Identification of a secretory protein c19orf10 activated in hepatocellular carcinoma

Title: Identification of a Secretory Protein c19orf10 Activated in Hepatocellular Carcinoma

Authors: Hajime Sunagozaka, Masao Honda, Taro Yamashita, Ryuhei Nishino, Hajime Takatori, Kuniaki Arai, Tatsuya Yamashita, Yoshio Sakai, and Shuichi Kaneko

Affiliation: Department of Gastroenterology, Kanazawa University Hospital, 13-1 Takara-Machi, Kanazawa, Ishikawa, 920-8641 Japan

Correspondence: Shuichi Kaneko, M.D.
Kanazawa University Hospital, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan
TEL: +81-76-265-2233
FAX: +81-76-234-4250
E-mail: skaneko@m-kanazawa.jp

Abstract

The identification of genes involved in tumor growth is crucial for the development of inventive anti-cancer treatments. Here, we have cloned a 17-kDa secretory protein encoded by *c19orf10* from hepatocellular carcinoma (HCC) serial analysis of gene expression libraries. Gene expression analysis indicated that *c19orf10* was overexpressed in approximately two-thirds of HCC tissues compared with the adjacent non-cancerous liver tissues, and its expression was significantly positively correlated with that of alpha-fetoprotein (AFP). Overexpression of *c19orf10* enhanced cell proliferation of AFP-negative HLE cells, whereas knockdown of *c19orf10* inhibited cell proliferation of AFP-positive Hep3B and HuH7 cells along with G1 cell cycle arrest. Supplementation of recombinant c19orf10 protein in culture media enhanced cell proliferation in HLE cells, and this effect was abolished by the addition of antibodies developed against c19orf10. Intriguingly, c19orf10 could regulate cell proliferation through the activation of Akt/mitogen-activated protein kinase pathways. Taken together, these data suggest that c19orf10 might be one of the growth factors and potential molecular targets activated in HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers with an estimated worldwide incidence of 1,000,000 cases per year [1]. Most HCCs develop as a consequence of chronic liver disease such as chronic viral hepatitis due to hepatitis C virus (HCV) or hepatitis B virus (HBV) infection [2-7]. Liver cirrhosis patients with any etiology are considered to be at an extremely high risk for HCC [8-10]. Indeed, approximately 7% of liver cirrhosis patients with HCV infection develop HCC annually [8, 11], and the advancement of reliable HCC screening methods for high-risk patients is crucial for the improvement of their overall survival [12].

Currently, imaging diagnostic techniques such as ultrasonography, computed tomography, magnetic resonance image, and angiography are the gold standards for the early detection of HCC [13, 14]. In addition, tumor markers such as alpha-fetoprotein (AFP) and des-gamma carboxyl prothrombin (DCP) have been used for the screening of HCC [15-18], although their sensitivity and specificity are not sufficiently high. Recently, a gene expression profiling approach shed new light on Glypican 3, a heparin-sulfate proteoglycan anchored to the plasma membrane, as a potential HCC marker, and its clinical usefulness as a molecular target as well as a tumor marker is presently under investigation [19].

There are several options available for the treatment of HCC, including surgical resection, liver transplantation, radio-frequency ablation, transcatheter arterial chemoembolization, and chemotherapy, while taking the HCC stage and liver function into consideration. Recently, molecular therapy targeting the Raf kinase/vascular endothelial growth factor receptor (VEGFR) kinase inhibitor sorafenib improved the survival of patients with advanced HCC [20, 21], emphasizing the importance of deciphering the molecular pathogenesis of HCC for the development of effective treatment options.

Here we investigated the gene expression profiles of HCC by serial analysis of gene expression (SAGE) to discover a novel gene activated in HCC [22-25]. We identified a gene, *c19orf10*, overexpressed in HCC, and determined that the encoded 17-kDa protein (c19orf10) is a secretory protein. Murine *c19orf10* was originally discovered to encode a cytokine interleukin (IL)-25/stroma-derived growth factor (SF20) in 2001 [26]. The gene *c19orf10* was mapped in the H2 complex region of mouse chromosome 17 between *C3* and *Ir5*, and the hypothetical protein was predicted as globular protein [26]. However, the subsequent study failed to reproduce its proliferative effect on lymphoid cells and the paper was retracted by the authors in 2003 [26, 27]. Nevertheless, independent studies revealed that c19orf10 was indeed produced by synoviocytes, macrophages, and adipocytes, although the function of c19orf10 remained elusive [28, 29]. In this study, we identified

that *c19orf10* was overexpressed in AFP-positive HCC samples. Our data imply that c19orf10 could activate the mitogen-activated protein kinase (MAPK)/Akt pathway and enhance cell proliferation in HCC cell lines, suggesting that c19orf10 may be a growth factor produced by tumor epithelial cells and/or stromal cells, and, therefore, would be a good target for the treatment of HCC.

Material and Methods

SAGE and HCC samples

HCC and normal liver SAGE libraries that we had constructed were re-analyzed using SAGE 2000 software. The size of each SAGE library was normalized to 300,000 transcripts per library. Monte Carlo simulation was used to select genes whose expression levels were significantly different between the two libraries. Each SAGE tag was annotated using the gene-mapping website SAGE Genie database (http://cgap.nci.nih.gov/SAGE/) and the SOURCE database (http://smd.stanford.edu/cgi-bin/source/sourceSearch) as previously described [30]. An additional 15 SAGE libraries of normal and cancerous tissues from various organs were retrieved using the National Center for Biotechnology Information (NCBI) SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE/).

