Silencing HSF4 can inhibit the proliferation of HCC cells

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Abstract

Purpose: To explore the regulatory role of HSF4 in hepatocellular carcinoma stemness property maintaining and analysis the clinical significance of HSF4 in hepatocellular carcinoma.

Materials and methods: Tumor spheroid formation assay was conducted to assess stemness property and enrich hepatocellular carcinoma stem-like cells. qRT-PCR and Western Blot was used to detect genes mRNA and protein expression level. KM-plotter database wad used to analyze the correlation between HSF4 and overall survival (OS) in HCC patients.

Result: mRNA level of HSF4, as well as stemness-associated genes, was significantly higher in hepatocellular carcinoma tissue then in adjacent normal tissue. Positive correlation between expression level of HSF4 and SOX (r=0.1668, p<0.05), NANOG (r=0.7, p<0.05), POUSF1(r=0.7362, p<0.05), CD44(r=0.0128, p<0.05) was observed. The KM plotter showed that there is no significant difference between HSF4 high patients and HF4 low patients in term of overall survival (OS)(p=0.48). However, significant difference in terms of progression-free survival (PFS)(p=0.019) and relapse-free survival (RFS) (p=0.005) between these two groups was observed. In vitro assay results also suggest the positive correlation between HSF4 and stemness-associated genes. Increased HSF4 expression confers HCC enhanced tumor spheroid formation ability.

Conclusion: HSF4 is significantly increased in liver cancer stem-like cells, indicating the possible contribution of HSF4 to stemness properties maintenance in liver cancer stem-like cells. Silencing HSF4 inhibits proliferation of hepatoma cells.

Introduction

Liver cancer is among the leading causes of cancer deaths globally. Figures show that liver cancer is the fifth most common malignancy in the world and the third leading cause of cancer death in humans. Less than 20% of the liver cancer patients are diagnosed at early stages and could be radically cured by surgery, liver transplantation or radiofrequency ablation. But most patients with liver cancer are diagnosed at an advanced stage, and the median survival time of liver cancer patients is only 3–6 months. Therefore, it is very important to develop effective methods to treat liver cancer. As we know hepatocellular carcinoma (HCC) is the most frequent primary liver malignancy. Although some progress has been made in basic and clinical studies of HCC, including the identification of multiple diagnostic markers and the detection of genes associated with HCC invasion and metastasis. However, the pathogenesis of liver cancer is still unclear.

Heat shock factor 4 (HSF4) is a member of the heat shock transcription factor family that contains conserved DNA binding and trimerization domains[5]. As a stress response transcription factor widely expressed in various human tissues, HSF4 can promote cell proliferation and protect cell senescence [", It has been reported that HSF4 can affect the function of caspase, which is beneficial to the survival of lens epithelial cells. Recent studies have found that the dysregulation of HSF4 is related to cancer, and it has

been reported that HSF4 may promote cell proliferation and differentiation[6,7], Study also shows that the deletion of HSF4 is related to the increased expression of cyclin-dependent kinase inhibitors p21 and p27 in mouse embryonic fibroblasts[8]. HSF4 deletion suppressed Arf/P53-mediated tumorigenesis through inhibiting proliferation and inducing cellular senescence in a P21 and P27-dependent manner[8]. However, the regulatory role of HSF4 in liver cancer stemness properties remains to be elucidated.

Cancer stem cells (CSCs), also known as tumor-initiating cells, are cells with the ability to self-renew and differentiate into a variety of non-stem cells. They play important roles in tumor growth, metastasis, recurrence, and drug resistance. Tumor stemness has been found in many cancers, including liver cancer, colon cancer, pancreatic cancer, lung cancer, ovarian cancer and prostate cancer[13]. Previous work had revealed that liver cancer stem cells (LCSCs) play a crucial role in liver cancer progression, and maintenance of stemness properties in liver cancer can be attributed to several stemness-associated protein coding genes, such as BMI1, OCT4 and Nanog.

The stemness of hepatocellular carcinoma is characterized by various stem cell markers and totipotent factors, which are regulated by various signaling pathways[14]. Some studies indicate that the major stem markers in liver cancer include CD44, and CD45[16,,]. The totipotency factors related to liver cancer include Sox2, Nanog, Oct4, and SALL4. One study revealed that CARM1-mediated GAPDH methylation is a key regulatory mechanism of glucose metabolism in liver cancer[13].

MiRNA and lncRNA, are two major classes of ncRNAs, can regulate Liver CSCs by many ways like Affecting the Wnt/β-Catenin Cascade and PTEN/PI3K/AKT/Bad Signaling Pathway. miRNA and lncRNA can also affect Tumor-Associated Genes. Targeted therapy for liver cancer stem cells seems to be a promising anti-cancer strategy, so further research on the key mechanisms of liver cancer stem cell characteristics may paving the way for targeted treatment of liver cancer.

