression of FOLR1 is positively correlated with sorafenib resistance in HCC. Finally, our results revealed that CAFs induce sorafenib resistance in HCC cells through the CXCL12/CXCR4/FOLR1 pathway.

Generally, CAFs promote the malignant progression of cancer cells by secreting cytokines, chemokines, and pro-angiogenic factors, including CXCL12, TGF-β1, VEGF, PDCF, IL-6 and CXCL16, etc[26–28]. Among them, SDF-1 and TGF-β1 are two of the most-powerful and widely investigated molecules in various solid tumors, including HCC, pancreatic carcinoma, colorectal cancer, etc[26–28]. Our results also suggested that compared with NFs, CAFs secreted higher level of CXCL12, which specifically binding to CXCR4 in HCC cells.

CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is an extracellular homeostatic chemokine that often binds to CXCR4, which could regulate cancer cell malignant progressions[29–31]. Relevant studies have shown that the high expression of CXCL12 could promote drug resistance in pancreatic cancer, breast cancer, and acute lymphoblastic leukemia[16–18]. However, the underlying mechanism of CXCL12 regulates sorafenib resistance in HCC cells remains unclear. In this study, we found that CXCL12, secreted by CAFs, induced sorafenib resistance in HCC cells by upregulating FOLR1.

Folate receptor 1 (FOLR1), a protein receptor for transporting folate into cells, is a highly restricted expression in normal epithelial cells[19, 32–35], but is abnormally upregulated in HCC, ovarian cancer, and pancreatic cancer[19, 32–35]. Studies found that FOLR1 plays a crucial role in various types of malignant cancers [19, 21, 36, 37]. Furthermore, the upregulation of FOLR1 was correlated in drug resistance of cancer cells[19]. However, the mechanism of FOLR1 upregulation in HCC cells is still unclear, especially in the tumor environment. As reported in our study, we found that FOLR1 is upregulated by CAFs-secreted CXCL12. These results suggested that CAFs secrete CXCL12 to induce sorafenib resistance of HCC cells by up-regulating FOLR1 expression. However, further research is needed to explore the molecular mechanism of FOLR1 effect on cancer cell behavior.

Sorafenib resistance is a persistent clinical challenge for HCC therapy. CAFs acted as a key player in HCC cells evasion of chemotherapy drugs[11, 38]. In this study, we revealed that CAFs secreted CXCL12 to induce sorafenib resistance in HCC cells. CXCL12 has been revealed to take part in the development of chemotherapy resistance in various tumors[18, 22, 39]. However, there is no study on the correlation between CXCL12 and FOLR1 in sorafenib resistant HCC. Our study emerged that CAF secreted CXCL12 to induce sorafenib resistance in HCC cells by upregulating FOLR1.

Conclusion

In summary, we demonstrated that the activation of CAFs is increased in HCC tissues, which is significantly to regulate sorafenib resistance in HCC cells. CAFs secret CXCL12 to induce sorafenib resistance in HCC cells by up-regulating the expression of FOLR1. Our study provides the first evidence that the CXCL12/CXCR4/FOLR1 axis is associated with sorafenib resistance in HCC, suggesting a potential new target for improving the efficacy of sorafenib and the prognosis of HCC patients.

Abbreviations

HCC: Hepatocellular carcinoma; CAFs: Cancer-associated fibroblasts; α-SMA: α-smooth muscle actin; CXCL12: Stromal cell-derived factor-1 (SDF-1); CXCR4: CXC receptor 4; FOLR1: Folate receptor 1; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; ERK1/2: Extracellular signal-related kinases 1 and 2; BAFF: B cell activating factor; NF-κB: Nuclear factor-κB; IL6: Interleukin-6; IL17A: Interluekin-17A; IGF1: Insulin-like growth factor 1; ChIP-qPCR: Chromatin immunoprecipitation quantitative PCR assays; SHH: Sonic Hedgehog; FAP: Fibroblast activation protein; SPP1: Plasma-

secreted phosphoprotein 1; IKKβ: Inhibitor of nuclear factor kappa-B kinase subunit beta; SULF2: Sulfatase 2; GEO: Gene Expression Omnibus.