Fifteen HCC tissues (4 HBV-related and 11 HCV-related) and the corresponding non-cancerous liver tissues were obtained from HCC patients who received hepatectomy. Four normal liver tissues were obtained from patients undergoing surgical resection of the liver for the treatment of metastatic colon cancer. Additionally, 36 HCC tissues (17 HBV-related and 19 HCV-related) were obtained from HCC patients undergoing hepatectomy. These samples were snap-frozen in liquid nitrogen immediately after resection and used for quantitative real-time detection PCR (RTD-PCR). Total RNA was extracted using a ToTALLY RNA^{TM} kit (Ambion, Austin,TX).

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the institutional ethical review board committee. All patients provided written informed consent for the analysis of the specimens.

Laser Capture Microdissection (LCM) and RNA isolation

LCM was performed as previously described [31]. Briefly, 20 HCV-related surgically resected HCC tissues were frozen in OCT compound (Sakura Finetech, Torrance, CA) [32]. Inflammatory cells and cancerous cells in HCC tissues were separately excised by LCM using a Laser Scissors CRI-337 (Cell Robotics, Inc. Albuquerque, NM) under a microscope. Total RNA was isolated from these cells using a microRNA isolation kit (Stratagene, La Jolla, CA) in accordance with the supplied protocol, with slight modifications [31].

Construction of *C19ORF10* Expression Plasmid and Recombinant Adenovirus Vector

PCR was performed on a Marathon cDNA library from Huh7 cells using the following primers:

sense primers; 5′-GACCCTAGTCCAACATGGCGGCGCCC-3′ (the first PCR), 5′-ATGGCGGCGCCCAGCGGAGGGTGGAACGGC-3′ (the nested 2nd PCR), and anti-sense primers; 5′-CACCGGAGATGAGAAGGTGCCACCCGC-3′ (the first PCR), 5′-CAGGGCTGCTGGTCACAGCTCAGTGCGCG-3′ (the nested 2nd PCR). The 5′ and 3′ends of the cDNA were isolated using a SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA) according to the manufacturer's recommendations. The PCR products were cloned into a TA vector (Invitrogen, Carlsbad, CA) to generate the pcDNA3.1- *c19orf10* expression plasmid. Using this plasmid, a C-terminally FLAG-tagged construct of *c19orf10* was generated and inserted in a pSI mammalian expression vector (Promega, Madison, WI), which was driven by the SV40 promoter (pSI- *c19orf10*).

The replication-incompetent recombinant adenovirus vector expressing FLAG-tagged *c19orf10* (Ad. *c19orf10-FLAG*) was generated by homologous recombination using the AdMax system (Microbix, Toronto, Canada) as previously described [33]. The generated recombinant adenovirus was purified by limiting dilution and the titer of viral aliquots was determined by the 50% tissue culture infectious dose (TCID) method as previously described [34].

RTD-PCR

RTD-PCR was performed as previously described [31]. Briefly, template cDNA was synthesized from 1 μg of total RNA using SuperScript™ II RT (Invitrogen). RTD-PCR of *c19orf10* (Hs. 00384077_m1), *AFP* (Hs00173490_m1), *GPC3* (Hs01018938_m1), *KRT19* (Hs00761767_s1), and the *ACTB* internal control (Hs99999903_m1) was performed using a TaqMan® Gene Expression Assay kit (Applied Biosystems, Foster City, CA). The expression of selected genes was measured in triplicate by ΔΔCT method using the 7900 Sequence Detection System (Applied Biosystems).

Cell Lines and Transfection of Plasmids

Human liver cancer cell lines HuH1, Huh7, Hep3B, HLE, and HLF, as well as HEK293 and NIH3T3 were cultured in Dulbecco's modified eagle medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) in 5% CO_2 at 37°C. Transfection of plasmids was performed using FuGENE™ 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction. Briefly, 5×10^5 cells were seeded in a six-well plate 12 hours before transfection, and 3 μg of plasmid DNA was used for each transfection. All experiments were repeated at least twice.

Purification of c19orf10-FLAG Fused Protein and Production of Anti-c19orf10 Antibody

Approximately 500 ml of culture supernatant obtained from HEK293 cells infected with Ad.*C19ORF10-FLAG* at a multiplicity of infection of 20 was applied to an anti-FLAG affinity gel column (Sigma-Aldrich, St. Louis, MO). The column was subjected to elution by competition with FLAG peptide (5 μg/ml), and each 1 ml fraction of the eluted aliquot was collected to obtain the most concentrated c19orf10-FLAG protein in accordance with the manufacturer's protocol. The anti-c19orf10 antibodies were developed by immunizing rabbits with repeated intradermal injections of oligo-peptides of c19orf10 (155-VAKAARSEL-163). Protein concentration was measured by the Bradford method.

Silencing Gene Expression by Short Interfering RNA (siRNA)

The selected siRNA targeting *C19ORF10* (Si-*C19ORF10*; Silencer Select siRNAs s31855) and the irrelevant control sequence (Si-*Control*; Silencer Select siRNAs 4390843) were obtained from Applied Biosystems. Transfection of these siRNAs was performed using FuGENE^{TM} 6 (Roche Diagnostics) as previously described [30]. Breifly, 2×10^5 cells were seeded in a six-well plate 12 hours before transfection. A total of 100 pmol/L of siRNA duplex was used for each transfection. The experiments were performed at least twice.

