PCGF2 and PCGF4 Opposite Drive Stem-like Properties in Hepatocellular Carcinoma

Jinjing Hu
Lanzhou University
Yongqiang Zhou
Lanzhou University
Huan Feng
Lanzhou University
Yi Xie
Lanzhou University
Kuo Qi
Lanzhou University
Yonglin Chen
Lanzhou University
Wenbo Meng
Lanzhou University
Xiaojun Ma
Lanzhou University
Yongjian Wei
Lanzhou University
Fei Lu
Lanzhou University
Jia Yao
Lanzhou University
Bo Cheng
Lanzhou University
Xun Li (lix@lzu.edu.cn)
Lanzhou University https://orcid.org/0000-0001-6862-7692

Primary research

Keywords: Hepatocellular carcinoma, liver cancer stem cells, PCGF2, PCGF4

Posted Date: September 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-850411/v1

Abstract

Background: PCGF4 is highly expressed in liver cancer and can be used as a marker for liver cancer stem cells. However, PCGF2, a homologue of PCGF4, is not clear whether it is expressed in HCC, and whether it regulates the stemness of liver cancer stem cells.

Methods: IHC and Western blot were used to detect the expression of PCGF2 and PCGF4 protein in human HCC tissues and cell lines. Flow cytometry and sphere formation were performed to detect the effect of PCGF2 or PCGF4 on the stem-like properties of liver cancer stem cells. Kaplan-Meier curves were conducted for OS and DFS. Cell viability was measured at the indicated time points using Cell Counting Kit-8. We performed KEGG analysis on these target genes through the cluster profiler package of the R language.

Results: IHC results showed that PCGF2 was lower expressed in HCC, while PCGF4 was higher expressed in HCC compared with matched paracancerous, and this higher expression exhibited poor prognosis in HCC. Up regulation of PCGF2 was also accompanied with decreased stem-like properties and sphere formation in HCC cell lines. Interestingly, down regulating PCGF4 got the similar results as up regulating PCGF2. Furthermore, up regulating PCGF2 or down regulating PCGF4 cells performed more sensitivity to sorafenib. We also found that PCGF2 and PCGF4 oppositely regulated the drives stem-like properties by p38 MAPK signal pathway.

Conclusion: PCGF2 was a novel negative regulator of LCSCs that inhibiting the stem cell population, reducing the sphere formation ability of liver cancer stem cells, and increasing the sensitivity of sorafenib by targeting p38 MAPK signaling. PCGF2 was supposed to be a novel therapeutic target for Liver cancer stem cell.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors with high morbidity and mortality [1]. According to the statistics, the recurrence rate of HCC is as high as 60 % to 70 % [2, 3]. Radical resection is the first choice of the treatment of HCC, but the 5-year survival rate of postoperative patients is only 30–50% [4]. Meanwhile, the chemotherapy resistance reduces the efficacy of chemotherapeutic drugs to a great extent, such as sorafenib. The malignant behaviors such as easy recurrence, poor prognosis and chemotherapy resistance of HCC blame on the existence of liver cancer stem cells (LCSCs)[5]. Exploring the relevant diagnosis and treatment schemes for LCSCs means great significance to defeat HCC.

B lymphoma Mo-ML V insertion region 1 homolog (BMI1), also known as PCGF4, is a member of the polycomb repressive complex1(PRC1) family proteins[6], PRC1 contains histone H2A-specific E3 ubiquitin ligase activity and cooperation with PRC2 to inhibit epigenetics. Some studies suggested that PCGF4, as a specific marker of cancer stem cells, is involved in the recurrence, metastasis and chemotherapy resistance in many kinds of tumors. Published articles have shown that CSCs are the main factor

influencing tumor recurrence after treatment, and some proto oncogenes in CSCs, such as PCGF4, are activated to cause tumor recurrence. [7, 8]. It has also been found that PCGF4 is abnormally expressed, such as in HCC, ovarian cancer, breast cancer, pancreatic cancer, lung cancer, and gastric cancer. Moreover, PCGF4 plays a key role in the EMT (epithelial-mesenchymal transition), cancer stem cells, and chemotherapy resistance.[9–18] .In head and neck squamous cell carcinoma, they found that $PCGF4^+$ CSC shows cisplatin resistance and may relapse after chemotherapy [9]. Atlasy found that PCGF4 was highly expressed in CD133 and EpCAM positive colorectal cancer CSCs [19]. In HCC, others found that the expression of PCGF4 was up-regulated and promoted the ability of HCC cells in proliferation, invasion and metastasis[20]. Recent studies have demonstrated that the carcinogenesis driven by PCGF4 was related to the inhibition of TGFβ-2/SMAD signal transduction axis [21]. Targeting PCGF4, could inhibit the proliferation and increase the radio sensitivity of HCC cell [22]. Down-regulation of PCGF4 inhibited the stemness of $CD133^+$ LCSC by blocking the NF-κB signaling pathway [11]. Other work indicated that HCC resistance can be overcome by co-delivering PCGF4 siRNA and cisplatin in cationic nanocapsules [23]. PCGF4 is most likely to promote the invasion and migration of $CD133^+$ HepG2 cells by inducing EMT [24].The polycomb gene PCGF4 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma[25].

Another PRC1 member, Melanoma nuclear protein18 (Mel18), as known as PCGF2, is a homologous isomer of PCGF4. PCGF2 plays different roles in various cancers. In some cancers, PCGF4 and PCGF2 synergistically promote cancer development [26–30]. It was found that the expression of PCGF4 and PCGF2 in cancer tissues was abnormally up-regulated, inhibiting the expression of REG3B protein, thereby affecting the STAT3 tumor suppressor signal pathway, promoting cell proliferation and reducing cell apoptosis in colorectal cancer [31]. In gastric cancer, the low expression of PCGF2 is related to the poor prognosis of patients, but negatively correlated with gastric cancer stem cell markers Oct4, Sox2 and Gli1[32]. Tao's study explored that the expression of PCGF2 in colorectal cancer was significantly lower than that in non-cancerous mucosa, and the patients with high expression of PCGF2 had longer disease-free survival time than others [33]. Won found that PCGF2 is a negative regulatory gene of breast cancer CSCs, which can inhibit stem cell population and self-renewal in vitro and in vivo by inactivating wnt mediated notch signal [34]. PCGF2 controls the enrichment of tumor-initiating cells in SP (side population) fraction in mouse breast cancer [35]. PCGF2 negatively regulates stem cell-like properties through down regulation of miR-21 in gastric cancer cells [32]. There have been a large number of reports in the published work that PCGF4 is highly expressed in liver cancer and promotes the formation of liver cancer stem cells, but the function of PCGF2 in liver cancer is unknown? In this study, we found that PCGF2 is down-regulated in HCC and is highly expressed in adjacent tissues. We also found that the expression of PCGF4 is highly expressed in cancer tissues and cancer cell, PCGF2 has the opposite effect to PCGF4. Further experiments revealed that PCGF2 and PCGF4 have opposite properties in regulating liver cancer stem cells. Finally, a preliminary exploration showed that PCGF2 and PCGF4 opposite drived stem-like properties by p38 MAPK pathway in Hepatocellular carcinoma.

