# CFTR, one membrane protein, inhibits proliferation and metastasis of hepatocellular carcinoma through new pathways

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# Abstract

**Background:** CFTR, a chloride channel protein expressed on cell membrane, plays an important role in the transdermal transport of salt and the regulation of intracellular ion concentration, and may be closely related to the occurrence and development of cancer.

**Methods:** In this study, the expression level of CFTR in hepatocellular carcinoma (HCC) and its correlation with metastasis and prognosis were predicted by TGCA, GEPIA and GTEx databases. By constructing MHCC97H with stable overexpression of CFTR and HepG2 with stable interference expression of CFTR cell lines, the expression level of CFTR and its correlation with proliferation and metastasis of HCC cells were preliminarily verified by Western Blot, MTT, Transwell and PI staining. In order to further clarify the potential related mechanism, it was predicted through String and Genecards databases, and the expression level of related proteins was verified by Western Blot. And it was further verified by orthotopic HCC transplanted tumor models combined with *in vivo* imaging system, western blot and immunohistochemical. Finally, CLUE Command database and autodock was used to predict the potential anti-HCC drugs targeting CFTR.

**Results:** The results showed that the expression of CFTR was significantly lower in human HCC tissues (predicted by clinical data) and HCC cells (HepG2, MHCC97L, MHCC97H), and the expression level was negatively correlated with clinical stage, prognosis (predicted by clinical data) and metastatic potency of HCC cells. CFTR could regulate the metastasis and proliferation of HCC by regulating the expression of MMP2, MMP9, E-cadherin, N-cadherin, and CyclinB1 proteins *in vitro* and *in vivo*. CFTR could regulate the expression of HSP90, TERT, and DNAJC6, which meant that HSP90, TERT, and DNAJC6 might build a bridge for CFTR to regulate proliferation and metastasis proteins. Parecoxib might be one potential anti-HCC drug by targeting CFTR.

**Conclusions:** The above studies further clarify a potential new mechanism of HCC occurrence and metastasis, and provide potential new targets and treatment schemes for the diagnosis and treatment of HCC.


# Introduction

Liver is one of the most important tissues in human body, and many physiological activities mainly occur in liver, such as glycogen synthesis and decomposition, gluconeogenesis, ketone body production, urea cycle. Liver cancer ranks third in cancer-related deaths worldwide. Hepatocellular carcinoma (HCC), which accounts for more than 90% of primary liver cancer, is characterized by difficult treatment, high morbidity and high mortality [1, 2]. At present, the therapeutic method of HCC mainly includes surgical resection, liver transplantation, local regional therapy and chemotherapy, and molecular targeted drug therapy of liver cancer. Although the treatment of liver cancer is improving, the overall survival rate of patients has not changed significantly, and one of the key reasons affecting the prognosis of liver cancer is the metastasis. HCC cells are active, invasive and rich in peripheral blood sinuses, which easily invade

capsules and blood vessels, resulting in local diffusion and distant metastasis. However, this recurrence and metastasis seriously restricts the long-term survival of patients.

Ion channel, a kind of hydrophilic transmembrane protein, participates in maintaining the various of life activities. They are closely related to the occurrence and development of many diseases, such as pain, epilepsy, tumor. In recent years, the relationship between ion channels and tumors has attracted more and more attention as a protein on cell membrane, and the abnormal expression level and function were occurred in several kinds of tumor cells. It seems to be used as an upstream factor to regulate tumor proliferation and apoptosis, and participate in tumor angiogenesis, deterioration and metastasis [3–5].

Cystic fibrosis transmembrane transduction regulator (CFTR) belongs to ATP binding cassette transporter family, which is a cAMP-dependent chloride channel protein [6]. CFTR is composed of 1480 amino acids with a molecular weight of about 170kDa, which plays an important role in transepithelial transport of salts, regulation of ion concentration and liquid flow. More and more studies have shown that the abnormal expression or function of CFTR may lead to many diseases, such as pulmonary fibrosis, chronic pancreatitis and cancers [7–9].

In recent years, the correlation between the expression level and function of CFTR and tumors has been paid more and more attention, and its expression tends to be down-regulated in many cancers, such as intestinal cancer, lung cancer, breast cancer, prostate cancer and pancreatic cancer [10–14], which is speculated to play the role of "tumor suppressor gene". However, the expression of CFTR is up-regulated in some other cancers, such as gastric cancer, ovarian cancer and cervical cancer [15–18]. This abnormal expression and bidirectional changes in various tumors suggest that CFTR may be a new potential regulatory factor in tumor pathogenesis, and its regulatory role varies with different tumor types. However, it is rarely reported about the molecular mechanism of abnormal expression of CFTR on tumor biological behavior.

In this study, the expression level of CFTR in HCC and its correlation with metastasis and prognosis were predicted. By constructing MHCC97H and HepG2 hepatocellular carcinoma cell lines with stable overexpression and interference of CFTR expression, the expression level of CFTR and its correlation with proliferation and metastasis of HCC cells were preliminarily verified. Further, the potential mechanism of CFTR regulating HCC proliferation and metastasis was further predicted by informatics and verified through HCC cells and HCC BALB/c nude mice. Finally, the potential therapeutic drugs for HCC by targeting CFTR were predicted by bioinformatics method. Through this study, it could further improve the mechanism of occurrence and metastasis of HCC, and find potential new targets for diagnosis and treatment of HCC.

## Materials And Methods

### Databases and Molecular docking

CFTR expression data in normal tissues and hepatocellular carcinoma were downloaded from The Cancer Genome Atlas (TCGA) (https://gdc.cancer.gov/about-data/publications/pancanatlas), Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/), and Genotype-Tissue Expression (GTEx) (https://www.gtexportal.org/home/) databases. String database (https://cn.string-db.org/) and Genecards database (https://www.genecards.org/) were used for related protein searching. CLUE Command (https://clue.io/command?q=/home) were used for targets prediction.