Cell Proliferation Assay

Cell proliferation was evaluated in quadruplicate using a Cell Titer 96 MTS Assay kit (Promega). After incubation with MTS/PMS solution at 37°C for 2 h, the absorbance at 450 nm was measured. The experiments were performed at least twice.

Cell Cycle Analysis

Cells were fixed using 80% ice-cold ethanol and incubated with propidium iodide (PI) for 10 min. DNA content was analyzed using a FACS Caliber flow cytometer (BD Biosciences, San Jose, CA) counting 10,000 stained cells. The distribution of cells in each cell-cycle phase was determined using FlowJo software (Tree Star Inc., Ashland, OR).

Western Blotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and the extracts were subsequently electrophoresed on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels and transferred onto protean nitrocellulose membranes. The blots were then incubated for 1 h with an

appropriate primary monoclonal antibody: phospho-PI3K (#4228), phospho-Akt (#4060), phospho-GSK-3β (#9323), phospho-c-Raf (#9427), phospho-MEK1/2 (#9154), phospho-p44/42 MAPK (Erk1/2) (#4370), Cdk4 (CDK4 (#2906), Cdk6 (#3136), cyclinD1 (#2926), cyclinD3 (#2936) , phospho-Rb (#9308), phospho-P53 (# 9286), phospho-cdc2 (#9111), and β-actin (#4970) (Cell Signaling Technology, Allschwil, Switzerland) and anti-FLAG antibodies (Sigma-Aldrich, St. Louis, MO). The blots were washed and exposed to peroxidase-conjugated secondary antibodies, such as anti-mouse or rabbit IgG antibodies, and visualized using the ECL™ kit (Amersham Biosciences Corp., Piscataway, NJ). All experiments were performed at least twice.

Statistical Analyses

Unpaired t-tests and Kruskal-Wallis tests were performed on the RTD-PCR and cell proliferation data using GraphPad Prism software (www.graphpad.com).

Results

Identification of C19ORF10 overexpression in HCC by SAGE

To comprehensively explore the candidate novel genes activated in HCC, we re-analyzed two SAGE libraries derived from HCC tissues and normal liver tissues [30]. After normalization of each SAGE library size to 300,000 tags, we compared the HCC and normal liver libraries to obtain the list of genes overexpressed in HCC. We identified 79 genes significantly overexpressed in the HCC library by more than 10-fold when compared with the normal liver library (Supplemental Table 1). Among them, we explored expressed sequence tags (ESTs) as candidates for novel HCC-related genes to identify 8 unique tags corresponding to 7 ESTs (Table 1). We especially focused on the EST chromosome 19 open reading frame 10 (*c19orf10*) because the sequence presumably encoded a secretory protein with a signal peptide sequence (Fig. 1A).

When we examined the expression profiles of *c19orf10* using retrieved SAGE data from various cancers and their normal counterparts, we identified that *c19orf10* was abundantly expressed in human HCC (Fig. 1B). We further examined the publicly available EST profiles of *c19orf10* (http://www.ncbi.nlm.nih.gov/unigene), and confirmed its tendency to be overexpressed in HCC compared with the normal liver (data not shown). We validated the overexpression of *c19orf10* in 15 independent HCC tissues and adjacent non-cancerous liver tissues by RTD-PCR. Gene expression of *c19orf10* was significantly higher in the HCC tissues than in the normal liver tissues and adjacent non-cancerous liver tissues ($P = 0.014$ and 0.048, respectively; Fig. 1C). *C19orf10* expression was elevated in HCC tissues compared with the adjacent non-cancerous liver tissues in 10 of 15 patients (66.7%; Fig. 1D).

Overexpression of C19ORF10 in AFP-positive HCC

As HCC is a heterogeneous mixture of cancer epithelial cells and stromal cells, and a previous report indicated that *c19orf10* is expressed in fibroblast-like synoviocytes. We therefore evaluated the expression of *c19orf10* in tumor epithelial cells and stromal cells separately using LCM and RTD-PCR in 20 HCC tissues (Fig. 2A). Although tumor stromal cells expressed *c19orf10* at some level, the expression levels were significantly higher in tumor epithelial cells than in stromal cells ($P = 0.006$) (Fig. 2B).

To explore the relationship of *c19orf10* with other established HCC markers, we investigated the gene expression of *c19orf10*, *AFP* (alpha-fetoprotein), *KRT19* (cytokeratin 19), and *GPC3* (glypican 3). Because only one of15 HCC tissues analyzed above (Fig. 1D) was AFP-positive (data not shown), we further investigated the expression of *c19orf10* in an additional

36 HCC tissues using RTD-PCR. Interestingly, *c19orf10* expression was significantly positively correlated with *AFP* (r = 0.44, *P* = 0.008), but not with *KRT19* (r = 0.08, *P* = 0.66) nor *GPC3* (r = 0.11, *P* = 0.54) (Figs. 2C–E). Furthermore, when we examined the expression of *c19orf10* in AFP-positive (HuH1, HuH7, and Hep3B) and –negative (HLE and HLF) HCC cell lines, we identified the overexpression of *c19orf10* in AFP-positive HCC cell lines (Fig. 2F). These data suggested that *c19orf10* is overexpressed and may play some role in AFP-positive HCCs.