Declarations

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Authors' contributions

Jiali Zhao designed the research, did most of experiments, analyzed the data, interpreted the results and wrote the original draft. En Lin, Zirui Bai, Yingbin Jia, and Bo Wang analyzed the data and interpreted the results. Yihua Dai, Wenfeng Zhuo, Guifang Zeng took part in sample collection. Xialei Liu provided funding support and took part in sample collection. Jian Li, Baojia Zou, Peiping Li, Chaonong Cai provided funding support and revised the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and are also available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This work was approved by the Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University (NO. 00337). Human HCC tissues were obtained from patients undergoing hepatectomy at the Department of Hepatobiliary Surgery, the Fifth Affiliated Hospital of Sun Yat-sen University. All HCC patients agreed to provide HCC tissue specimens for this study. All subjects signed an informed consent form. Meanwhile, animal experiment in this study was approved by the ethics committee of the Fifth Affiliated Hospital of Sun Yat-sen University (NO. 00337). The study was carried out in accordance with the ARRIVE guidelines. All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

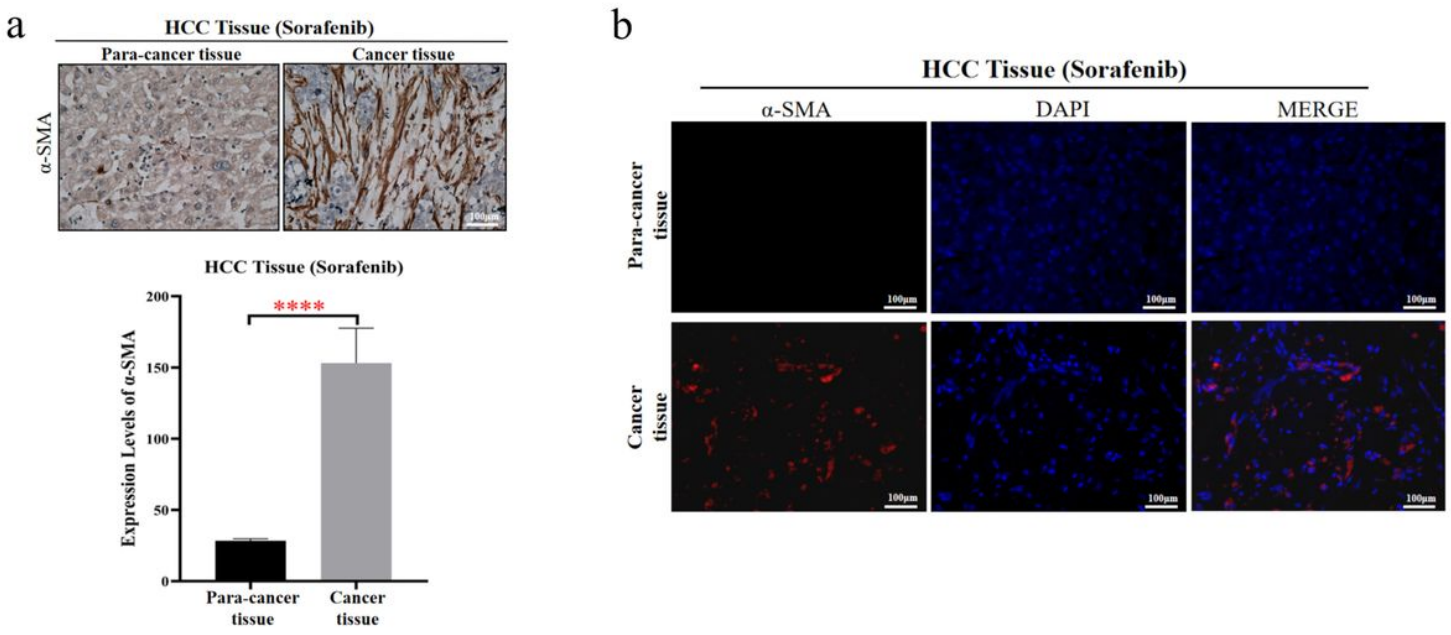

Figure 1

CAFs were significantly activated in cancer tissues compared with para-cancr tissues in HCC samples. **(a)** Representative images of fibroblasts with the expression of α-SMA in cancer tissues and para-cancer tissues of HCC (upper). Statistical plot of fibroblasts expressing α-SMA in cancer tissues and para-cancer tissues of HCC (below). **(b)** Immunofluorescence representative images: the expression of α-SMA in fibroblasts in cancer tissues and para-cancer tissues of HCC. The data presented mean +/- SEM. ****$p<$ 0.00001.