2. Material And Methods

2.1 Tissue microarrays-Patients and tissues

Purchased tissue microarrays (Outdo Biotech Co., Ltd, Shanghai, China, LivH180Su06) containing recurrence time, survival time, biochemical indicators, and other clinical data to obtain tumor samples: including tissues and adjacent tissues from 90 patients who underwent surgical resection from February 2006 to May 2007 and were confirmed to be HCC by post operative pathology. All patients were followed up for 5–6 years. The study was approved by the Hospital Ethics Committee and informed consent was obtained from each patient under Institutional Reviewer Board protocols.

2.2 Immunohistochemistry-IHC

The same set of tissue microarray, the HCC tissue microarray from the same patient was separately stained with anti-PCGF2 (Santa Cruz biotechnology, H115) antibody and anti-PCGF4 antibody (Cell Signaling Technology, Beverly, 6964, USA) according to the previous description. Taking into account the intensity and percentage of staining, a semi-quantitative evaluation of the protein expression levels of PCGF2 and PCGF4 was performed by three experienced pathologists and for data analysis was referenced this work [36].

2.3 Cell culture and transfect

Human HCC cell lines Huh7 and HCCLM3 cells were purchased from Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Gibco, USA) containing 10% fetal bovine serum (Hyclone, Logan, Utah, USA), 1% penicillin and streptomycin in a 37℃ incubator with 5% CO_2. PCGF2 over expression, PCGF4 knockdown and the corresponding negative control virus were produced from Genechem (Shanghai, China) and transfected according to the manufacturer's instructions, the stably transfected cells were cultured for subsequent experiments.

2.4 Cell proliferation assay

Cell proliferation was detected using cell Counting Kit-8 (CCK8, Dojindo, Japan). In short, the above stably transfected cells were trypsinized (0.25% Trypsin EDTA, BI) and seeded at 2,000 cells / well on a 96-well plate (Corning, USA). Each group was set up with 6 replicates. After the cells were completely adhered, replace the complete medium with 100 μL of serum-free medium containing 10% (v/v) CCK8. After 2hr, the OD at 450 nm was measured and recorded as 0 hr. Thereafter, the process was repeated every 24 hr until the 96 hr.

2.5 Protein extraction and western blot analysis

Cells treated as above and harvested at 48 h were washed three times in phosphate-buffered saline (PBS: pH 7.4; 0.15 mol/L). Total protein (~ 60 μg), extracted in RIPA buffer from each subset, was exposed to SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skim milk in Tris Buffered Saline and 1% Tween 20 (TBST) and incubated (overnight, 4°C) with primary antibody (1:1000) in TBS-T containing 1% skim milk. After three washings in TBS-T, membranes were incubated (room temp, 1 h) with horse radish peroxidase-conjugated secondary antibody (1:4000; Santa Cruz

Biotechnology, Santa Cruz, CA, USA) and exposed to enhanced chemiluminescence reagent (Chemicon International, Temecula, CA, USA). To confirm equal protein loading, anti-β-actin antibody (1:2000; Sigma) was used to re-probe. All experiments were done in triplicate.

2.6 RT-PCR

RNA, primary cells was prepared by Trizol and reverse transcribed using TAKARA cDNA synthesis kit. The primers were designed and synthesized by Sangon Biotech (Shanghai, China), and the sequence is as follows: PCGF4, forward 5'-CCAGGGCTTTTCAAAAATGA-3', reverse 5'- GCATCACAGTCATTGCTGCT-3', PCGF2, forward 5'-GCATCTTGCCAAGTTTCTCC-3', reverse 5'-TCTGCAGGCAGTTCAAGCTA-3', respectively. GAPDH were used as internal controls. All experiments were done in triplicate.

2.7 Flow cytometry

Liver cancer stem cell markers such as anti-CD133 antibody, anti-CD13 antibody and anti-EpCAM antibody were screened by flow cytometry. In order to analyze the characteristics of samples, fluorescence activated cell sorting (FACS) analysis was carried out. For each well, 100 μL 1×10^6 cells were incubated with 10 μL anti-CD133-PE (cat. no.130-110-962), EpCAM-APC (cat. no. -111-000) and CD13-PE-Vio770 (cat. no. 130-120-727), purchased from Miltenyi Biotec (Bergisch Gladbac,Germany) in the dark at 4°C for 10 minutes. The cells were then washed twice with PBS and suspended in 500 μL PBS for analysis by flow cytometry.

2.8 Tumor sphere formation

Huh7 and HCCLM3 cells at 10^3 cells/well were placed to the 6 well ultra low attachment plates (Corning Inc.) adding sphere medium for 5 day incubation. Sphere cells with diameter > 80 μm were counted. Sphere medium: DMEM-F12 medium supplemented with B27 (Invitrogen), 20ng/mL basic fibroblast growth factor, 20ng/mL epidermal growth factor) respectively.

2.9 Statistics

The data are expressed as average ± standard error. The data were analyzed using the Social Science Statistics package (SPSS, Chicago, Illinois, USA) version 17.0. Pearson and Spearman correlation coefficients were used to evaluate the correlation between gene expression and clinical and histopathological parameters. One-way ANOVA and t-test were used to compare between groups. The survival curve was drawn by Kaplan-Meier method and compared with logarithmic rank test. In all statistical analysis, $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Expression of PCGF4 and PCGF2 inversely correlates in HCC cell lines and HCC tumors

To determine PCGF2 and PCGF4 expression in HCC, we used IHC to test in the tissue microarray. The proportion of patients whose expression of PCGF2 in adjacent tissues is higher than that in cancer tissues is 66.26%. On the contrary, the expression level of PCGF4 in cancer tissues is higher than that of adjacent patients is 51.76%. (Fig. 1A). HCC tumor tissues expressed higher PCGF4 and lower PCGF2. (Fig. 1B, Fig. 1C). As was expected, PCGF4 was up-regulated, and PCGF2 was down-regulated in HCC tissues. The same results were also found in cell lines. We used western blot to detect the expression of PCGF4 or PCGF2 between in normal liver cell L02 and HCC cell lines Hep3B, Huh7, HCCLM3 cell lines. The results further revealed that the expression of PCGF4 was significantly higher than L02, while PCGF2 showed the opposite results (Fig. 1D). According to above results, PCGF2 has a negative role in the HCC. In order to further explore the role of PCGF2 in LCSCs, we constructed different cell lines to study their functions.