The purpose of our study was to investigate the regulatory role of HSF4 in the maintenance of hepatocellular carcinoma dryness, and to analyze the clinical significance of HSF4 in hepatocellular carcinoma.

Results

HSF4 expression level in liver cancer patients and its prognostic value

Analyzing the data retrieved from TCGA dataset, we found that HSF4 mRNA expression level is significantly increased in liver cancer tissue compared with paired adjacent peritumoral tissues (Fig. 1A). In order to evaluate the prognostic value of HSF4 in liver cancer, we utilized Online KM plotter database (http://kmplot.com/analysis) to conducted the Kaplan-Meier survival plots regarding HSF4 high expression patients and HSF4 low expression patients. The results showed that there is no significant difference between HSF4 high patients and HF4 low patients in term of overall survival with p value being

0.48 (Fig. 1B). However, significant difference in terms of progression-free survival (PFS) and relapse-free survival (RFS) between these two groups was observed (Fig. 1C, Fig. 1D).

HSF4 expression level is significantly increased in liver cancer stem-like cells

Tumor sphere formation assay was commonly used to enrich liver cancer stem-like cells. We carried out tumor sphere formation assay using 7721 cell line to enrich liver cancer stem-like cells. In order to confirm whether these tumor sphere cells were liver stem-like cells, we detected stemness-associated genes in these tumor sphere cells, and the result suggested that mRNA expression stemness-associated genes in tumor sphere cells was significantly increased compared with parental adherent 7721 cells (Fig. 1A). CD44 is regarded as a surface marker for liver cancer stem-like cells. Immunofluorescence staining assay confirmed the increased expression of CD44 in tumor sphere cells compared with parental adherent 7721 cells (Fig. 2B). These results suggested that tumor sphere cells possessed stemness properties and represent liver cancer stem-like cells. We further analyzed the mRNA expression level of HSF4 in tumor sphere cells. The result suggested that HSF4 in significantly increased in liver cancer stem-like cells (Fig. 2C), indicating the possible contribution of HSF4 to stemness properties maintenance in liver cancer stem-like cells.

Establishment of HSF4 stably knockdown cell lines

HSF4 mRNA and protein expression level was significantly downregulated in cells transfected with sh-HSF4 vector compared with cells transfected blank control vector (Fig. 3A, Fig. 3B). Colony formation assay suggested that knockdown of HSF4 could significantly inhibit viability of liver cancer cells (Fig. 3C). We further investigated the effect of HSF4 on migration ability of liver cancer cells using wound scratching assays, the result revealed that HSF4-knocking down significantly inhibit the migration ability of liver cancer cell (Fig. 3D). These results elucidated the successful establishment of HSF4 knocking down cell line, and HSF4 knocking down exert significant inhibitory effect on viability and migration ability of liver cancer.

HSF4 contribute to stemness property maintenance in liver cancer stem-like cells

We further investigate the regulatory role of HSF4 in stemness properties in liver cancer cell. qRt-PCR test revealed that knocking down HSF4 in 7721 cell line significantly inhibited stemness-associated genes expression, such as Sox2, OCT4, Nanog and CD44 at both mRNA and protein level (Fig. 4A, Fig. 4B). Besides that, HSF4 knocking down significantly inhibited tumor sphere formation ability in terms of quantity and size of tumor sphere (Fig. 4C, Fig. 4D), suggested the contribution of HSF4 to stemness properties maintenance in liver cancer.

Correlation between HSF4 expression and stemness-associated genes in liver cancer

In order to analyze the correlation between HSF4 and stemness-associated genes in liver cancer patients, CHIP Base V2.0 online database (http://rna.sysu.edu.cn/chipbase/index.php) was utilized. The result suggested that there is positive correlation between HSF4 and stemness-associated genes, such as

SOX2, Nanog, OCT4 and CD44. This further suggested the possible contribution of HSF4 to stemness property maintenance in liver cancer cells.

Conclusion

HCC is one of the most common primary cancer, and ranks second in the leading causes of cancer-related death worldwide. Poor prognosis in patients with liver cancer may be mainly due to distant metastasis and tumor recurrence. In recent years, liver cancer stem cells are considered to be the main cause of tumor recurrence and distant metastasis. Targeted treatment of liver cancer stem cells seems to be a promising anti-cancer strategy. Therefore, studying the mechanism of stem cell dryness of liver cancer will lay a foundation for targeted therapy of LCSC. In this study, we investigated whether there is a contribution of HSF4 to stemness-maintaining in liver cancer stem cells. As a stress responsive transcription factor that is widely expressed in many human tissues, HSF4 stimulates cell proliferation and protects cells from aging [,,]. In addition, its upregulation enhances HIF-1a expression, which has well-characterized effects on carcinogenesis, angiogenesis, tumor metastasis, tumor resistance therapy [,]. All these suggested that HSF4 might act as an oncogene to promote malignant phenotypes transformation of tumor cells. However, little is known about the role of HSF4 in liver cancer stem cells, our study aimed to investigate the regulatory effect of HSF4 in maintaining stemness properties in liver cancer stem-like cells.