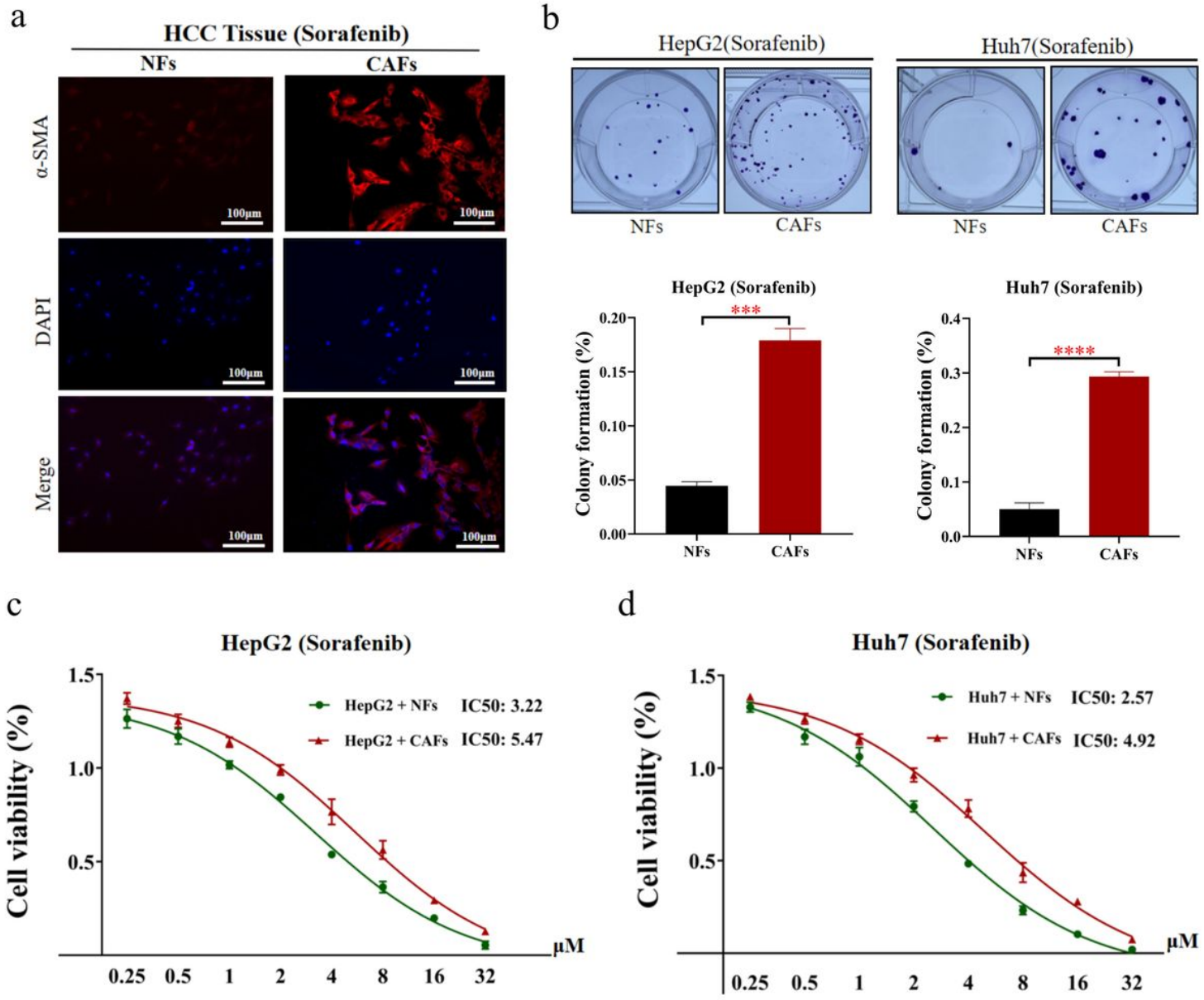

Figure 2

Compared with NFs, CAFs significantly promoted sorafenib resistance in HCC cells. **(a)**CAFs were isolated from HCC tissues. Meanwhile, para-cancer normal fibroblasts (NFs) were isolated from non-tumor tissues adjacent to the HCC. **(b)** The colony forming assay of HCC cells (HepG2 and Huh7), which were treated with the cellular supernatant of CAFs and NFs, and sorafenib. **(c, d)** Cell viability assay: compared with NFs, CAFs significantly increased sorafenib resistance in HCC cells (HepG2 and Huh7). The data presented mean +/- SEM. ***p< 0.0001; ****p < 0.00001.