3.2 PCGF4 KD reduces tumor spheres formation and cell proliferation, Tumor sphere formation capacity is reduced after PCGF2 OE in HCC cells

To evaluate the effect of PCGF2 or PCGF4 on the ability of proliferation, PCGF4 expression was knocked down using siRNA in HCCLM3 and Huh7 cells. The knockdown of PCGF4 (PCGF4 KD) was confirmed by Western blotting (Fig. 2A). At the same time, PCGF2 is low expressed in cancer cell lines, so we constructed PCGF2 over expression (PCGF2 OE) cell lines and verified PCGF2 over expression by Western blotting (Fig. 2B). CCK8 assay were used to analyze cell proliferation, it was found that after PCGF4 KD, the cell proliferation rate decreased (Fig. 2C, 2D). However, proliferation ability was not significantly suppressed after PCGF2 OE. Based on these associations, we explored the effect of PCGF4 or PCGF2 on the tumor sphere formation and found that PCGF4 KD decreased the size of tumor spheres in HCCLM3 ($P < 0.05$) and Huh7 cells ($P < 0.05$) (Fig. 2E, F). Moreover, the sphere formation was decreased by PCGF2 OE in HCCLM3 ($P < 0.05$) and Huh7 cells ($P < 0.05$) (Fig. 2E, F). Therefore, these data indicated that PCGF4 blockade-mediated weakened tumor sphere formation in vitro and over expression of PCGF2 also weakens the ability of sphere formation. These results indicate that sphere formation as a characteristic of cell with CSC phenotype was reduced by PCGF4 KD or PCGF2 OE.

3.3 PCGF2 and PCGF4 opposite drives liver cancer stem cell population

In order to explore how PCGF4/PCGF2 affects the ratio of liver cancer stem cells, we used the LCSCs surface marker (EpCAM/CD13/CD133) to perform flow cytometric analysis to detect the ratio. In the HCCLM3 cell line, it was found that the number of $CD13^+/EpCAM^+/CD133^+$ cells was significantly down-regulated by flow cytometric analysis in PCGF4 KD cells ($P < 0.01$, $P < 0.01$, $P < 0.001$) (Fig. 3A). In Huh7 cells, when PCGF4 expression was down-regulated, the number of $CD13^+/EpCAM^+/CD133^+$ cells was down-regulated (Fig. 3B). In addition, flow cytometric analysis of CD13, EpCAM positive cell populations found that PCGF2 OE had the same outcome as PCGF4 knockdown, and the number of $CD13^+$, $EpCAM^+$

cells decreased. But the number of $CD133^+$ cells does not verify significantly (Fig. 3A). This shows that the change of PCGF2 was not obviously regulated to CD133, a marker of liver cancer stem cells in HCCLM3 or Huh7 cell lines (Fig. 3A,3B). Finally, we found that the subpopulation of $CD13^+$, $EpCAM^+$ cells increased in the cell line after PCGF2 knockdown (Data not show). In summary, these results indicate that PCGF4 KD or PCGF2 OE significantly reduces the expression of LCSC surface markers.

3.4 PCGF4 KD or PCGF2 OE enhances the sensitivity of cells to sorafenib

CSC is a major cause of clinical drug resistance to chemical agents. It has been reported that LCSCs are resistant to sorafenib [37, 38]. Therefore, we compared the effect of sorafenib on the cell viability of PCGF4 KD or PCGF2 OE cells. According to the results of Fig. 2A, the proliferation rate of PCGF4 KD or PCGF2 OE cells within 24 hours is the same as NC cells. Therefore, sorafenib was immediately added after the cells adhere to the wall. Within 24 hours, the cell viability was detected. As was expected, PCGF4 KD or PCGF2 OE cells were more sensitive to sorafenib at concentrations of 10 μM in HCCLM3 ($P < 0.001$) and Huh7 cell lines ($P < 0.001$) (Fig. 4A, 4B). In summary, these results revealed that PCGF2 or PCGF4 reversely regulates the stemness of liver cancer stem cells.

3.5 Higher PCGF4 but not PCGF2 exhibits poor prognosis in HCC

Liver cancer stem cells are a factor to the poor prognosis of HCC. The high expression of PCGF4 in many cancers predicts poor prognosis for patients, weather PCGF4 or PCGF2 affect the prognosis of liver cancer patients. We examined the prognosis of patients with HCC. A total of 76 patients with HCC (details in the material) were examined for tissue microarray, and the expression levels of PCGF4 and PCGF2 were detected by IHC. Analysis of the relationship between PCGF4 expression and clinicopathological characteristics showed that the up-regulation of PCGF4 is related to TNM staging (Table 1) Kaplan-Meier survival curves of patient demonstrate that high expression of PCGF4 significantly correlates with poor prognosis, and the os (overall survival) was lower than patients with lower expression of PCGF4 (Fig. 5A). Univariate analyses revealed that high expression of PCGF4 was significantly associated with decreased os but not high risk of postoperative recurrence in HCC patients (Fig. 5B, Table 2). But PCGF2 were not independent prognostic indicators of overall survival (Data not shown). In a word, PCGF4 may be an independent factor of prognosis of HCC rather than PCGF2.

Table 1
Relationship between tumor PCGF4 expression and clinicopathologic features of HCC patients

Characteristics		**PCGF4**		
		Negative	Positive	P
Gender	Female	10	3	0.054
	Male	30	33	
Age	< 51	19	17	0.981
	> 51	21	19	
HBsAg	Negative	4	36	0.243
	Positive	7	29	
HCVAb	Negative	39	35	0.480
	Positive	0	1	
Cirrhosis	No	5	5	0.858
	Yes	35	31	
AFP (ng/mL)	< 20	14	9	0.343
	> 20	26	27	
ALT (U/L)	≤ 75	19	21	0.315
	> 75	13	23	
Tumor size (cm)	≤ 5	23	17	0.168
	> 5	15	21	
Tumor number	Single	34	6	0.274
	Multiple	9	27	
Vascular invasion	No	17	23	0.512
	Yes	18	18	
TNM	I	30	10	0.044*
	II-III	20	16	

Abbreviations: HCC: hepatocellular carcinoma. HBsAg, hepatitis B surface antigen. HCVAb, hepatitis C virus antibody; AFP, α-fetoprotein; ALT, alanine transaminase; TNM, tumor-nodes-metastases; * P value < 0.05 was considered statistically significant. The Pearson Chi-square test was used.