Our study showed that HSF4 is significantly increased in liver cancer stem-like cells, indicating the possible contribution of HSF4 to stemness properties maintenance in liver cancer stem-like cells. To further understand the role of HSF4 in the development of liver cancer,we establish HSF4 stably knockdown cell lines. Results showed that HSF4 knocking down exert significant inhibitory effect on viability and migration ability of liver cancer.

We further investigate the regulatory role of HSF4 in stemness properties in liver cancer cell. Tumor sphere formation assay is a common method for enriching cancer stem-like cells. In our study, HSF4 knocking down significantly inhibited tumor sphere formation ability in terms of quantity and size of tumor sphere. The stemness of liver cancer is characterized by several stem markers, totipotency factors and stemness-associated signaling pathways. It was reported that CD44 could act as surface marker for liver cancer stem cells[12]. Besides that, the totipotency factors, such as Sox2, Nanog, Oct4, BMI1 also contributed to stemness-maintaining in liver cancer stem cells[[10,13]]. The expression of these markers in HCC is also related to a higher frequency of extrahepatic metastasis and might serve as an indicator for overall survival in liver cancer patients. Our results revealed that knocking down HSF4 in 7721 cell line significantly inhibited stemness-associated genes expression, such as Sox2, OCT4, Nanog and CD44 at both mRNA and protein level, suggesting the contribution of HSF4 to stemness properties maintenance in liver cancer.

In conclusion, HSF4 is significantly increased in liver cancer stem-like cells, indicating the possible contribution of HSF4 to stemness properties maintenance in liver cancer stem-like cells. HSF4 knocking down exert significant inhibitory effect on viability and migration ability of liver cancer. We will collect

liver cancer patients in the hospital, study the relationship between HSF4 and the prognosis of patients with liver cancer, and further explore the regulation mechanism of HSF4 in liver cancer.

Materials And Methods

Cell culture

SMMC-7721 cell line was obtained from Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells grew at 37°C in 5% CO2 atmosphere in DMEM medium (Gibco) containing 10% fetal bovine serum, as well as 100 IU/ml penicillin and 100 μg/mL streptomycin. Cells in the logarithmic growth phase were used for the subsequent experiments.

Knockdown of HSF4

Two shRNA against HSF4 were used to knock down HSF4 expression. The target sequence was: Sh1: CCGGCCAACTCAACATGTACGGTTTCTCGAGAAACCGTACATGTTGAGTTGGTTTTT; sh2: CCGGCATCTCTGACATCCCAGAAGACTCGAGTCTTCTGGGATGTCAGAGATGTTTTTTG. shRNA was cloned into lentiviral vector pLKO.1, and were co-transfected into 293T cells with lentivirus packaging vectors using Lipofectamine 3000. 48 hours after transfection, lentiviral particles were harvested and used for cell transfection. HSF4 stably knocking down cells were achieved by screening with puromycin for 2 weeks.

Cell proliferation

Cells were seeded into 96-well plate for proliferation assay using Cell Counting kit-8. Colony formation assay were carried out by seeding 6000 single cells into 6 cm dish, and staining with Crystal Violet Staining Solution for further observation.

Wound scratching assays

Nearly 500000 cells were seeded in 6-well plates. After forming confluent monolayer, cells were scratched using Pipette tips and washed to remove cell debris. Wound healing was observed right after scratch, as well as after 24 hours growth in serum-free DMEM medium.

Spheroid Colony Formation Assay

7721 cells, cultured as monolayer, were harvested as single cell suspension and resuspended in tumor sphere medium (serum-free DMEM/F12 medium supplemented with 2% B27, as well as 20 ng/ml human recombinant basic fibroblast growth factor (bFGF, PeproTech) and 20 ng/ml human recombinant epidermal growth factor (EGF, Millipore)). Cells were then seeded in ultra-low attachment 96-well plates (Corning) at a density of 400 cells per well. After two weeks, spheroid colonies in each well were counted using light microscope.

qRT-PCR

Trizol reagent was utilized to extract the total RNA of cultured cell lines and tissue samples according to the manufacture's protocol. PrimeScript RT Reagent Kit (TaKaRa) and SYBR Premix Ex Taq (TaKaRa) were used to detect mRNA expression level following the manufacturer's instructions. The primers for qRT-PCR were summarized in table 1.