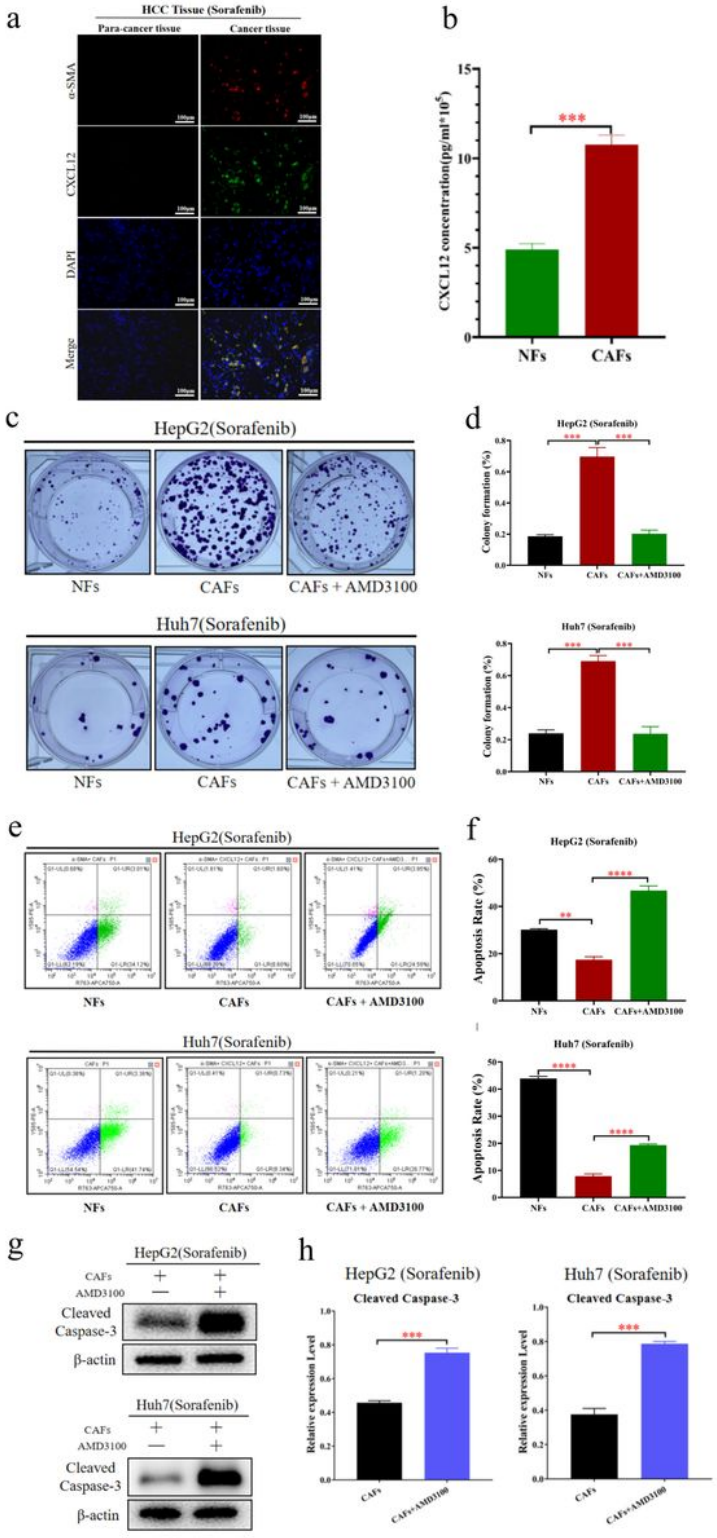

Figure 3

CAFs induce sorafenib resistance in HCC cells by secreting CXCL12. **(a)** The results of immunofluorescence showed that the expression of CXCL12 in CAFs of HCC tissues was significantly higher than that in para-cancer tissues of HCC. **(b)** Elisa showed that CAFs secreted higher level of CXCL12 than NFs. **(c,d)** Colony forming assay detected the sorafenib resistance of HCC cells (HepG2 and Huh7), after treated with the cellular supernatant of CAFs and NFs, sorafenib, and AMD3100. **(e, f)** Flow

cytometry apoptosis assay detected the sorafenib resistance of HCC cells (HepG2 and Huh7), after treated with the cellular supernatant of CAFs and NFs, sorafenib, and AMD3100. **(g, h)** Western blotting was performed to detect the expression of β-actin, and Cleaved Caspase-3 in HCC cells (HepG2 and Huh7), which were treated with the cellular supernatant of CAFs, sorafenib, and AMD3100. The data presented mean +/- SEM. *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$; ****$p < 0.00001$.