C19orf10 regulates MAPK/Akt pathways and activates cell proliferation

To explore the functional role of c19orf10 in HCC, we performed *c19orf10* overexpression and knockdown studies using *c19orf10*-low HLE cells, and *c19orf10*-high Hep3B and HuH7 cells, respectively. When we transfected HLE cells with pcDNA3.1 or pcDNA3.1- *c19orf10* plasmids, we identified an approximately 6-fold overexpression of *c19orf10* when compared with the control 48 hours after transfection (P < 0.0001) (Fig. 3A). Interestingly, cell proliferation was modestly, but significantly, enhanced compared with the control 72 hours after transfection (P = 0.0015) (Fig. 3B).

We also transfected siRNAs targeting an irrelevant sequence (Si-*Control*) or *c19orf10* (Si-*c19orf10*) in Hep3B and HuH7 cells. We observed an approximately 50% decrease in *c19orf10* expression in Hep3B cells transfected with Si- *c19orf10* compared with the control 48 hours after transfection with statistical significance (P < 0.0001). In this condition, cell proliferation was suppressed to 50% compared with the control 72 hours after transfection (P < 0.0001) (Figs. 3C, D). When we performed cell cycle analysis of HuH7 cells transfected with Si-*Control* or Si- *c19orf10*, we identified an increase of G1-phase cells and a decrease of S- and G2-phase cells by *c19orf10* knockdown, suggesting the G1 cycle arrest was caused by the knockdown of *c19orf10* (Fig. 3E).

We examined the representative MAPK/Akt pathway-associated proteins and cell cycle regulators using Western blotting 72 hours after siRNAs transfection (Fig. 3F). Interestingly, phosphorylation of c-Raf, MEK, MAPK, PI3K, and pAkt was inhibited by knockdown of *c19orf10*, suggesting the involvement of *c19orf10* in the MAPK/Akt pathways. Furthermore, phosphorylation of Rb, CDK4, and CDK6 was also inhibited by knockdown of *c19orf10*, consistent with the observation of G1 cell cycle arrest by *C19ORF10* knockdown. PTEN, p53, and phosphorylated CDC2 protein expression was not affected by knockdown of *c19orf10*.

C19orf10 encodes the secretory protein and stimulates cell proliferation

As the sequence of *c19orf10* suggested that it encodes a secretory protein, we transfected pSI-

c19orf10-FLAG in NIH3T3 cells and examined the culture supernatant. Immunoprecipitation of the collected culture supernatant 48 h after transfection using anti-FLAG antibodies indicated the existence of a 17-kDa protein (c19orf10), compatible with the molecular weight of the 142 amino acids protein encoded by *c19orf10* (Fig. 4A). We purified c19orf10-FLAG protein from the supernatant of HEK293 cells infected with Ad. *c19orf10-FLAG* using an anti-FLAG column. Supplementation of purified c19orf10-FLAG into the culture media for 72 h enhanced the proliferation of HLE cells in a dose dependent manner with statistical significance, whereas control FLAG peptides and BSA had no effects on cell proliferation (Fig. 4B). Western blot analysis of HLE cells cultured with purified c19orf10-FLAG (40 ng/ml) or BSA control (40 ng/ml) indicated the immediate strong phospholyration of Akt peaked 5 minutes after supplementation (Fig. 4C). The modest phospholyration of GSK3β (Ser9) and p44/42 MAPK also followed and peaked 60 minutes after c19orf10 supplementation. These data suggest that Akt pathway might be directly involved in the c19orf10-mediated cell proliferation signaling with the subsequent activation of MAPK pathway. Furthermore, addition of antibodies against c19orf10 peptides to the culture media abolished the cell proliferation induced by c19orf10, whereas control IgG had no effects (Fig. 4D). Taken together, these data suggest that c19orf10 may be a growth factor overexpressed in AFP-positive HCCs and activates the Akt/MAPK pathways, potentially through the activation of an unidentified c19orf10 receptor.

Discussion

SAGE facilitates the measurement of transcripts from normal and malignant tissues in a non-biased and highly accurate, quantitative manner. Indeed, SAGE produces a comprehensive gene expression profile without *a priori* gene sequence information, leading to the identification of novel transcripts potentially involved in the pathogenesis of human cancer [19]. In this study, we identified seven SAGE tags potentially corresponding to novel genes activated in HCC. Among them, we identified the secretory protein c19orf10 activated in a subset of HCCs.

Several serum markers including AFP, DCP, and Glypican 3 are currently used for the detection and/or the evaluation of the treatment for HCCs in the clinic [15-18, 35]. These markers are known as oncofetal proteins, that is, expressed in the fetus, transcriptionally suppressed in the adult organ, and reactivated in the tumor. We identified that the expression of *c19orf10* positively correlated with *AFP* expression, but did not correlate with the expression of *GPC3* or the biliary marker *KRT19*. As *c19orf10* was rarely detected in the normal liver, it is possible that c19orf10 is also an oncofetal protein activated in HCC. We are currently developing a system to detect serum c19orf10 in HCC patients, and the significance of the serum c19orf10 value as an HCC marker should be clarified.

Recent advancement in molecular biology has revealed the considerable diversity of transcription initiation and/or termination of genes altered in the process of carcinogenesis. Indeed, using 5' SAGE approach, we recently discovered the novel intronic transcripts activated in HCC [36]. Interestingly, when we investigated the transcription initiation of *c19orf10* using the 5' SAGE database, we identified a potential 5' splice variant initiated from the second exon of *c19orf10* (data not shown). Although we have not yet validated the presence of 5' splice variants in *c19orf10* by PCR, examination of 5' EST database also suggested the presence of the similar splice variants (GenBank Accession Number CR980295, BQ680744, BQ648461, etc). Alteration of transcription initiation/termination in *c19orf10* might affect the abundance or function of c19orf10 protein, and the details of 5' splice variants in *c19orf10* should be clarified in future studies.