Table 1: Sequence of the primers used in qPCR.

	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')
HSF4	GCCTTCCTCGGCAAGCTATG	AAACGGCTCTGGTCGCTTAC
SOX2	GCCGAGTGGAAACTTTTGTCG	GGCAGCGTGTACTTATCCTTCT
NANOG	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
CD44	CTGCCGCTTTGCAGGTGTA	CATTGTGGGCAAGGTGCTATT
OCT4	GAGTGAGAGGCAACCTGGAGAAT	ACCGAGGAGTACAGTGCAGTGAA

Western blot

Antibody against CD44, GAPDH were obtained from Abcam, and antibodies against OCT4, Nanog, SOX2 and HSF4 was purchased from Santa Cruz Biotechnology. Western blotting analysis were carried out as previously described[1].

Immunofluorescence staining

Adherent cells: Washed with PBS for three times after removing the culture medium. Then it was incubated with CD44 antibody labeled with FITC (Bioligand) at 4 ℃ for 1 hour. After being washed for three times, it was observed under inverted fluorescent microscope.

Tumor sphere cells: Tumor spheres were collected in 1.5 ml centrifugal tube, and incubated with CD44 antibody labeled with FITC (Bioligand) at 4 ℃ for 1 hour. After being washed for three times, tumor spheres were resuspended. Drop the suspension on the slide and observe under inverted fluorescent microscope.

Co-expression analysis of HSF4 and stemness-associated genes

Co-expression relationship between HSF4 and stemness-associated genes in liver cancer was analyzed using CHIP Base V2.0 online database (http://rna.sysu.edu.cn/chipbase/index.php).

Evaluation of prognostic value of HSF4 using Online KM plotter database

Online KM plotter database (http://kmplot.com/analysis) were used to evaluate the prognostic value of individual HSF mRNA expression for overall survival (OS) in liver cancer.

Statistical analysis

Data was shown as Mean + SD, calculated by the two-tailed Student's T test (graph pad software). Differences were regarded significant when $p < 0.05$.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: Please contact author for data requests

Conflict of Interest: The named authors have no conflict of interest, financial or otherwise.

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Authors' contributions: CF, YLF, WXF, CJX and ZY are all involved in cell culture, gene editing, blot hybridization, and PCR. CF organizes test results, analyzes data, and writes articles. Zhu-XN conceived of the study, Chen-F participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Figures