Table 2
Univariate analyses of factors associated with overall survival of HCC patients

Variables	HR	95% CI	P
PCGF4 (High vs. low)	1.930	(1.094–3.404)	0.023*
Gender (male vs. female)	1.662	(0.746–3.702)	0.214
Age,years (> 51vs.≤51)	1.247	(0.706–2.202)	0.447
Tumor differentiation (poor vs. well)	0.868	(0.477–1.578)	0.642
Tumor size (cm) (> 5 vs.≤5)	1.692	(0.959–2.975)	0.048
Tumor number (multiple vs. single)	1.313	(0.670–2.573)	0.428
Vascular invasion (yes vs. no)	0.910	(1.052–3.289)	0.743
TNM (I vs. II-III)	1.860	(0.519–1.597)	0.033*
AFP ng/mL (≤ 20 vs.>20)	1.122	(0.603–2.085)	0.717
ALT(U/L) (≤ 75 vs.>75)	2.090	(1.155–3.781)	0.015*
Cirrhosis (Yes vs. No)	0.738	(0.330–1.648)	0.458
HCVAb (Negative vs. Positive)	1.780	(0.243–13.036)	0.570
HBsAg (Negative vs. Positive)	0.676	(0.316–1.445)	0.312
Cox proportional hazards regression model.			
HR, hazard ratio. CI, confidential interval. * P value < 0.05 was considered statistically significant.			

3.6 PCGF2/PCGF4 affected the Stem-Like Properties in HCC cells is mediated by p38 MAPK signaling

PCGF2 and PCGF4 belong to the polycomb protein family PRC1 components, and PRC1 is considered as a polycomb inhibitory complex. PRC1 is a type of transcriptional repressor that regulates the expression of target genes through chromatin modification. To clarify the downstream transcriptional regulatory network caused by PCGF2 or PCGF4, a comprehensive database of human transcription factor target genes hTF target and GTRD were applied. The number of overlapping PCGF4 (4496 genes, the number of regulated target genes, Fig. 6A) is much more than that of PCGF2 (939 genes, Fig. 6A), suggesting that PCGF4 shares a higher compatibility in modulating biological events. PCGF4 and PCGF2 were mainly enriched in MAPK signaling pathway (Fig. 6A). The MAPK pathway has three main branch routes: ERK, JNK and p38 MAPK. Among them, JNK and p38 have similar functions and are related to inflammation, apoptosis, and growth; PCGF4 or PCGF2 induced phosphorylation of p38 MAPK in HCC cells by Western blot (Fig. 6B).

4. Discussion

This study suggested that the two homologous proteins PCGF2 and PCGF4 reversely regulate the stem cell population, the mechanism of PCGF2 or PCGF4 regulates liver cancer stem cells depending on the p38 pathways (Fig. 6C). The expression of PCGF2 and PCGF4 showed an opposite trend in gastric cancer and breast cancer [39, 40]. However, the regulation of PCGF2 or PCGF4 on the activity of LCSCs has not been verified. In this study, it has revealed that PCGF2 and PCGF4 negatively regulate the activity of liver cancer stem cells. It was also found that expression of PCGF4 protein can be used as an independent prognostic indicator of overall survival, but PCGF2 can not.

The reliable result determined that low expression of PCGF4 or high expression of PCGF2 can inhibit the population of liver cancer stem cells and the sphere formation. Like HCC cells, liver cancer stem cells also have heterogeneity. Considering this, the study used different liver cancer stem cell markers to compare the effects in the expression of PCGF2 and PCGF4 based on the changes in liver cancer stem cell populations. It is remarkable that the low expression of PCGF4 will reduce the ratio of $CD13^+$, $EpCAM^+$, $CD133^+$ cells, but the high expression of PCGF2 only affects the ratio of $CD13^+$, $EpCAM^+$ cells. In line with our findings, the currently reported markers of liver cancer stem cells are not specific to liver cancer stem cells, and general LCSC is unlikely to appear. In order to facilitate the separation of LCSC, various types of markers should be examined [41].These results indicate that PCGF2 or PCGF4 can alter the population and self-renewal ability of liver cancer stem cells. To test if the low expression of PCGF2 will lead to poor prognosis of patients, we conducted IHC. Results showed that PCGF2 is lower expressed in HCC tissues compared with para cancer tissues, but PCGF2 cannot be used as an independent prognostic marker for patients in HCC. For HCC cell line, PCGF4 affects the proliferation of HCC cells, which is the same as the results of Wu et al [9, 10, 16, 23, 42, 43]. The overexpression of PCGF2 has no significant effect on the proliferation of HCC cells, but enhanced the sensitivity to sorafenib. These results suggested that targeting PCGF2 and PCGF4 may slow down the progression of HCC and improve the prognosis of patients.

For stemness experiments, we found that PCGF4 and PCGF2 negatively regulate the tumor sphere formation. The negative regulation of LCSCs by PCGF2 offered the same results in gastric cancer and breast cancer. It is suggested that PCGF2, as a tumor suppressor gene, also affects the maintenance of LCSCs stemness.

5. Conclusion

PCGF2 is a novel negative regulator of LCSCs that inhibits the stem cell population. PCGF2 and PCGF4 opposite drive stem-like properties and PCGF4 is associated with poor prognosis in HCC.

Abbreviations

HCC: Hepatocellular carcinoma; IHC: Immunohistochemistry; OS : Overall survival; DFS：Disease free survival; CCK8: Cell CountingKit-8; KEGG: Kyoto Encyclopedia of Genes and Genomes; LCSCs: Liver cancer stem cells; PRC1: Polycomb Repressive Complex1; BMI1: B lymphoma Mo-ML V insertion region 1 homolog; Mel18: Melanoma nuclear protein 18; AFP: Alpha-fetoprotein; DMEM: Dulbecco's modified Eagle medium; GAPDH: Glyceraldehyde-3-phosphatedehy drogenase; PBS: Phosphate buffered solution; qRT-PCR: Quantitative real-time polymerase chainre action; HBsAg: Hepatitis B surface antigen; HCVAb: Hepatitis C virus antibody; ALT: Alanine transaminase; TNM: tumor-nodes-metastases.