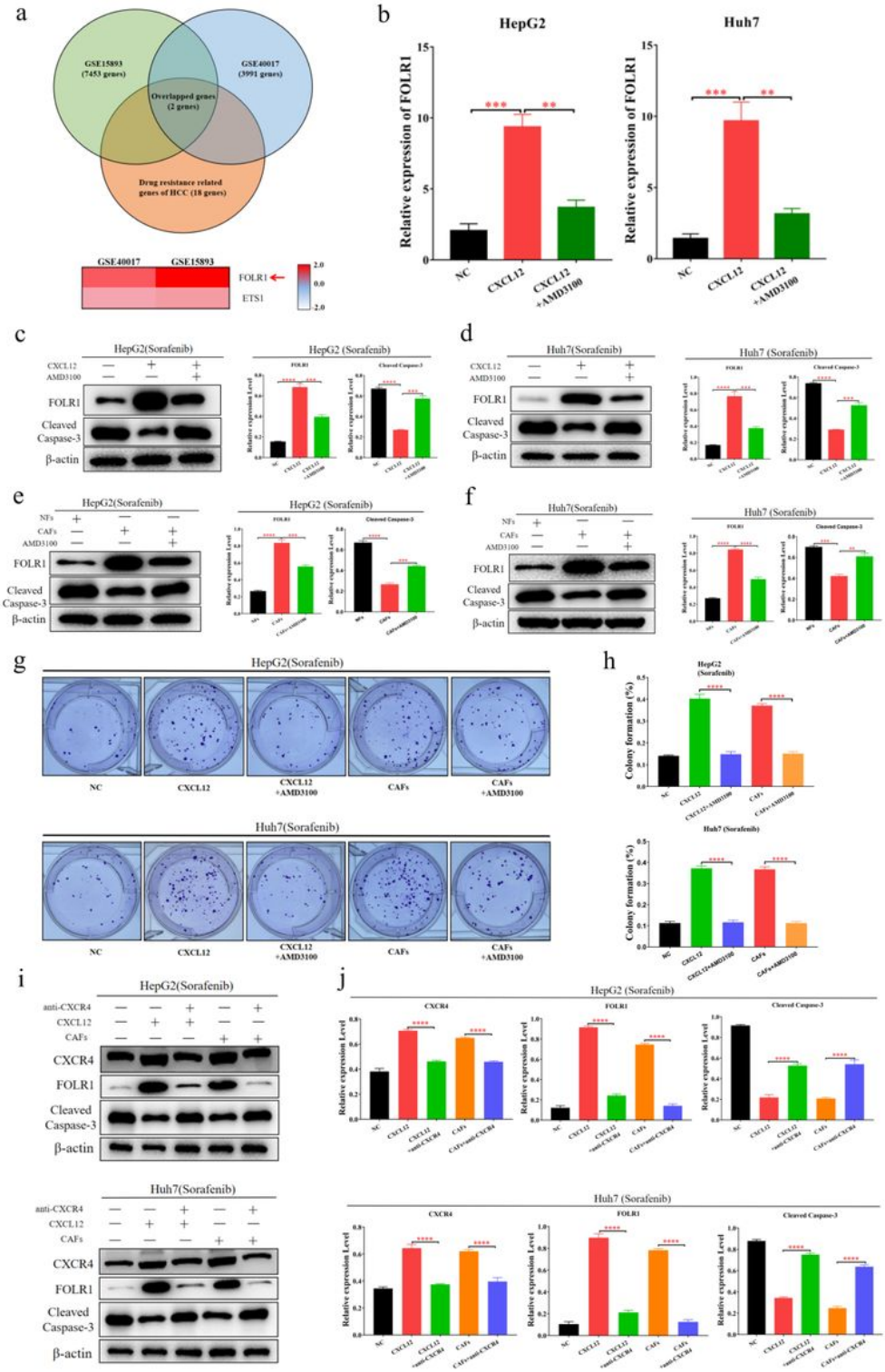

Figure 4

CXCL12 induces sorafenib resistance in HCC cells by up-regulating the expression of FOLR1. **(a)** We found two datasets of cancer cells treated with CXCL12 protein (GSE15893 and GSE40017) in the GEO database. We intersected these two datasets and combined them with the reported drug-resistant genes of HCC to obtain two genes. FOLR1 was the most significantly upregulated drug-resistant gene. **(b)** The qPCR was performed to detect the level of FOLR1 in Huh7 and HepG2, which treated with CXCL12 protein and AMD3100. **(c, d)** Western blotting was performed to detect the expression of β-actin, FOLR1, and Cleaved Caspase-3 in Huh7 and HepG2, after treated with sorafenib, CXCL12 protein, and AMD3100. **(e, f)** Western blotting was performed to detect the expression of β-actin, FOLR1, and Cleaved Caspase-3 in Huh7 and HepG2, after treated with sorafenib, AMD3100, the supernatant of CAFs, and NFs. **(g, h)** Colony forming assay detected the sorafenib resistance of HCC cells (HepG2 and Huh7), after treated with sorafenib, CXCL12 protein, the supernatant of CAFs, and AMD3100. **(i, j)** Western blotting was performed to detect the expression of β-actin, FOLR1, CXCR4, and Cleaved Caspase-3 in Huh7 and HepG2, after treated with sorafenib, anti-CXCR4, CXCL12 protein, and the supernatant of CAFs. The data presented mean +/- SEM. ** $p < 0.001$; *** $p < 0.0001$; **** $p < 0.00001$.