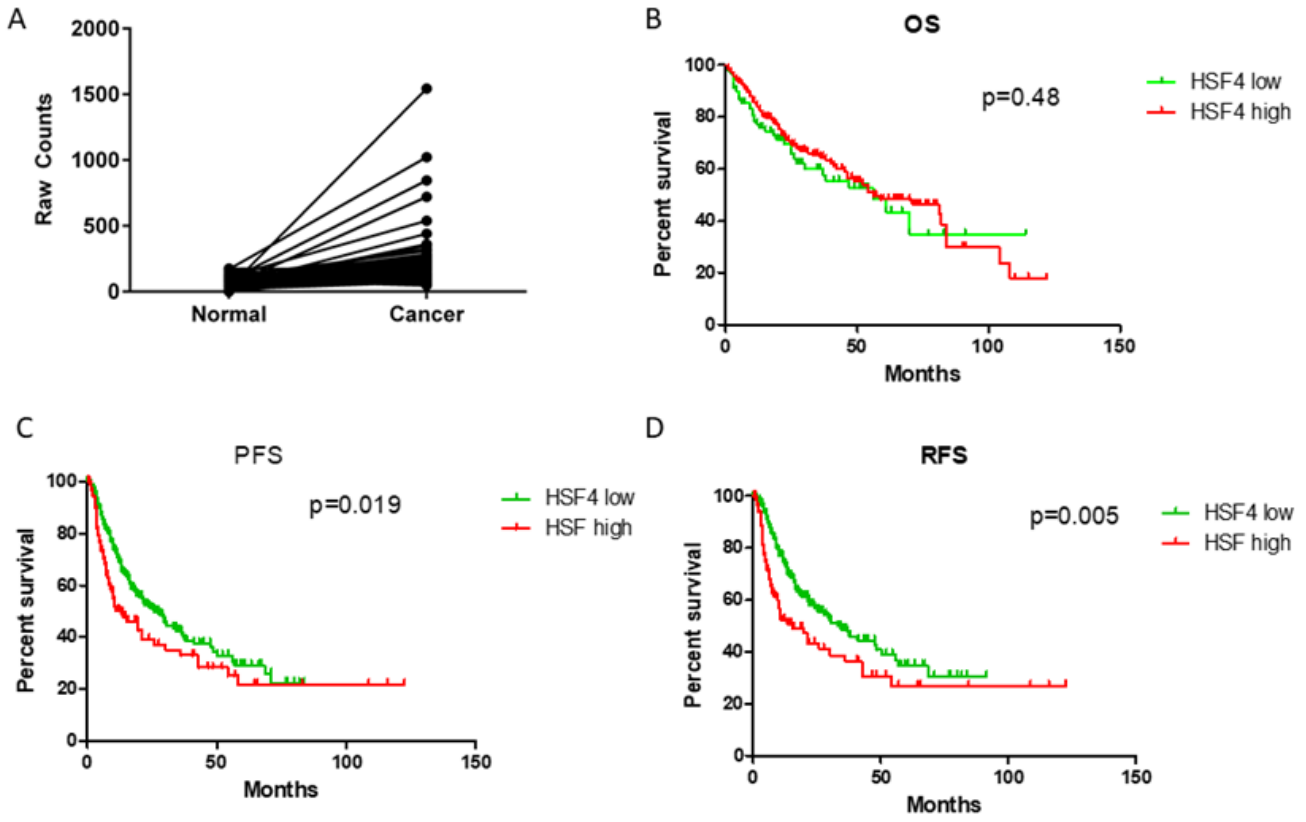

Figure 1

Clinical significance of HSF4 in liver cancer. (A) HSF4 expression is significantly increased in liver cancer. (B) Correlation between OS of liver cancer patients and HSF4 expression level. (C) Correlation between PFS of liver cancer patients and HSF4 expression level. (D) Correlation between RFS of liver cancer patients and HSF4 expression level.