Molecular targeting therapy has rapidly emerged for solid tumors as well as for leukemia [37-39]. Sorafenib is a multi-kinase inhibitor targeting Raf kinase in the MAPK pathway as well as VEGFR and the platelet-derived growth factor-receptor [40, 41]. In this study, we identified that c19orf10 activates the MAPK and Akt/PI3K pathways, and contributes to the proliferation of HCC cell lines, although we still could not discover the potential receptor of c19orf10. Development of a neutralizing c19orf10 antibody may provide novel therapeutic options for HCC patients to inhibit these signaling pathways, and its efficacy should be evaluated in the future.

Recently, c19orf10 was found to be expressed in fibroblast-like synoviocytes in the synovium using a proteomics approach [29]. In addition, a recent paper indicated that c19orf10 was expressed in pre-adipocyte cells and involved in adipogenesis using two-dimensional electrophoresis mass spectrometry analysis [28]. Thus, c19orf10 may have pleiotropic effects on various lineages of normal organs in various developmental stages, and the clarification of its distribution and biological properties in the whole body may provide more detailed information about the function of c19orf10.

In conclusion, we have identified the protein c19orf10 that regulates the Akt /MAPK pathways and cell cycle through an unidentified mechanism in HCC. Although further studies should be conducted to detect the potential c19orf10 receptor or signaling molecules binding to c19orf10, the current study suggests that c19orf10 may be a novel growth factor, a potential tumor marker, and also a potential target molecule for HCC treatment.

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Figure legends

Fig. 1

A. Structure of a *c19orf10* gene and a c19orf10 protein. The DNA sequence of *c19orf10* and amino acid alignment of the encoded c19orf10 protein are shown. C19orf10 is predicted to have a molecular weight of 17 kDa and contain a signal peptide cleavage site (indicated as a black arrow). **B.** *C19orf10* gene expression profiles in various tissues by SAGE. Y axis indicates the number of tags corresponding to *c19orf10* in each tissue. **C & D.** RTD-PCR analysis of *c19orf10*. RNA was isolated from 34 tissue samples: 15 HCC, 15 corresponding non-cancerous liver samples, and 4 normal liver samples. Differential expression of each gene among normal liver tissues, non-cancerous liver tissues, and HCC tissues was examined using the Kruskal-Wallis test and unpaired t-test. The mean value of gene expression data in each group is indicated (**C**). *C19orf10* was overexpressed in 10 of 15 examined HCC tissues compared with the non-cancerous liver tissues (**D**).

Fig. 2

A. Representative photomicrographs of an HCC tissue used for LCM (toluidine blue staining). Inflammatory mononuclear cells and stromal cells were separately captured (Left: Pre-LCM, right: Post-LCM). **B.** RTD-PCR analysis of *c19orf10* expression in inflammatory mononuclear cells and tumor epithelial cells in 20 HCV-related HCC tissues. Tumor-inflammatory mononuclear cells and stromal cells were isolated using LCM. RNAs were isolated from these cells as well as parenchymal tissues from the same liver, followed by RTD-PCR for *c19orf10* gene expression. Expression of the *c19orf10* gene was higher than that observed in HCC-infiltrating inflammatory mononuclear cells. *,$P < 0.05$. **C–E.** Scatter plot analysis of *c19orf10, AFP, KRT19,* and *GPC3* expression in HCC. RNA was isolated from 17 HBV-related HCC and 19 HCV-related HCC. **F.** RTD-PCR analysis of *c19orf10* in AFP-negative (HLE and HLF) and -positive (HuH1, HuH7, and Hep3B) liver cancer cell lines.

Fig. 3

A. RTD-PCR analysis of *c19orf10* expression in HLE cells transfected with pcDNA3.1 or pcDNA3.1-*c19orf10* plasmids. **B.** Cell proliferation assay of HLE cells transfected with pcDNA3.1 or pcDNA3.1-*c19orf10* plasmids. Cell proliferation was evaluated 72 h after each plasmid transfection. **C.** RTD-PCR analysis of *c19orf10* expression in Hep3B cells transfected with Si-*Control* or Si-*c19orf10*. Gene expression was measured in triplicates 48 h after transfection. **D.**

Cell proliferation assay of Hep3B cells transfected with Si-*Control* or Si-*c19orf10*. Cell proliferation was evaluated 72 h after siRNA transfection. **E.** Cell cycle analysis of HuH7 cells transfected with Si-*Control* or Si-*c19orf10*. Cell cycle was evaluated 72 h after siRNA transfection. A black arrow indicates the G2 phase peak. **F.** Western blotting analysis of Huh7 cells transfected with Si-*Control* or Si-*c19orf10*. Cells were lysed by RIPA buffer 72 h after siRNA transfection.

Fig. 4.

A. Coomassie blue staining and Western blotting of culture supernatant of NIH3T3 cells transfected with pSI-*c19orf10-FLAG*. A black arrow indicates the 17-kDa c19orf10 protein. **B.** Cell proliferation assay of HLE cells supplemented with recombinant c19orf10-FLAG. Cell proliferation was measured in quadruplicates 72 h after supplementation. **C.** Western blotting of HLE cells supplemented with c19orf10-FLAG (40 ng/ml). Cells were lysed at indicated time after c19orf10 supplementation. **D.** Cell proliferation assay of HLE cells supplemented with control BSA (40 ng/ml) (white bar), c19orf10-FLAG (40 ng/ml) (light grey bar), c19orf10-FLAG (40 ng/ml) +anti-c19orf10 antibodies (gray bar), and c19orf10-FLAG (40 ng/ml) + control mouse IgG (black bar).