Declarations

Acknowledgments

We would like to thank the other members of this laboratory for their help and support.We thank the Core Facility of the School Life Sciences, Lanzhou University.

Authors' contributions

JH, YZ, BC, XL: The overall design of the experiment, the completion of the experimental data. JH, YZ: Data analysis. JH, YZ, HF, YW: Draft manuscript. YX, KQ: Flow cytometry analysis. YC: IHC, immunohistochemical analysis. WM, BC: academic contributions and manuscript revision. XM, FL, JY: Technical guidance, experimental suggestions. XL: Fund support.

Funding

We acknowledge the National Natural Science Foundation of China (Projects 82060119 ,31471233 and 31970624), Science and Technology Major Project of Gansu province(1602FKDA001), Gansu Province Science and Technology Plan Project Mission Statement (18JR2TA018, and Gansu Province Health Industry Scientific Research Project (GSWSKY-2015-490). The Fundamental Research Funds for the Central Universities (Project lzujbky-2018-k05 and lzujbky-2020-kb05). Intra-hospital Fund of the First Hospital of Lanzhou University (ldyyyn-2014-01).

Availability of data and materials

All data supporting this research are included in the article.

Ethics approval and consent to participant.

The study was approved by the Hospital Ethics Committee and informed consent was obtained from each patient under Institutional Reviewer Board protocols.

Consent for publication

All authors read and approved the final manuscript.

Competing interests

All authors of this manuscript declare that they have no conflict of interest.

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Figures

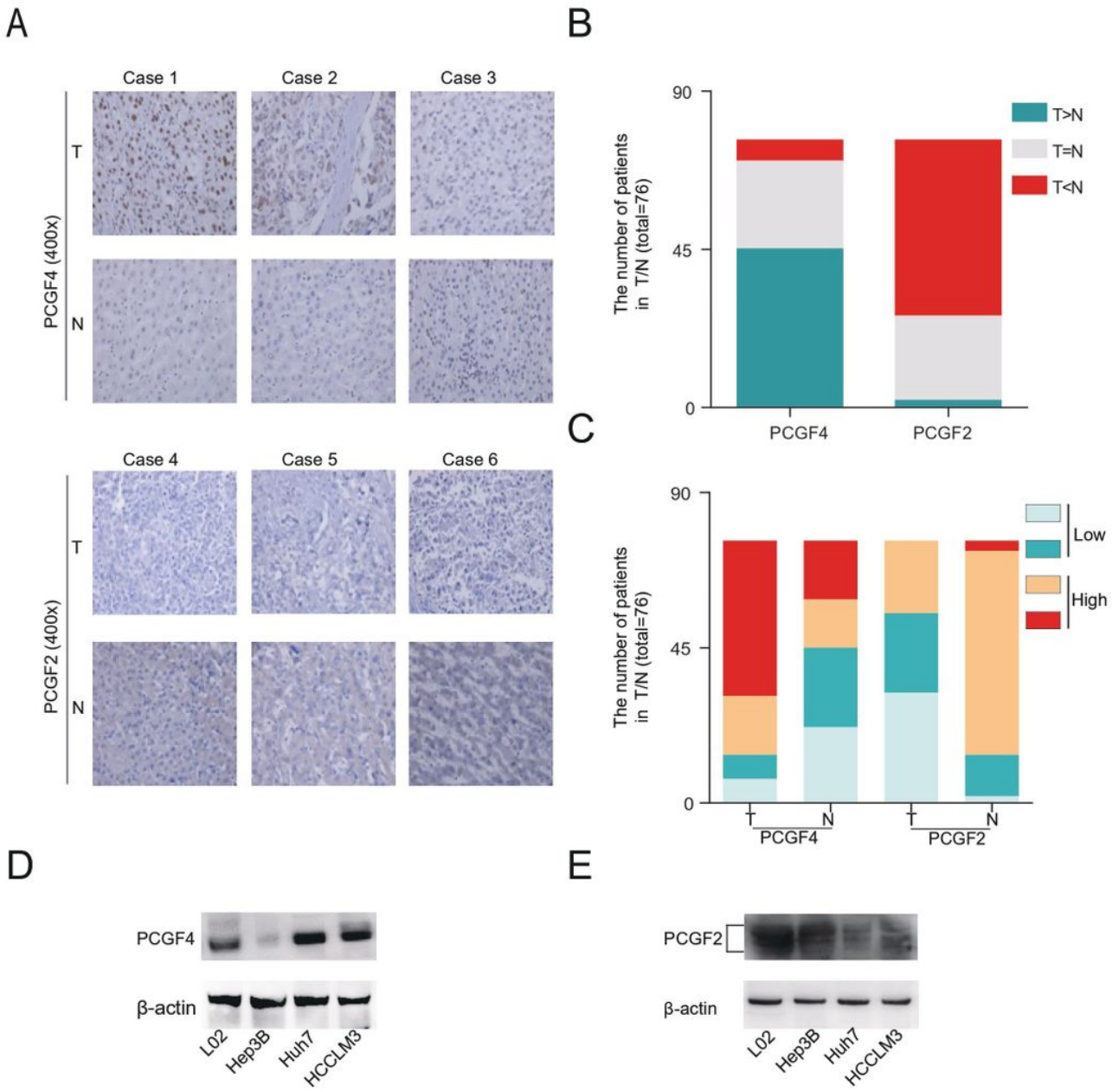

Figure 1

The expression of PCGF4 and PCGF2 was negatively correlated in HCC Patients and HCC Cell Lines. (A) Expression of PCGF2 and PCGF4 by IHC in 76 matched HCC and adjacent non-tumor tissues. (B, C) Bar graph shows statistics for staining intensity in training cohorts. (D-E) Western blot showed that PCGF2 is lowly expressed in cancer cells, while PCGF4 is highly expressed compared to L02.