AutoDock 4.2 [19] was used for automatic placement of compounds in the binding of CFTR (PDB: 5UAK) to obtain the initial structure of complexes performed with a maximum of $2.5 \times 10^7$ energy evaluations in the grid point spacing of 0.375 Å. Clusters were shown to choose the one includes the most conformations and the pose in strongest binding free energy.

# Cell culture

The hepatocellular carcinoma cell lines HepG2, MHCC97L, MHCC97H (American Type Culture Collection, USA) were grown in RPMI 1640 medium, with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, USA) at 37°C in a 5% CO2 incubator. HBLV-hCFTRshRNA-ZsGreen-PURO and control plasmid HBLV-ZsGreen-PURO were purchased by Wanze Bio (Shenyang, China). The cells were infected with MOI value of 30, and the resistance was screened by puromycin. Finally, monoclonal cell lines with stable interference with CFTR expression were screened. hCFTR-GV492 and control plasmid Ctrl-GV492 were purchased by Genechem (Shanghai, China). The cells were infected with MOI value of 30, and the resistance was screened by puromycin. Finally, monoclonal cell lines with stable CFTR over-expression were screened. Geldanamycin were obtained from MedChem Express (GA, USA).

# Cell viability assay

*In vitro* cell viability was determined using the MTT assay. Cells ($5 \times 10^3$/well) were seeded in 96-well culture plates. The cells were incubated for 24, 48, and 72 h at 37°C in a 5% CO2 incubator, after which 10 μL of the MTT solution (5 mg/mL) was added, then 100 μL of DMSO was added 4 hours later and each well was measured at 570 nm using a multi-mode plate reader (Molecular Devices, USA).

# Flow Cytometry for Cell Cycle Analysis

Cell cycle analysis was assessed using PI staining. Cells ($5 \times 10^4$/well) were seeded in 6-well culture plates. The cells were incubated for 48 h at 37°C in a 5% $CO_2$ incubator, after which the cells were stained with 50 μg/mL PI. Then, all labeled cells were analyzed by flow cytometry (BD Biosciences, USA).

# Transwell assays

Cell invasion and migration was assessed using Transwell Permeable Supports (Corning, USA). Cells were resuspended in 100 μL serum-free medium and plated onto Transwell filter inserts coated with Matrigel 1:8 (BD Biosciences, USA) for the invasion assay, and without Matrigel for migration assay. Cells on the bottom side were stained with platen blue and photographed.

# Western blotting

Total protein extraction was carried out using Total Protein Extraction kit (Kaiji, Nanjing, China). The concentration of protein was determined by using the BCA Protein Assay Kit (Kaiji, Nanjing, China). Each sample with 30 mg total protein were added to the polyacrylamide gel. Details of western blotting were as previously described [20]. After transferred to a PVDF membrane, blocked with 10% skim milk or goat serum, they were immersed in diluted primary antibody (CFTR antibody: Santa Cruz, sc-376683, 1:1000; MMP2 antibody: Invitrogen, 436000, 1:1000; MMP9 antibody: Invitrogen, MA5-15886, 1:1000; E-cadherin: Cell Signaling, 14472S, 1:1000; N-cadherin: Cell Signaling, 13116S; Cyclin B1 antibody: Santa Cruz, sc-752, 1:1000; Santa Cruz, sc-753, 1:1000; HSP90 antibody: Cell Signaling, 4877S, 1:1000; TERT antibody: Invitrogen, MA5-38639, 1:1000; DNAJC6: Invitrogen, PA5-56662, 1:1000; β-actin antibody, Invitrogen, MA5-15739, 1:1000); GAPDH antibody, Invitrogen, MA5-15738, 1:1000) at 4 ℃ overnight. β-actin and GAPDH was used as internal references. Each experiment was repeated at least three times to obtain the mean values.

# Immunohistochemistry

Tumor tissue samples of mice were embedded in paraffin and antigen retrieval was performed. Following the blockade of endogenous peroxidase activity, the samples were incubated with the primary antibodies of interest and the appropriate secondary antibodies and reacted with DAB detection reagents.

**Orthotopic hepatocellular carcinoma transplanted tumor model** in vivo

HepG2 sh-Ctrl, HepG2 cells sh-CFTR, MHCC97H Ctrl, MHCC97H CFTR cells were injected into the left lobe of the liver of BALB/c nude mice and then the wound was quickly sutured. The tumor growth and metastasis of BALB/c nude mice in each group were observed every two weeks by small animal in vivo imaging system (Bruker MI SE, Germany). After 6 weeks, the BALB/c nude mice were sacrificed, and the tumors of liver and other tissues were taken out, measured and weighed, and the tumor formation rate and metastasis rate were calculated. The tumor tissues were stored in liquid nitrogen or formalin solution. This protocol was approved by the Committee on the Ethics of Animal Experiments of the Shenyang Pharmaceutical University (SYPU-IACUC-C2021-9-30-102).

# Statistical analysis

Each experiment was repeated at least three times and pooled data were presented as the mean ± SEM. Data were analyzed by using GraphPad Prism and SPSS. Statistical comparisons were made by using Student's t-test and One-Way ANOVA.

# Results

### Low Expression of CFTR in Hepatocellular Carcinoma and Hepatocellular Carcinoma Cell and Its Correlation with Metastasis

The expression level of CFTR in HCC were predicted by TGCA, GEPIA and GTEx databases. According to the prediction of database, CFTR expression in HCC is significantly lower than that in normal adjacent

tissues (Fig. 1A-C). The correlation of CFTR expression with metastasis and prognosis were predicted by TGCA, GEPIA and Kaplan-Meier plotter databases. According to the prediction of database, it was further found that CFTR was negatively correlated with the histological stage of HCC and the clinical prognosis (Disease-free survival, DFS; Recurrence-free survival, RFS) (Fig. 1D-F).