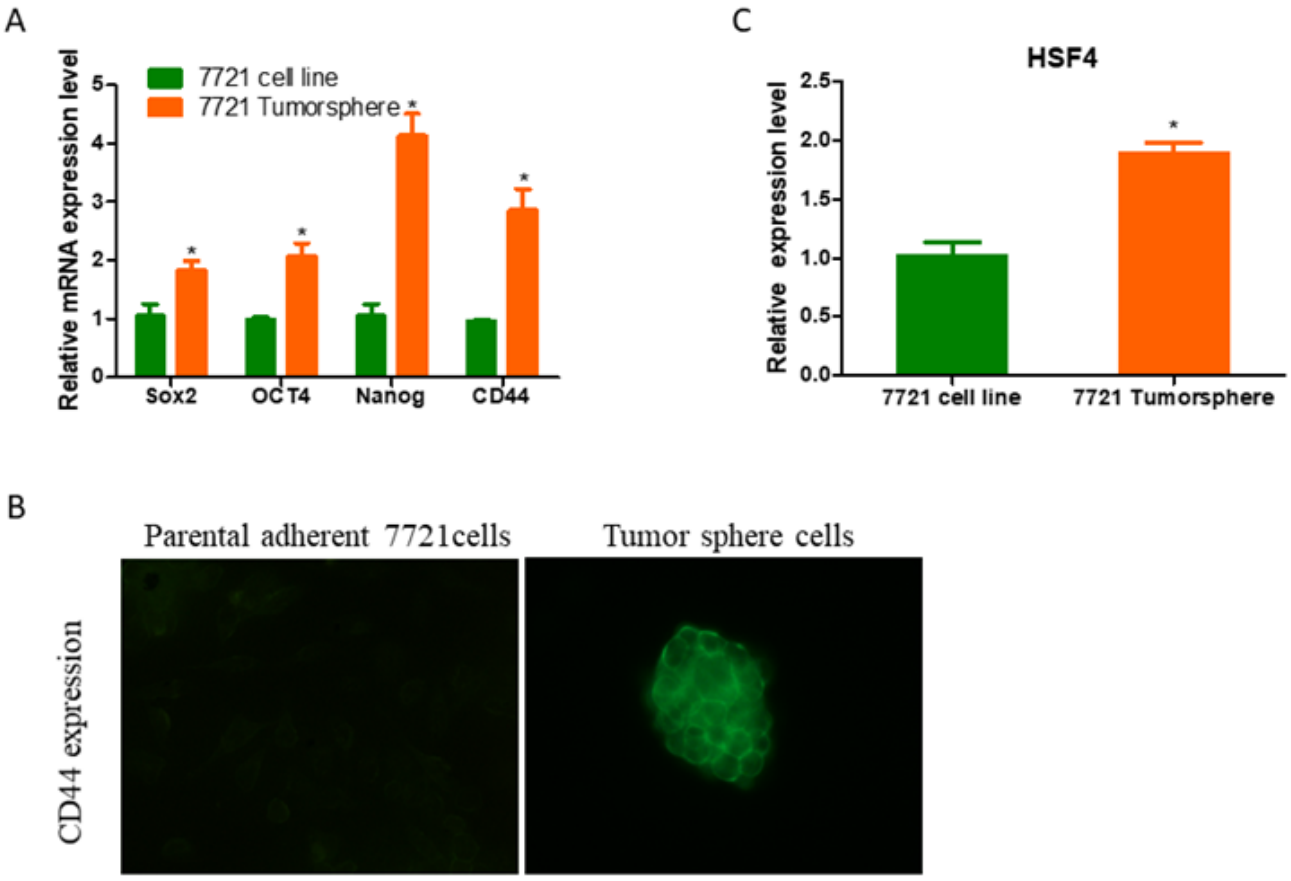

Figure 2

Serum-free suspension culture. (A) Tumor-sphere cells exhibit increased expression of stemness associated genes, such as Sox2, OCT4, Nanog, CD44. (B) Tumor-sphere cells shows increased expression of CSC marker-CD44. (C)Tumor-sphere cells exhibit increased expression of HSF4.