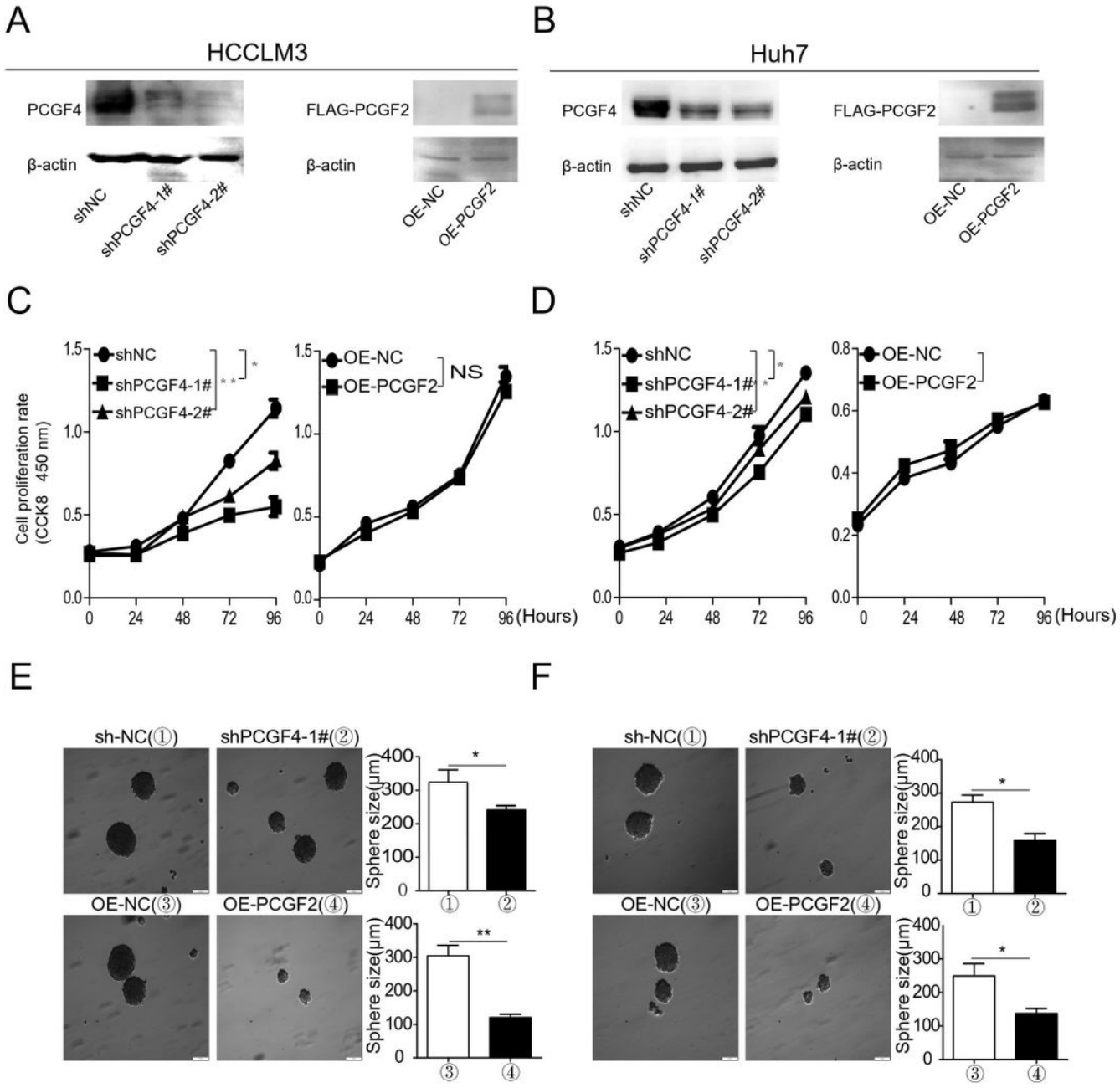

Figure 2

Loss PCGF4 inhibits cell proliferation, tumor spheres formation as well as PCGF2 up regulation suppresses cell stemness in HCC. (A-B) Western blot confirmed the protein expression of PCGF2 or PCGF4 in parental cells and stable cell lines (C-D) CCK8 assay was detected the PCGF2 up-regulation or PCGF4 down-regulation on the proliferation rate of Huh7 and HCCLM3 cell lines. (E-F) Cells are planted in low adhesion plates Count the number of sphere cells with a diameter greater than 80 μm. All experiments were performed in triplicate. Results are shown as mean ± SE. Scale bars =100 μm.