Then, HL7702 normal hepatocytes cells and HepG2, MHCC97L and MHCC97H HCC cells with different metastatic potential were selected to detect the expression of CFTR protein by Western Blot. The results showed that the expression level of CFTR in HepG2, MHCC97L and MHCC97H cells were lower than that in HL7702 cells, and its expression level was negatively correlated with the metastatic potential of hepatoma cells (MHCC97H lower than MHCC97L lower than HepG2) (Fig. 1G).

# CFTR negatively regulates the proliferation of hepatocellular carcinoma

In order to further verify the correlation between CFTR and HCC proliferation and metastasis, HepG2 sh-CFTR cell line was constructed by using HepG2 cells with low metastatic potential and high CFTR expression, and MHCC97H CFTR cell line was constructed by using MHCC97H cells with high metastatic and low CFTR expression. After preliminary verification of CFTR overexpression and interference, the effects of CFTR on proliferation of HCC cells were detected by MTT methods. The MTT results showed that the proliferation level of HepG2 sh-CFTR group was significantly higher than that of HepG2 sh-Ctrl group, while that of MHCC97H CFTR group was significantly lower than that of MHCC97H Ctrl group. The inhibitory effect of CFTR on HCC was time-dependent, and the cell proliferation effect of 72h was the most significant (Fig. 2A).

PI staining and flow cytometry were used to detect the HCC cell cycle. The results showed that over-expression of CFTR significantly increased the proportion of S phase cells and decreased the proportion of G2 phase cells in MHCC97H cells, which inhibited the transformation of MHCC97H cells from S phase to G2 phase. After interfering with CFTR expression, the proportion of S phase cells in HepG2 cells decreased significantly, while the proportion of G2 phase cells increased significantly, which promoted the transformation of MHCC97H cells from S phase to G2 phase, but had no significant effect on the proportion of G1 phase cells (Fig. 2B).

At last, the expression level of related cell cycle proteins was detected by Western Blot. The results showed that overexpression of CFTR inhibited the expression of Cyclin B1 in MHCC97H cells, and interference with CFTR promoted the expression of Cyclin B1 in HepG2 cells, while CFTR had no significant effect on the expression of Cyclin D1 in both MHCC97H and HepG2 cells (Fig. 2C).

# CFTR negatively regulates metastasis of hepatocellular carcinoma

Transwell was used to detect the effects of CFTR on invasion and migration of hepatocellular carcinoma. The results showed that overexpression of CFTR significantly inhibited the invasion of MHCC97H cells,

while interference with CFTR significantly promoted the invasion of HepG2 cells. However, it showed no significant effects on migration of both MHCC97H and HepG2 cells (Fig. 2D).

Therefore, expression level of invasion-related cell adhesion proteins and metastasis-related proteins were tested by Western Blot. The results showed that overexpression of CFTR inhibited the expression of MMP2, MMP9, and N-cadherin in MHCC97H cells, and interference with CFTR promoted the expression of MMP2, MMP9, and N-cadherin in HepG2 cells. Meanwhile, overexpression of CFTR promoted the expression of E-cadherin in MHCC97H cells, and interference with CFTR inhibited the expression of E-cadherin in HepG2 cells. (Fig. 2E)

# Further exploration of potential pathways of CFTR regulating proliferation and metastasis of hepatocellular carcinoma

In order to further explore the related mechanism of CFTR affecting the proliferation and metastasis of HCC, informatics methods were used to predict the potential mechanism. The differentially expressed genes of HCC were obtained through TCGA database, and at the same time, 20 direct (primary) related genes and 229 indirect (secondary) related genes of CFTR were obtained through String database. After intersecting the differential genes of HCC and indirect CFTR related genes, 11 genes were obtained. Two potential pathway genes, TERT and DNAJC6, were obtained by further screening with Genecards database and related researches (Fig. 3A and B).

To validate the predictive results, the protein expression levels of TERT, DNAJC6 and HSP90 (corresponding CFTR-related genes) in HCC cells were detected by Western Blot. The results showed that overexpression of CFTR inhibited the expression of TERT, DNAJC6, and HSP90 in MHCC97H cells, and interference with CFTR promoted the expression of them in HepG2 cells (Fig. 3C).

In order to further verify the mechanism of CFTR regulating of HCC, geldanamycin (GA), a double inhibitor of HSP90 and TERT, was selected. The results showed that the proliferation and invasion of HepG2 cells interfering with CFTR expression were significantly inhibited after GA administration by 100 nmol/L, 1μmol/L, and 10μmol/L (Fig. 3D and E). Compared with HepG2 sh-Ctrl group, the inhibitory effect of HepG2 sh-CFTR group was more significant. It is suggested that this inhibitory effect is closely related to the decrease of CFTR expression level (Fig. S1).

**Negative regulation of CFTR on proliferation and metastasis of hepatocellular carcinoma** in vivo

*In vivo* model of orthotopic HCC transplanted in nude mice was used to further verify the negative regulation of CFTR on proliferation and migration of HCC and its related mechanism. Firstly, four HCC cell lines (MHCC97H Ctrl, MHCC97H CFTR, HepG2 sh-Ctrl, HepG2 sh-CFTR) were transplanted into BALB/c nude mice in situ. After 6 weeks of tumor bearing, the liver and other tissues of mice were taken out, and the growth rate and metastasis rate of mice were counted, meanwhile, the tumor volume was measured and the tumor weight was weighed.

The results showed that MHCC97H CFTR group significantly reduced tumor weight, tumor volume and tumor metastasis rate in liver tissue of mice compared with MHCC97H Ctrl group. Compared with HepG2 sh-Ctrl group, the tumor weight, tumor volume and tumor metastasis rate in liver tissue of HepG2 sh-CFTR group were significantly increased, and most of the metastatic tissues were subcutaneous and intestinal metastasis (Fig. 4A-D).