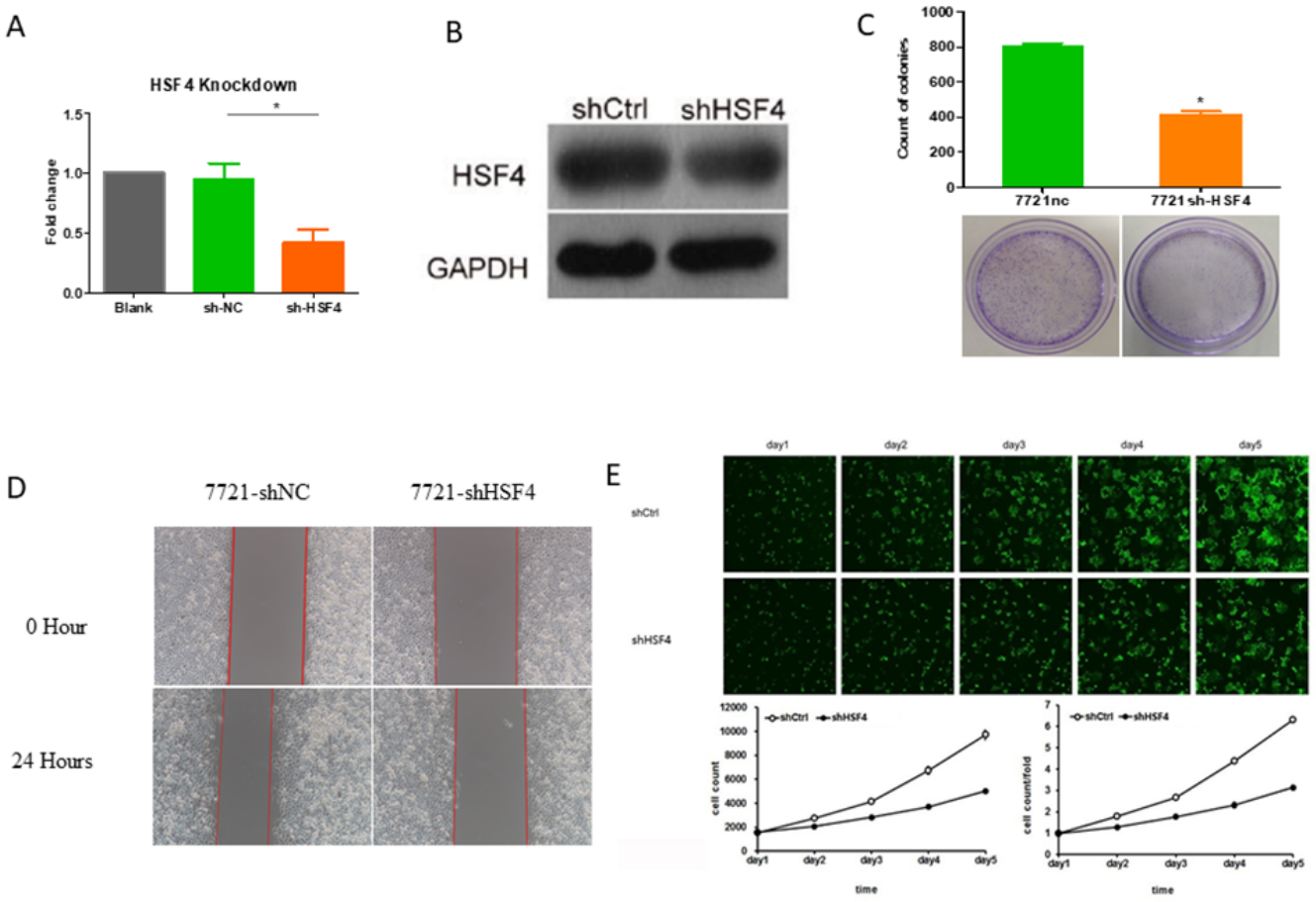

Figure 3

Establishment of stably HSF4-knockdown 7721 cell line. (A) Lentivirus-mediated downregulation of HSF4 mRNA expression. (B) Lentivirus-mediated downregulation of HSF4 protein level. (C) HSF4 knockdown inhibit 7721 colony formation ability. (D) HSF4 knockdown inhibit migration ability of 7721 cell line. (E) HSF4 knockdown inhibit 7721 proliferation ability.

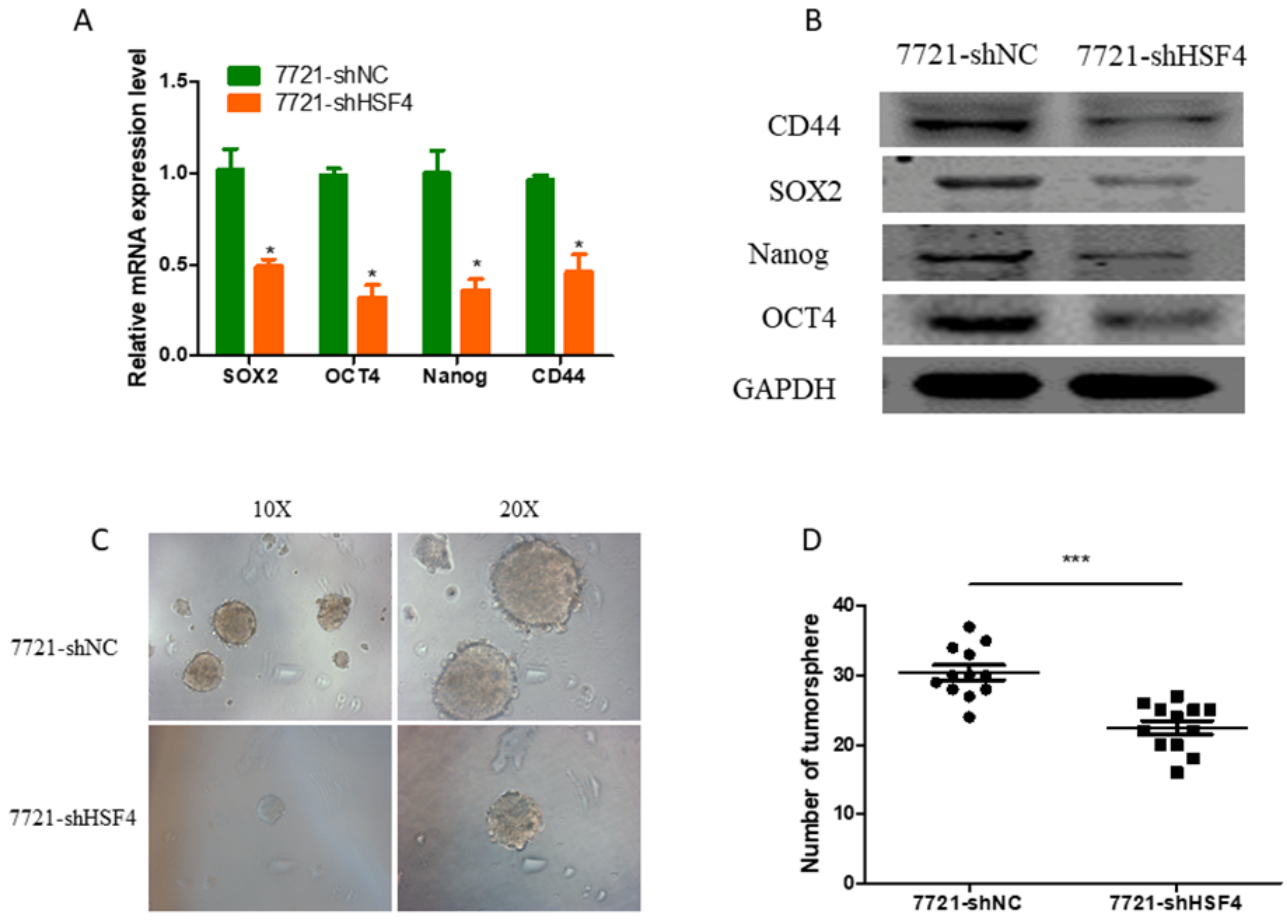

Figure 4

Contribution of HSF4 to stemness properties in liver cancer cells. (A) HSF4 knockdown lead to inhibited mRNA expression of stemness-associated genes, such as Sox2, OCT4, Nanog and CD44. (B) HSF4 knockdown lead to downregulated protein level of stemness-associated genes, such as Sox2, OCT4, Nanog and CD44. (C□D) SHF4 knockdown inhibited tumor-sphere formation ability of 7721 cell line.