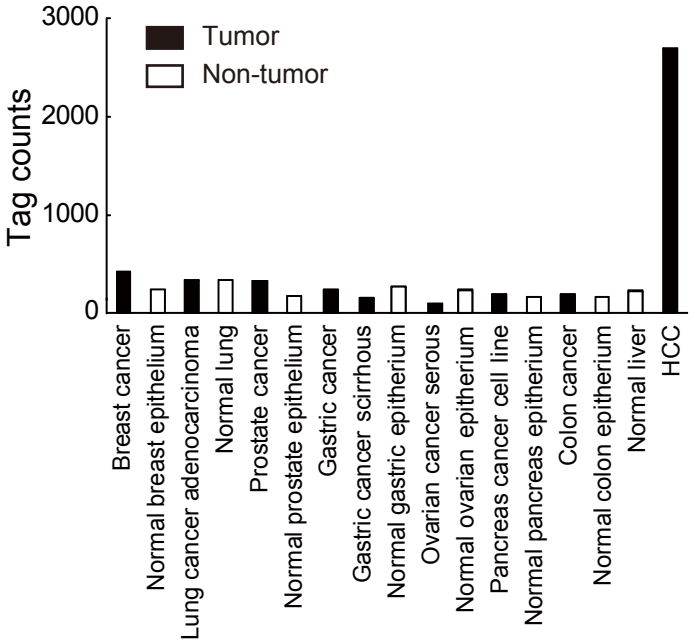
Table 1. ESTs overexpressed in the HCC library

Tag Sequence	P-value	HCC	normal liver	T/N ratio	Name	UniGene ID
TGGGCAGGTG	<0.00001	33	0	>33	Chromosome 5 open reading frame 13	Hs.483067
GCAAAATATC	<0.00001	31	2	15.5	Liver cancer associated non coding mRNA, partial sequer	Hs.214343
AGCCTGCAGA	0.0002	12	1	12	Chromosome 19 open reading frame 10	Hs.465645
TTGTGCACGT	0.000228	12	1	12	CDNA FLJ45284 fis, clone BRHIP3001964	Hs.514273
ACATTCTTGT	0.000042	12	0	>12	Transcribed locus, strongly similar to XP_496055.1	Hs.76704
ACAAGTACCC	0.001161	10	1	>10	Chromosome 5 open reading frame 13	Hs.483067
GAGGTGAAGG	0.000174	10	0	>10	KIAA1914	Hs.501106
GCTGGAGGAG	0.000114	10	0	>10	Transcribed locus	Hs.520115