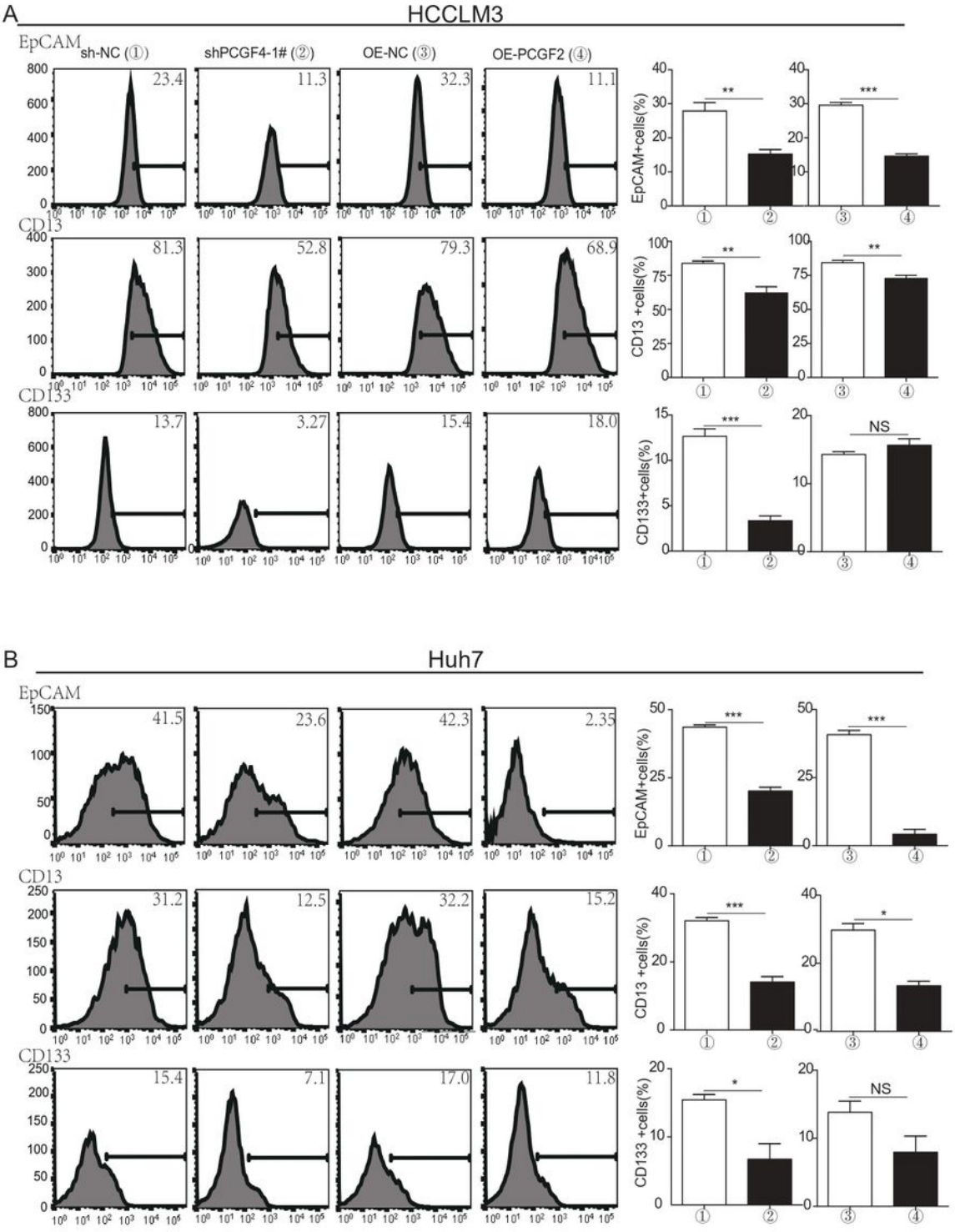

Figure 3

Decrease of the CD13+, EpCAM+ expressions via down regulated PCGF4 or up regulated PCGF2. (A-B) HCCLM3 and Huh7 cells were transfected with Control or shPCGF4, and the subpopulations of theCD13+/CD133+/EpCAM+ phenotype in these cells were measured by FACS analysis. These cell populations were also measured in PCGF2-over expressing in HCCLM3 or Huh7cells. Each plot is representative of 3 independent experiments. Results are shown as means± SD)

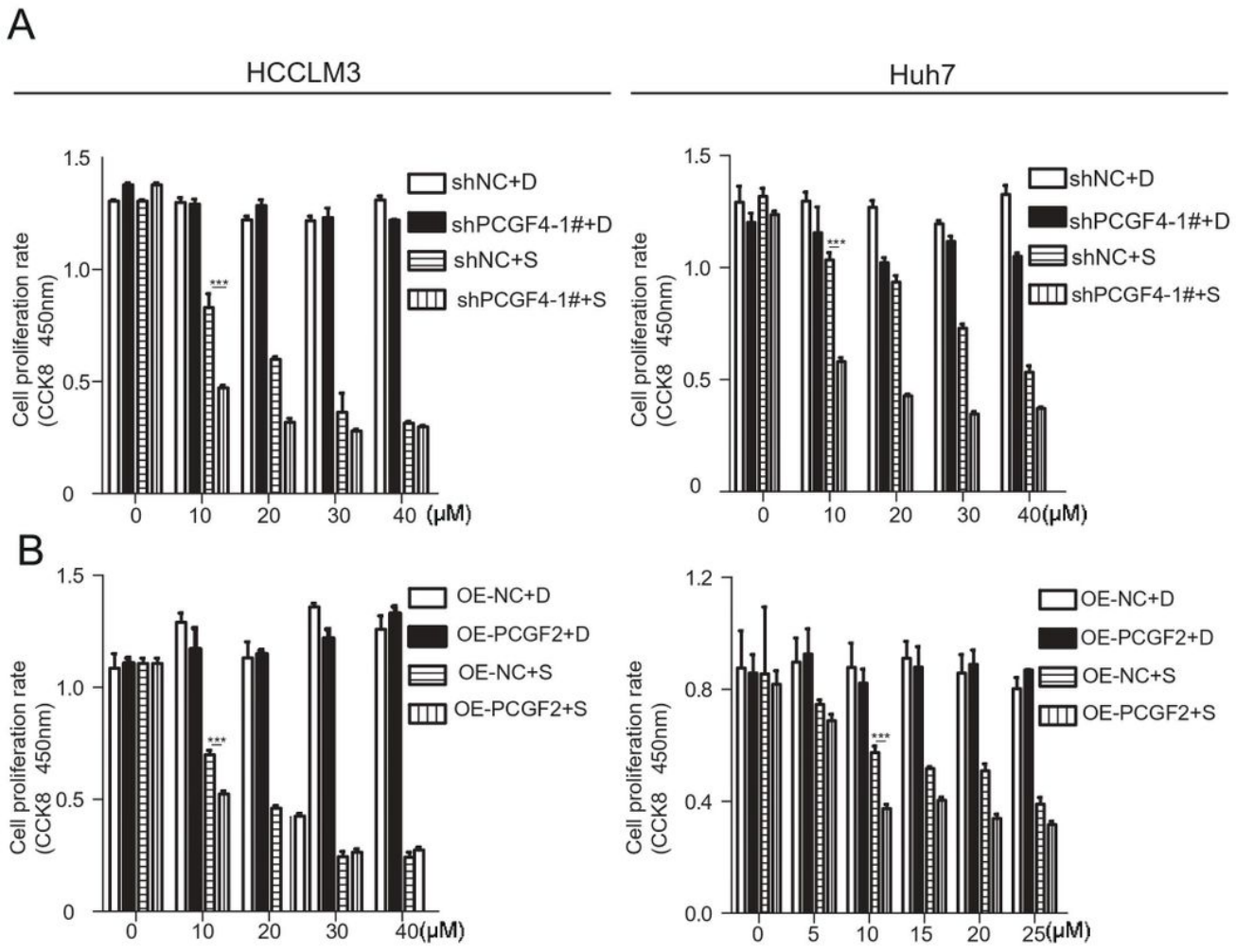

Figure 4

Cells with low PCGF4 expression or high PCGF2 expression are more sensitive to sorafenib. (A-B) The cell viability of Huh7 and HCCLM3 cells were treated with 0-40 μM sorafenib for 24 hours was evaluated by CCK8. D:DMSO and S:sorafenib.Results are shown as mean ± SD.