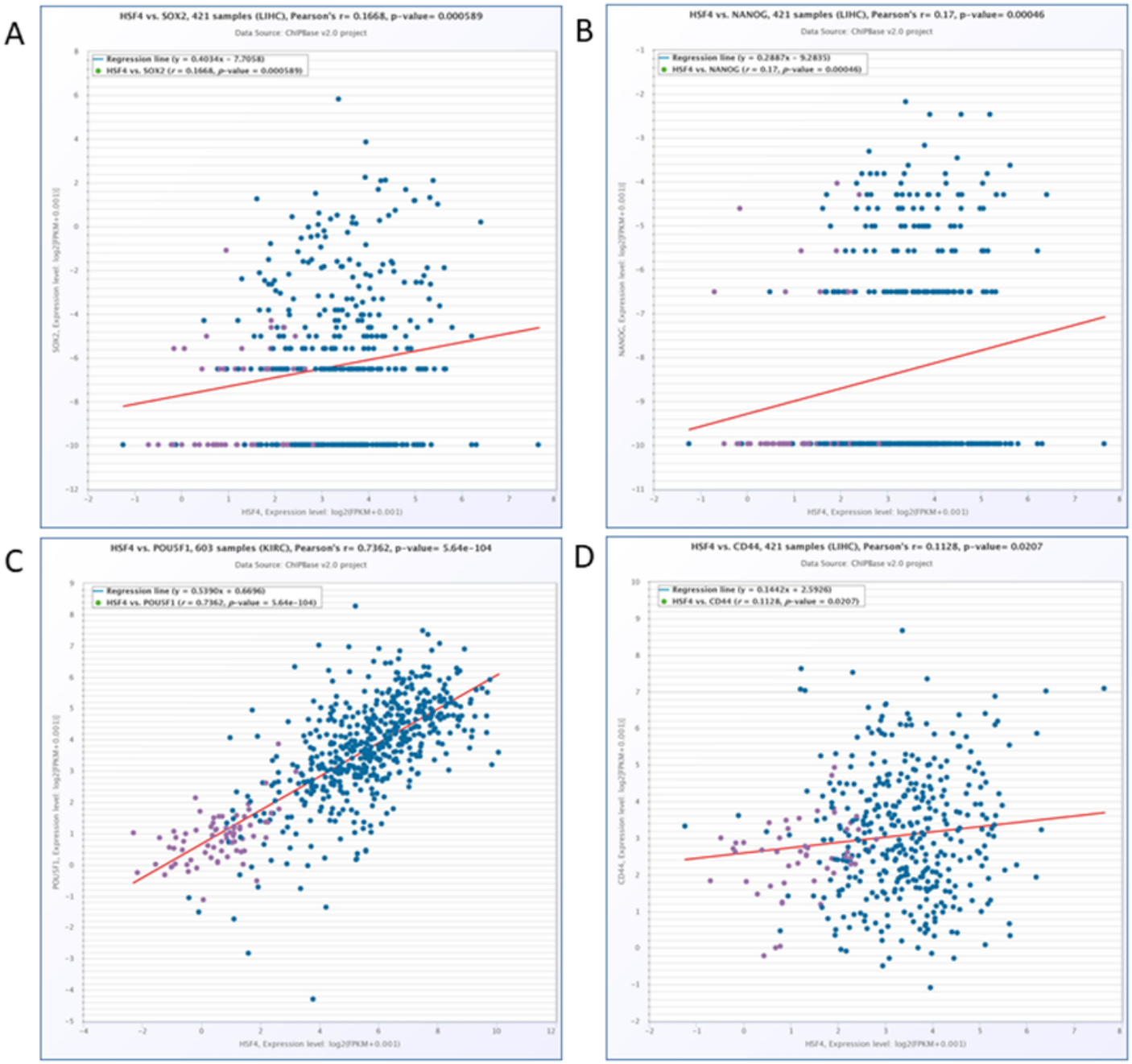

Figure 5

Correlation between HSF4 and stemness-associated genes in liver cancer patients. (A) Positive correlation between HSF4 and Sox2 mRNA expression in liver cancer patients. (B) Positive correlation between HSF4 and Nanog mRNA expression in liver cancer patients (C) Positive correlation between HSF4 and OCT4 mRNA expression in liver cancer patients. (D) Positive correlation between HSF4 and CD44 mRNA expression in liver cancer patients.