To further validate the potential mechanism results *in vitro*, the related protein expressions were detected by either Western Blot or Immunohistochemistry. The results showed that the expression level of MMP2, MMP9, N-cadherin, and Cyclin B1 in MHCC97H CFTR group were lower than that in MHCC97H Ctrl group, while the expression levels of these proteins in HepG2 sh-CFTR group were higher than that in HepG2 sh-Ctrl group. The expression levels of E-cadherin in MHCC97H CFTR group were higher than that in MHCC97H Ctrl group, while the expression levels of E-cadherin in HepG2 sh-CFTR group were lower than that in HepG2 sh-Ctrl group (Fig. 4E). The potential pathway proteins were also verified, and the results showed that the expression level of TERT, DNAJC6 and HSP90 in MHCC97H CFTR group were all lower than that in MHCC97H Ctrl group, while the expression levels of them in HepG2 sh-CFTR group were higher than that in HepG2 sh-Ctrl group (Fig. 4F).

# Prediction of drugs targeting CFTR to inhibit the growth and metastasis of hepatocellular carcinoma

Since CFTR seems to be one potential therapeutic target for HCC due to its significant regulatory effect on proliferation and metastasis of HCC. Drugs targeting CFTR might become potential drugs for the treatment of HCC. Therefore, potential drugs targeting CFTR were predicted through CLUE Command database. The results showed that 10 drugs (DMP-543, mesalazine, valaciclovir, otenzepad, parecoxib, nicorandil, cytarabine, tosufloxacin, midazolam, secnidazole) with potential effects were obtained from CLUE Command database, and all these 10 compounds were positively correlated with CFTR, which seemed to be potential activators of CFTR (Fig. 5A). Then, autodock was used for further prediction. The results showed that, all these 10 compounds could bind to CFTR with different binding energy, among which, parecoxib showed the least binding energy (Fig. 5B and C) (Fig. S2). Considering the binding energy, binding site and the type of bond, parecoxib might be supposed to a potential therapeutic for HCC by targeting CFTR.

# Discussion

HCC cells are active in growth, strong in invasiveness, abundant in peripheral blood sinuses, and easy to invade capsules and blood vessels. These characteristics lead to the metastasis of HCC and high mortality. At present, the treatment methods are constantly improving, but the overall survival rate of patients has not been significantly improved. Therefore, it is extremely urgent to find more effective diagnosis and treatment targets for liver cancer.

Ion channels are a kind of membrane proteins, which are responsible for regulating ion homeostasis inside and outside cells and have complex biological functions. Many studies have found that the

abnormal expression of ion channel protein is closely related to the occurrence and development of cancer [21, 22].

HCC is the main type of liver cancer, therefore, the expression level of channel protein was screened in the early stage of the experiment. It showed that compared with the normal tissues adjacent to cancer, the expression level of CFTR mRNA and protein in cancer tissues of HCC patients decreased significantly, and the expression level of CFTR was positively correlated with the differentiation level of HCC (The results have not been published publicly). Therefore, the expression level and the correlation with metastasis of CFTR in HCC was preliminarily confirmed through several clinical databases and HCC cells in this study. Then, MHCC97H cell line overexpressing CFTR and HepG2 cell line interfering with CFTR were constructed at HCC cell level to further prove the correlation between CFTR and proliferation and metastasis of HCC and its related mechanism. In this part of the results, an interesting discovery was appeared, CFTR showed a very significant inhibitory effect on the invasion of HCC, but had no effect on migration, which suggested that CFTR might cause metastasis of HCC by affecting some adhesion factors. Subsequently, this conjecture was verified by further experiments. CFTR could regulate the expression levels of MMP2, MMP9, E-cadherin and N-cadherin, which indicated that these proteins were involved in the regulation mechanism of CFTR on HCC metastasis. In order to further clarify the regulation mechanism of CFTR on proliferation and metastasis of HCC, two potential pathway proteins, TERT and DNAJC6 and their upstream protein HSP90, were screened through clinical database, and verified by *in vitro/in vivo* experiments, and the potential regulatory mechanism of CFTR was clarified, CFTR–DNAJC6–TERT/DNAJC6–MMP2/MMP9/E-cadherin/N-cadherin–CyclinB1 (Fig. 6).

These two pathway proteins have been found to be involved in the proliferation and metastasis of several cancer cells, and showed significant regulatory effects. The abnormal expression of TERT could lead to the reactivation of telomerase. During hepatocarcinogenesis, telomerase reactivation is required to enable the uncontrolled cell proliferation which could lead to development and metastasis of HCC [23]. DNAJC6, DNA/HSP40 homolog subfamily C member 6, could promote the proliferation and metastasis of HCC, and might affect tumor metastasis by regulating epithelial-mesenchymal transition [24, 25]. Considering that abnormal expression of CFTR lead to differential expression of these two proteins in both *in vitro* and *in vivo* tests, it was found that CFTR was probably one key upstream factor of them both.

To further compare the results of HCC tests *in vitro* and *in vivo*, after comprehensive consideration, it was found that although the expression level of CFTR showed rarely effects on the migration of hepatocellular carcinoma *in vitro*, the abnormal expression of CFTR played a significantly important role in both proximal subcutaneous metastasis and distal intestinal metastasis of HCC in BALB/c nude mice. Based on this, it was also suggested that some other mechanism participate in CFTR negatively regulating the metastasis of HCC, such as immune cells in matrix. However, this conjecture needs further verification. Therefore, although the mechanism of CFTR has not been fully understood at present, the negative regulation of CFTR on the metastasis level of HCC is quite significant.