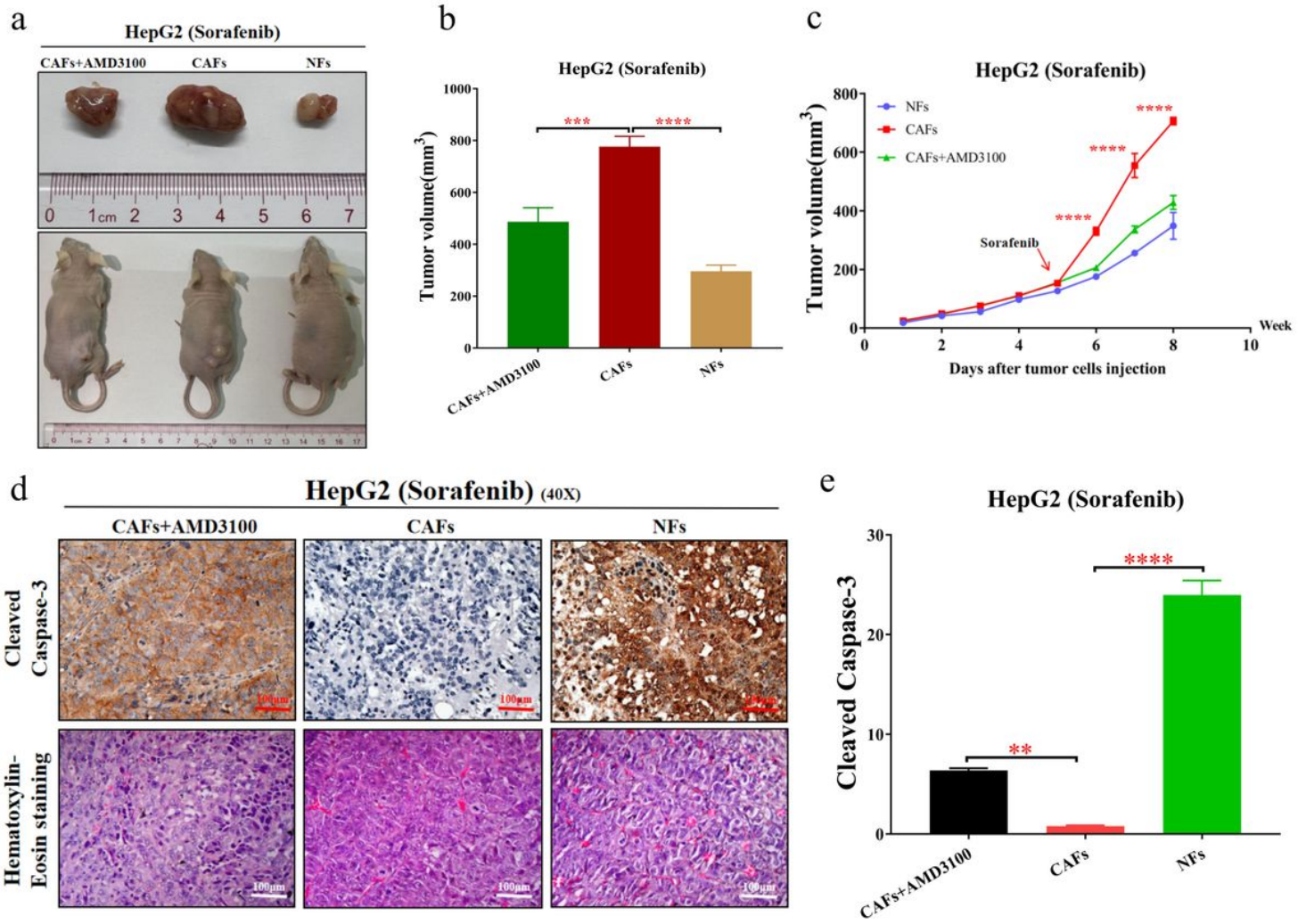

Figure 5

CAFs enhance sorafenib resistance of HCC cells through CXCL12 in vivo. **(a)** Representative images of tumors in mice of CAFs + AMD3100 group, CAFs group, and NFs group after different treatments. **(b)** The tumor volume in different treatment groups. **(c)** The tumor proliferation trend in different treatment groups. **(d)** Pathological validation of tumors under a microscope (40X), after H&E staining and Immunohistochemistry in tumor tissues. The immunohistochemistry staining to detect the expression of Cleaved Caspase-3 in different treatment groups from the tumor tissues of mice. **(e)** The expression level of Cleaved Caspase-3 in different treatment groups from the tumors of mice. The data presented mean +/- SEM. $^{**}p < 0.001$; $^{***}p < 0.0001$; $^{****}p < 0.00001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FullLengthWesteringblotsofFigure3and4.pdf