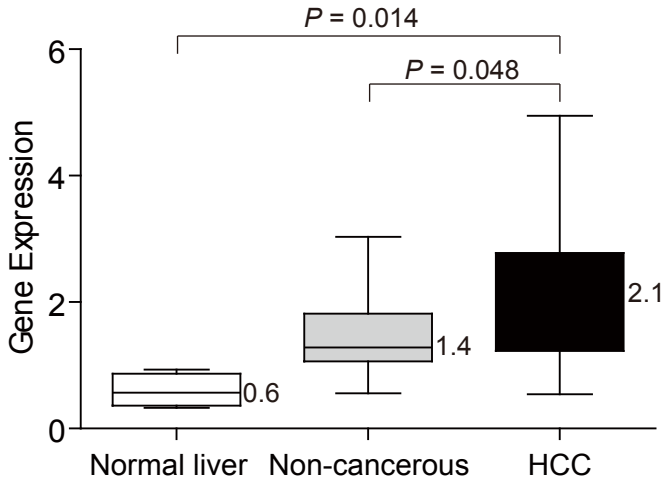
Figure 1

A

B



C



D

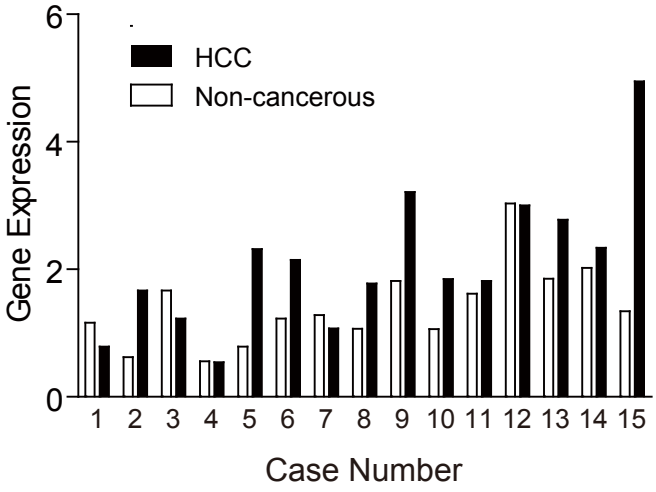


Figure 2

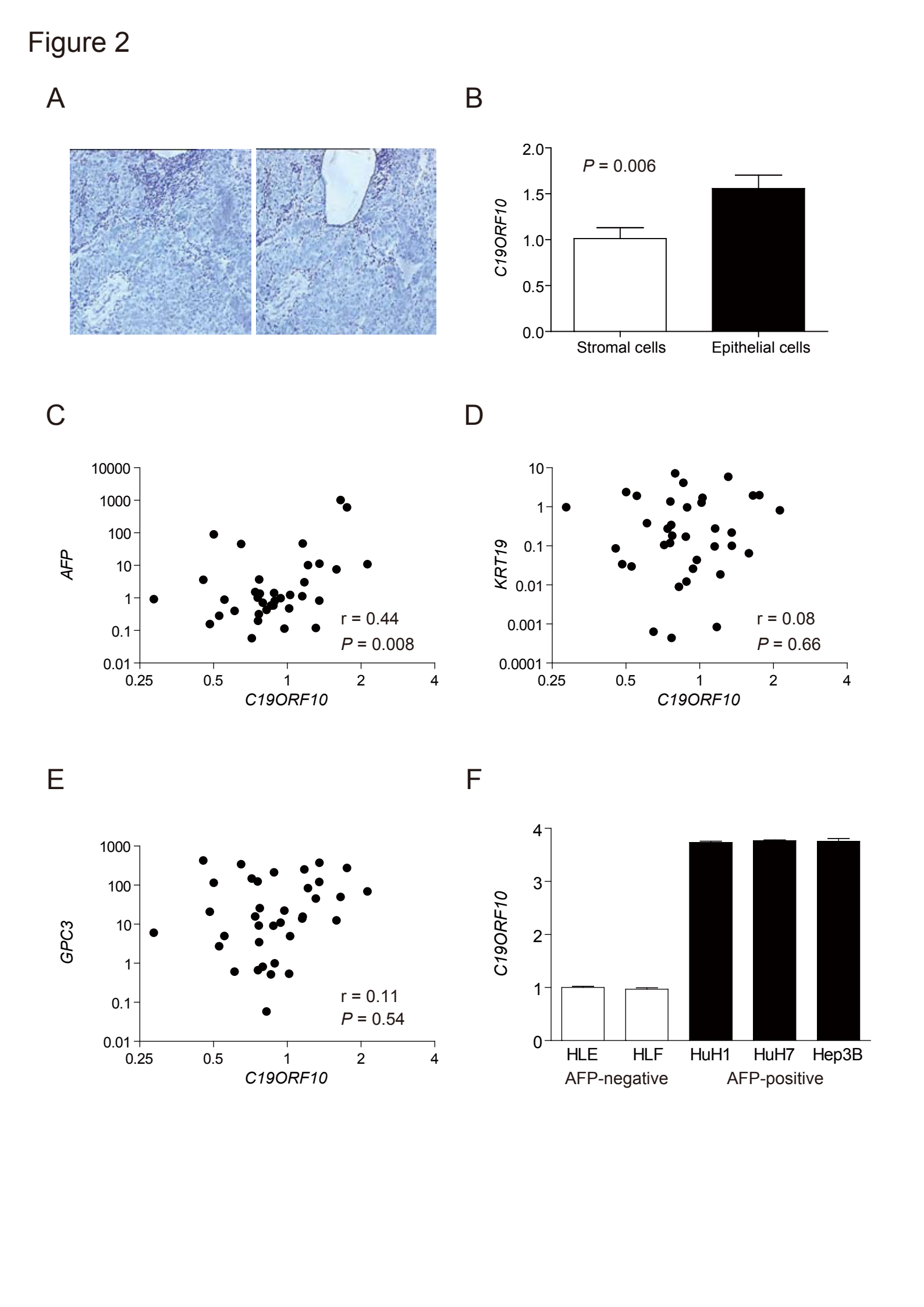

A
B
P = 0.006
C19ORF10
2.0
1.5
1.0
0.5
0.0
Stromal cells
Epithelial cells
C
AFP
10000
1000
100
10
1
0.1
0.01
r = 0.44
P = 0.008
0.25
0.5
1
2
4
C19ORF10
D
KRT19
10
1
0.1
0.01
0.001
0.0001
r = 0.08
P = 0.66
C19ORF10
E
GPC3
1000
100
10
1
0.1
0.01
r = 0.11
P = 0.54
C19ORF10
F
C19ORF10
4
3
2
1
0
HLE
HLF
HuH1
HuH7
Hep3B
AFP-negative
AFP-positive