Since the mechanism that CFTR negatively regulates the proliferation and metastasis of HCC is elucidated, which further suggests that CFTR may be a potential anti-hepatocellular carcinoma target. Based on this, the CLUE Command database was used to predict the compounds targeting CFTR, and 10 compounds with high correlation were obtained. In order to further predict the potential binding of compounds to CFTR, the binding energies and binding sites were obtained by molecular docking. It was showed that all the 10 compounds could bind to different amino acid residues of CFTR by hydrogen bond, hydrophobic bond or ionic bond. However, since CFTR is a transmembrane protein, it is bound to have a transmembrane domain, and the compounds which predicted to bound to this region seems very difficult to perform effectively. Considering this factor, only parecoxib, tosufloxacin and valaciclovir could bind to the lasso motif of CFTR (Fig. 5B). Meanwhile, by comparing the binding energy of these three compounds with CFTR, it was found that parecoxib is the most potential anti-hepatocellular carcinoma drug targeting CFTR.

In conclusion, through this study, it was deeply explored the biological function and molecular mechanism of a newly discovered potential metastasis inhibitor of liver cancer, supplemented the existing research theory of molecular mechanism of liver cancer metastasis, and provided potential targets and therapeutics for the research and development for liver cancer metastasis and recurrence.

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of supporting data

Please contact the corresponding author for all data requests.

### Competing interests

The authors declare that they have no competing interests.


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### Author contributions

Yijia Xu, Mingyi Zhao, Jinghai Zhang conceived and designed the experiments. Yijia Xu, Liying Yang, Mengying Zhang, Jianfang Sun, Ruojin Wen, Kaikai Lv, Ying Teng, and Yingxin Qiu conducted the experiments and draft the paper. Yijia Xu, Jianfang Sun, Xin Liu, Qi Wang, Shangfeng Zhao, Yang Su, and Yanfeng Liu conducted database prediction and analyzed the data. All authors revised the paper and approved the final manuscript.

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# Figures

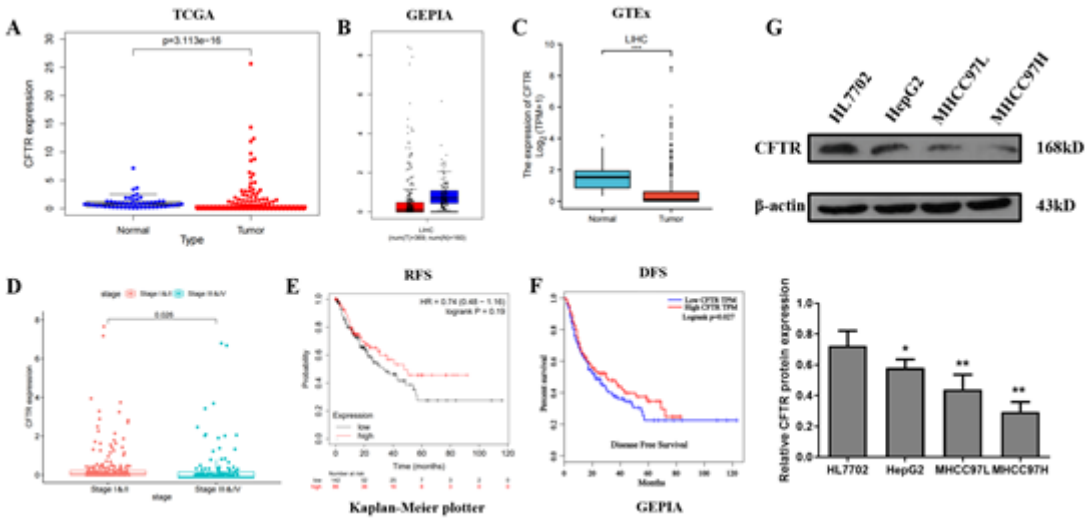
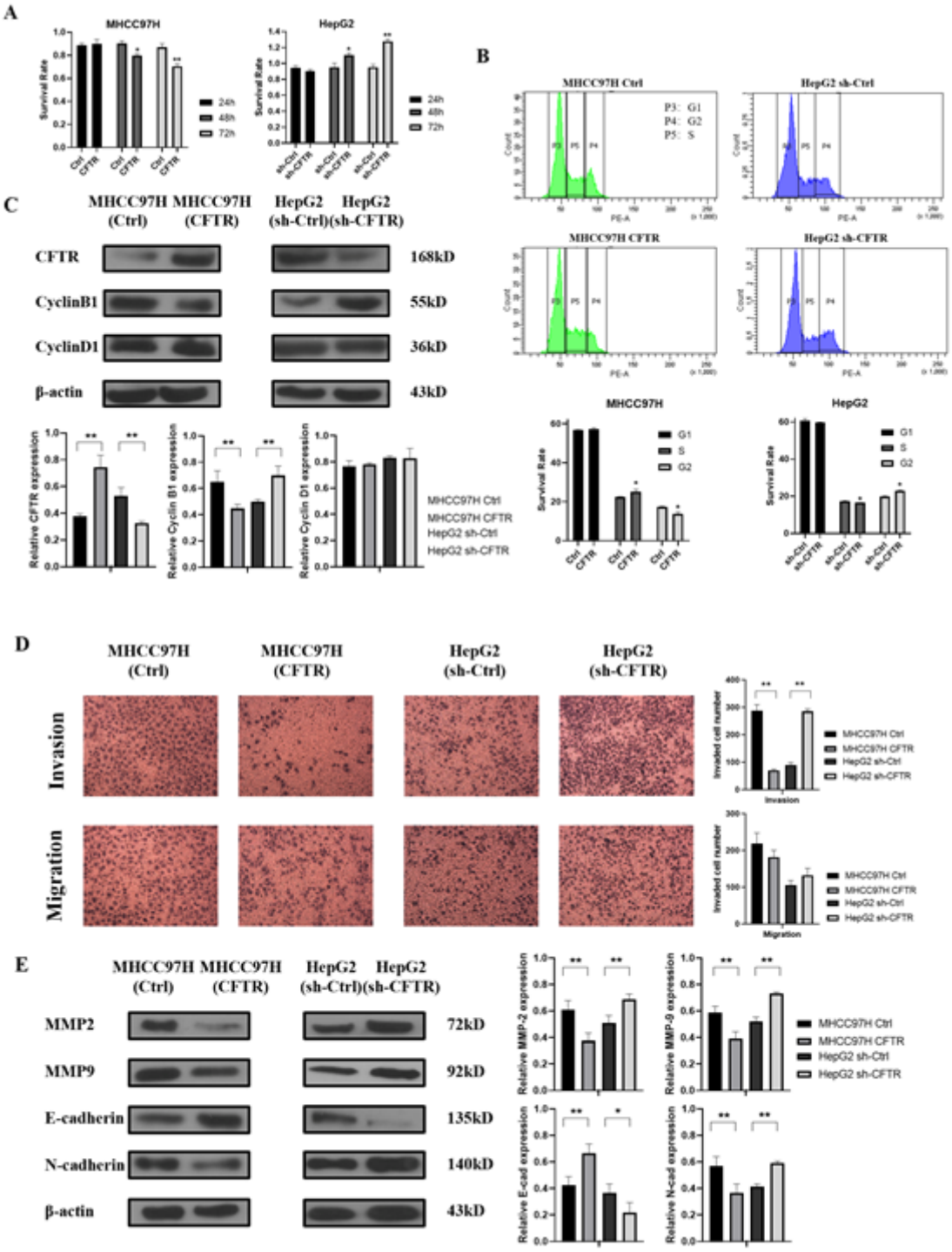