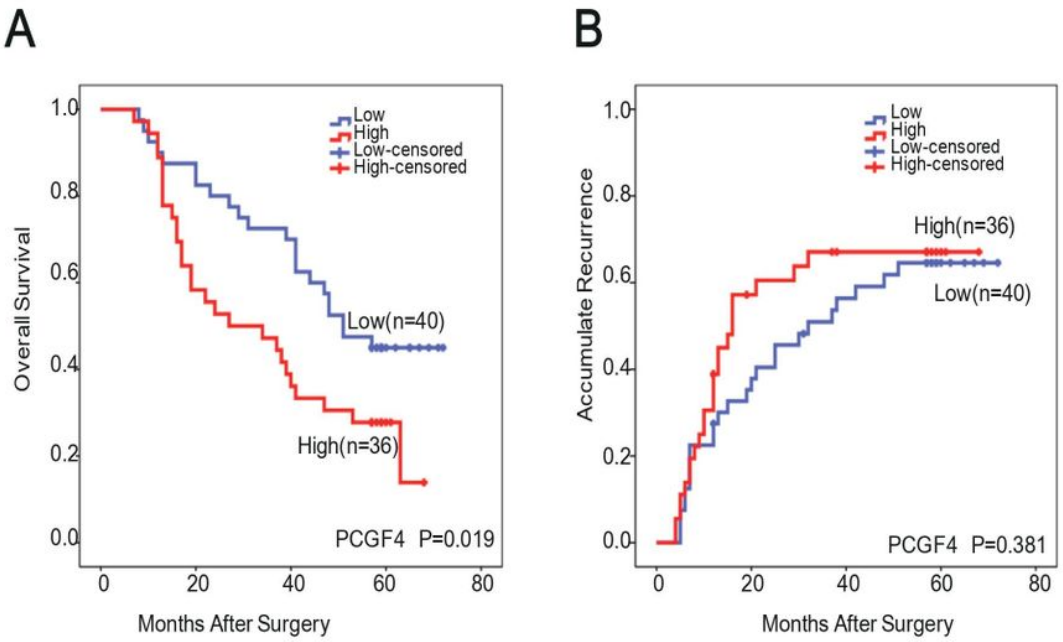

Figure 5

Higher PCGF4 exhibit poor prognosis in HCC. (A,B) Kaplan-Meier's curves for Overall Survival (P=0.019, Kaplan–Meier method (log-rank test))and Accumulate Survival according to PCGF4 expression. (P =0.381, Kaplan–Meier method (log-rank test)).

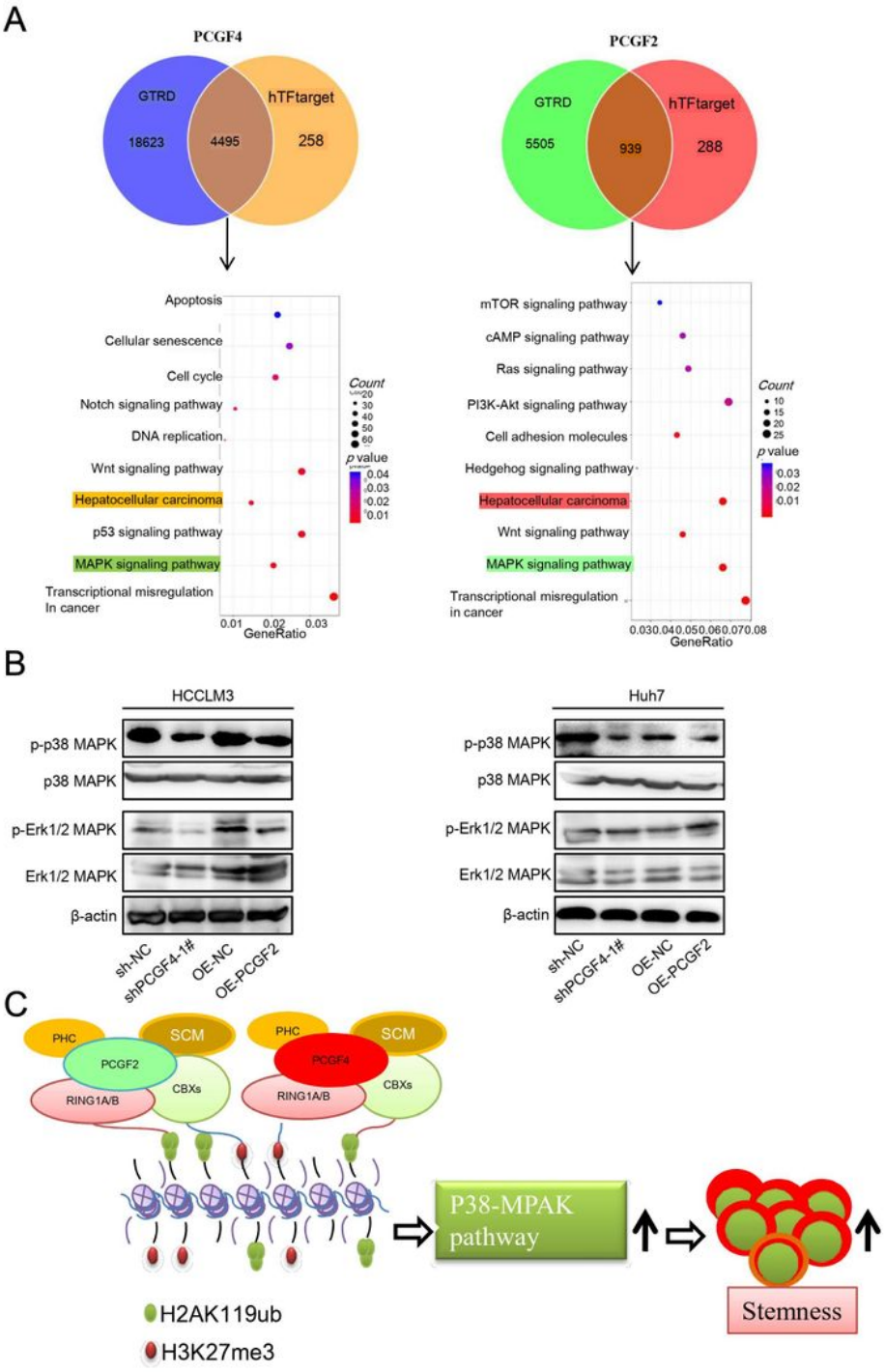

Figure 6

Functional enrichment analyze. (A) The hTF target (http://bioinfo.life.hust.edu.cn/hTFtarget) is an online platform for exploring human transcription factor target genes. The GTRD: a database on gene transcription regulation. We predicted the target genes of PCGF2 and PCGF4 through the hTF target and GTRD databases, and obtained 939 and 4496 target genes, respectively, and performed KEGG analysis on these target genes through the cluster Profiler package of the R language. (B) PCGF4/PCGF4 induced

phosphorylation of p38 MAPK in HCC cells. (C) Proposed model for the regulation mechanism of LCSCs by PCGF2 or PCGF4.