**Figure 1**

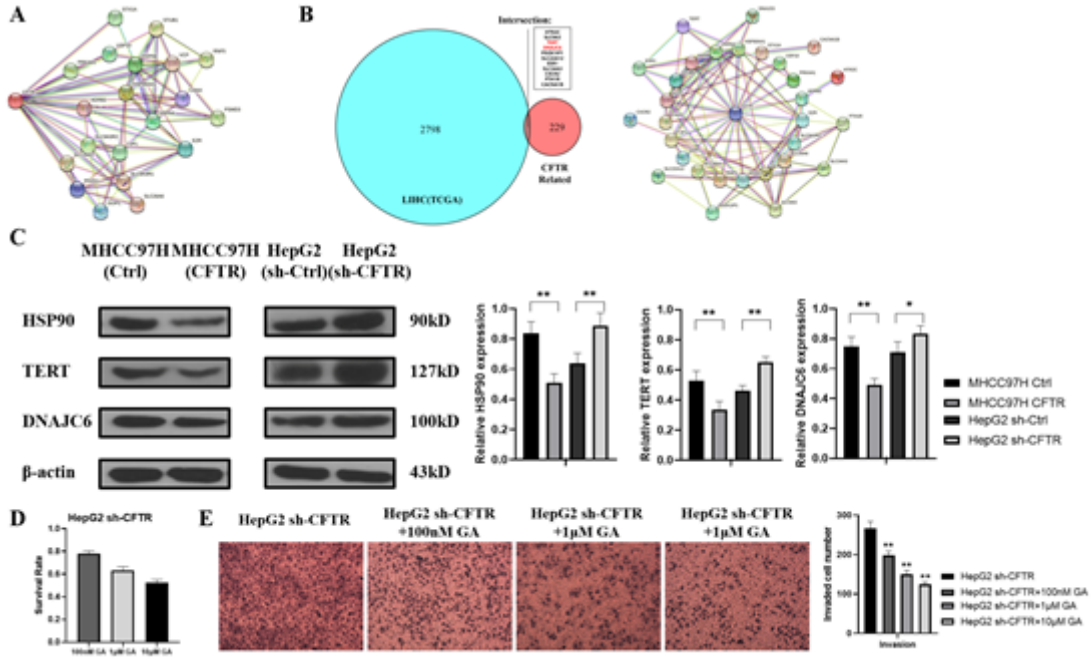
CFTR is lower expressed in human HCC tissues and HCC cells, and the expression level of CFTR was negatively related with metastasis and prognosis. A-C, the predicted expression of CFTR through TCGA, GEPIA, and GTEx databases. D, the predicted correlation of CFTR expression with metastasis through TCGA. E and F, the predicted correlation of CFTR expression with prognosis were predicted by GEPIA and Kaplan-Meier plotter databases. G, Western blot analysis of CFTR expression in normal liver cell and HepG2, MHCC97L, and MHCC97H HCC cells. Data are expressed as mean ± SEM of at least three independent experiments. Statistical significance was concluded at $*P < 0.05$, $**P < 0.01$.



## Figure 2

CFTR inhibited HCC growth and invasion.

A, MTT assays showed that overexpression of CFTR inhibited the growth of MHCC97H cells, and interference of CFTR expression promoted the growth of MHCC97H cells. B, overexpression of CFTR increased the proportion of S phase cells and decreased the proportion of G2 phase cells in MHCC97H cells. Interference of CFTR expression decreased the proportion of S phase cells and increased the proportion of G2 phase cells in HepG2 cells. C, Western Blot analysis of CFTR, Cyclin B1 and Cyclin D1 in overexpression MHCC97H cells and interference of CFTR expression HepG2 cells. D, MHCC97H cells with CFTR overexpression and HepG2 cells with interference of CFTR expression were subjected to Transwell assays. Both migration and invasion assays were performed to determine the effect of CFTR on HCC metastasis. E, Western Blot analysis of MMP2, MMP9, E-cadherin, and N-cadherin in overexpression MHCC97H cells and interference of CFTR expression HepG2 cells. Representative images and the statistical analyses were shown. Data are expressed as mean ± SEM of at least three independent experiments. Statistical significance was concluded at $*P < 0.05$, $**P < 0.01$.

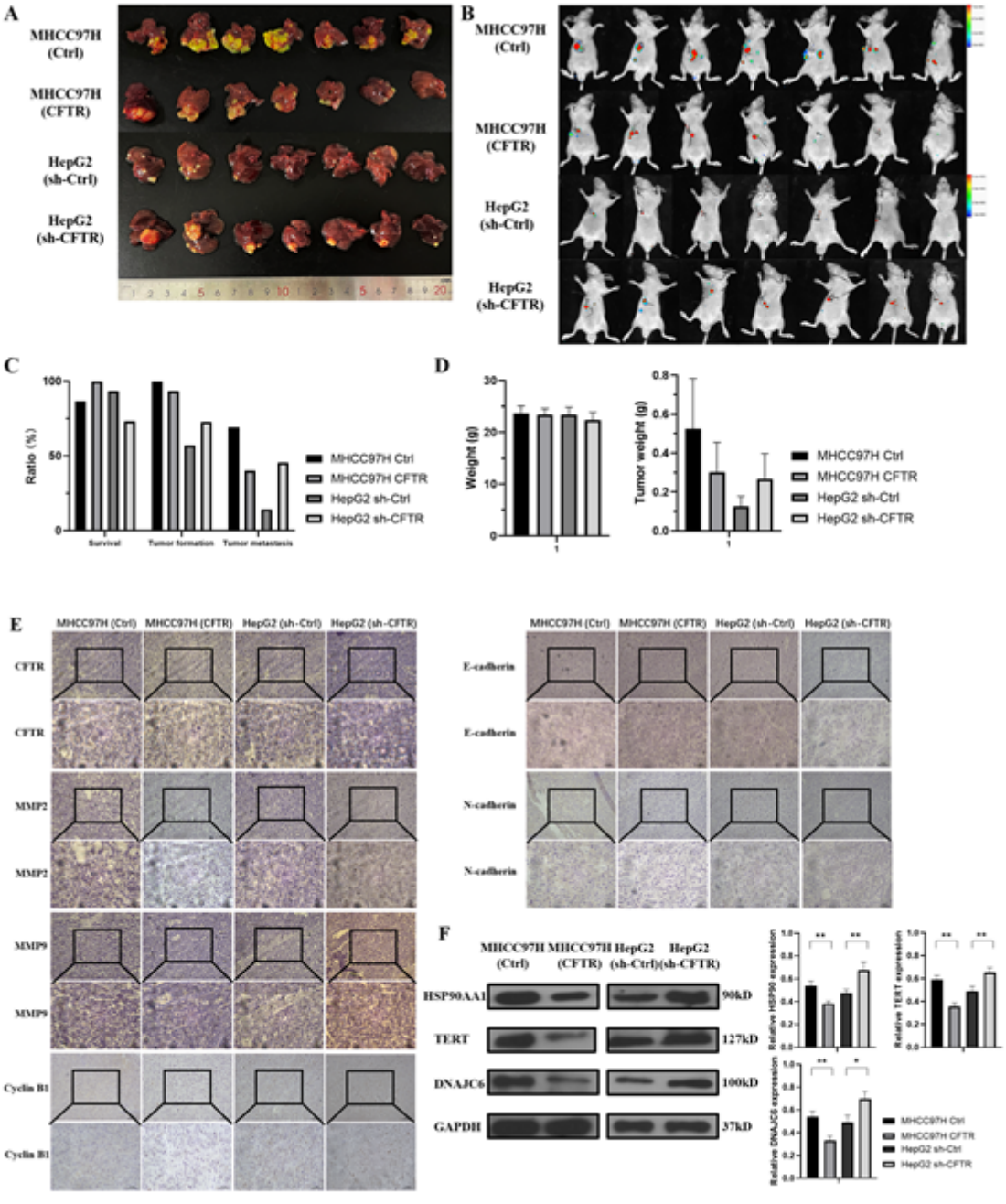



## Figure 3

The mechanism of negative regulation of CFTR on proliferation and metastasis of HCC cells.

A, 20 direct (primary) related genes were obtained through String database. B, 11 genes were obtained by intersecting the differential genes of HCC through TCGA database and 229 (secondary) indirect CFTR related genes and the relationship between 11 intersecting genes and direct related genes. C, Western Blot analysis of HSP90, TERT, and DNAJC6 in overexpression MHCC97H cells and interference of CFTR expression HepG2 cells. D, the MTT assays of HepG2 cells interfering with CFTR expression in the

presence of 100 nmol/L, 1μmol/L, and 10μmol/L GA. E, the invasion HepG2 cells interfering with CFTR expression in the presence of 100 nmol/L, 1μmol/L, and 10μmol/L GA was detected by transwell assays.

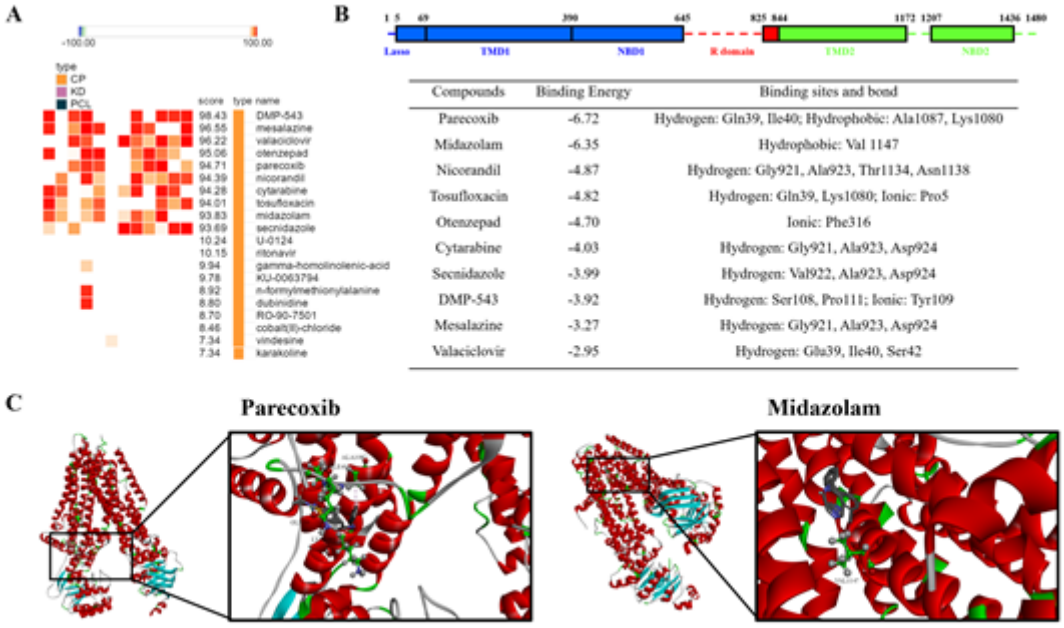



## Figure 4

CFTR inhibited HCC growth and metastasis *in vivo*.

A and B, representative imaging of orthotopic HCC tumors of MHCC97H Ctrl, MHCC97H CFTR, HepG2 sh-Ctrl, HepG2 sh-CFTR groups. C, survival, tumor formation, and tumor metastasis ratio of each group was calculated. D, the weight of tumor and mice of each group was weighed and calculated. E, Immunohistochemistry analysis of MMP2, MMP9, Cyclin B1, E-cadherin, and N-cadherin in cancer tissues

of BALB/c node mice in each group. F, Western Blot analysis of HSP90, TERT, and DNAJC6 in cancer tissues of BALB/c node mice in each group. Representative images and the statistical analyses were shown. Data are expressed as mean ± SEM of at least three independent experiments. Statistical significance was concluded at *$P$ < 0.05, **$P$ < 0.01.




| Compounds | Binding Energy | Binding sites and bond |
|---|---|---|
| Parecoxib | -6.72 | Hydrogen: Gln39, Ile40; Hydrophobic: Ala1087, Lys1080 |
| Midazolam | -6.35 | Hydrophobic: Val 1147 |
| Nicorandil | -4.87 | Hydrogen: Gly921, Ala923, Thr1134, Asn1138 |
| Tosufloxacin | -4.82 | Hydrogen: Gln39, Lys1080; Ionic: Pro5 |
| Otenzepad | -4.70 | Ionic: Phe316 |
| Cytarabine | -4.03 | Hydrogen: Gly921, Ala923, Asp924 |
| Secnidazole | -3.99 | Hydrogen: Val922, Ala923, Asp924 |
| DMP-543 | -3.92 | Hydrogen: Ser108, Pro111; Ionic: Tyr109 |
| Mesalazine | -3.27 | Hydrogen: Gly921, Ala923, Asp924 |
| Valaciclovir | -2.95 | Hydrogen: Glu39, Ile40, Ser42 |

## Figure 5

Prediction of anti-HCC drugs targeting CFTR

A, potential drugs targeting CFTR were predicted through CLUE Command database. B, 10 compounds (DMP-543, mesalazine, valaciclovir, otenzepad, parecoxib, nicorandil, cytarabine, tosufloxacin, midazolam, secnidazole) could bind to CFTR with different binding energy. C, The binding sites of parecoxib and midazolam with CFTR through autodock.

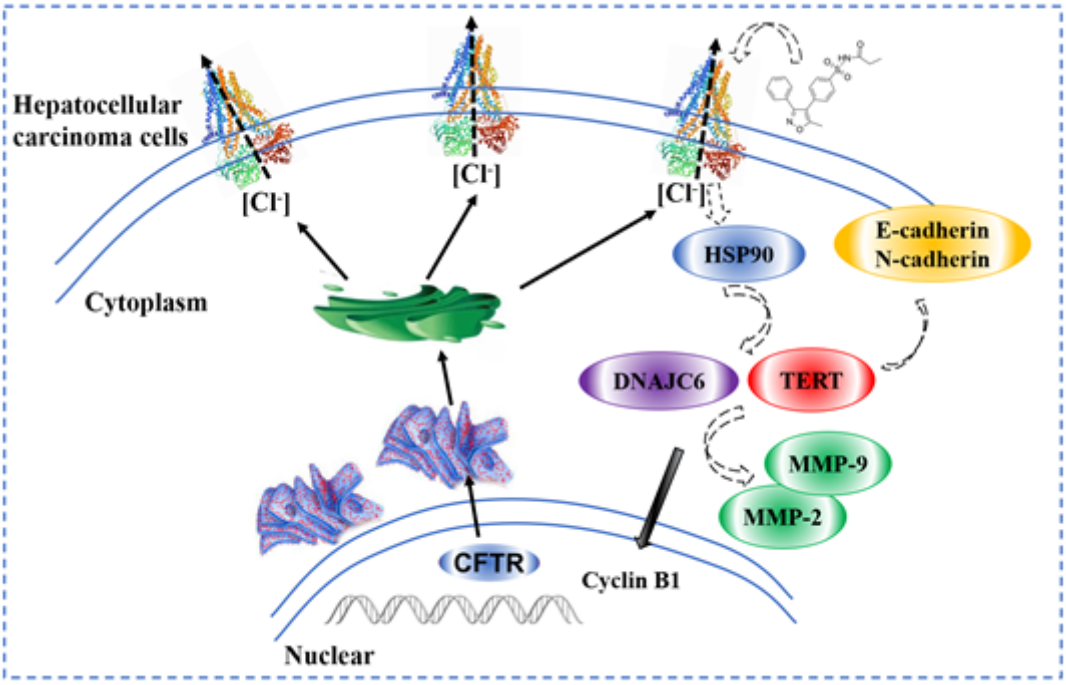



**Figure 6**

Potential signal pathway of CFTR negatively regulate the proliferation and metastasis of HCC.

## Supplementary Files

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- Supplementary.docx