Figure 3

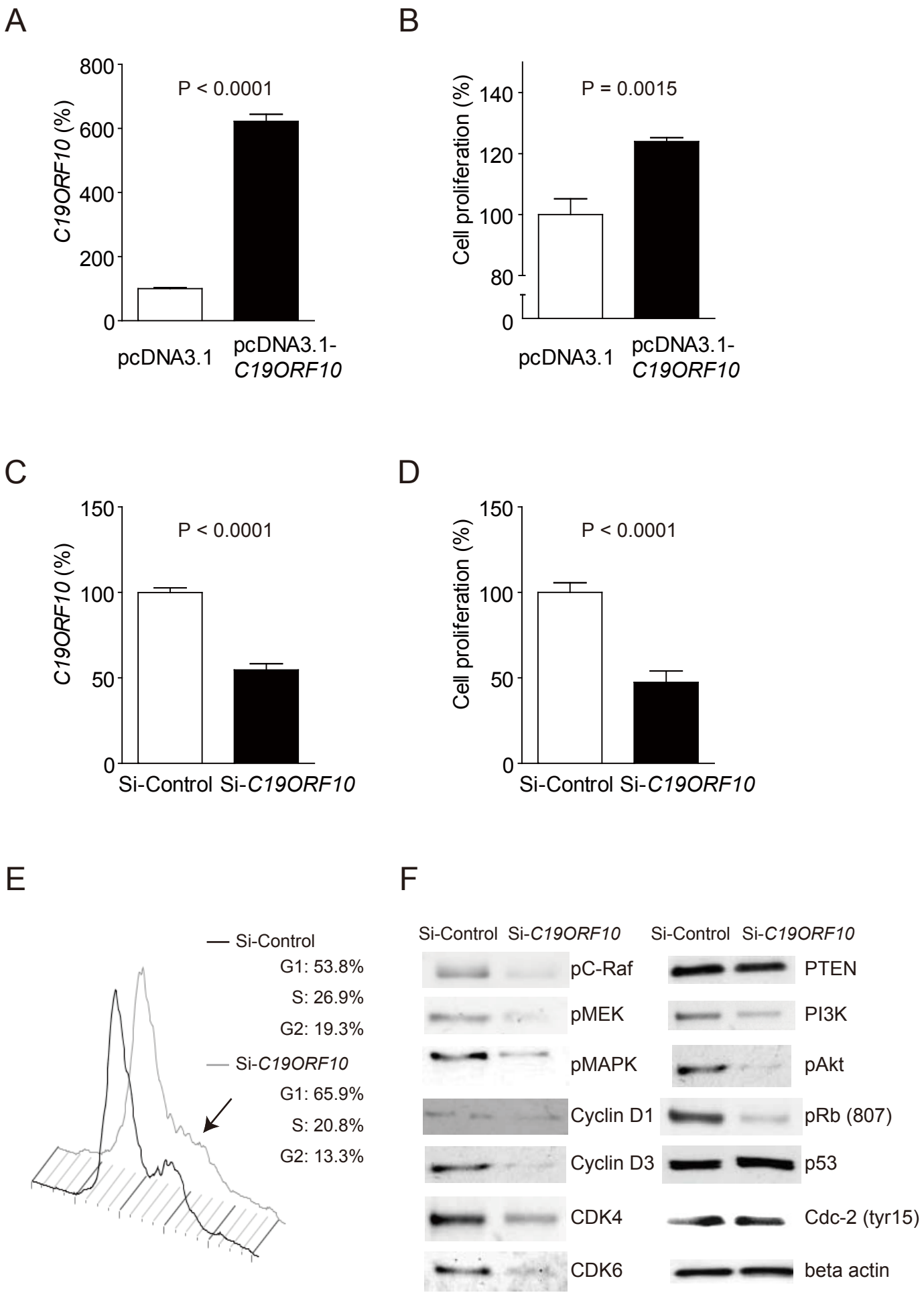
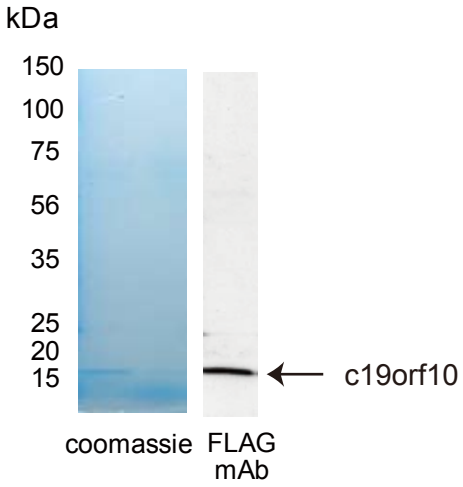
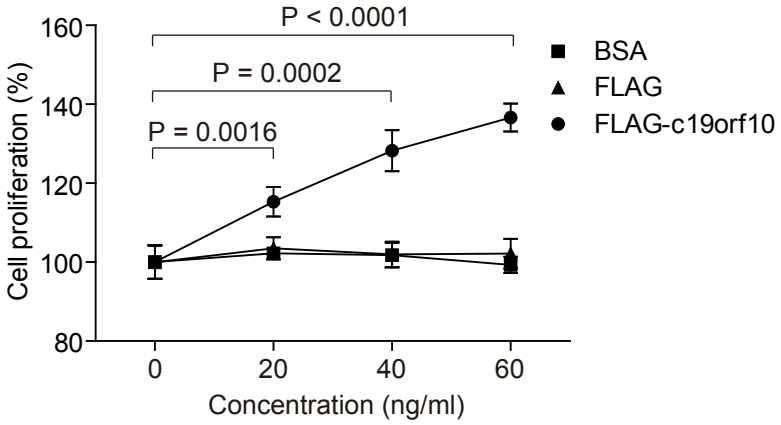

A
800
600
400
200
0
C19ORF10 (%)
P < 0.0001
pcDNA3.1
pcDNA3.1-
C19ORF10
B
140
120
100
80
0
Cell proliferation (%)
P = 0.0015
pcDNA3.1
pcDNA3.1-
C19ORF10
C
150
100
50
0
C19ORF10 (%)
P < 0.0001
Si-Control
Si-C19ORF10
D
150
100
50
0
Cell proliferation (%)
P < 0.0001
Si-Control
Si-C19ORF10
E
Si-Control
G1: 53.8%
S: 26.9%
G2: 19.3%
Si-C19ORF10
G1: 65.9%
S: 20.8%
G2: 13.3%
F
Si-Control
Si-C19ORF10
Si-Control
Si-C19ORF10
pC-Raf
pMEK
pMAPK
Cyclin D1
Cyclin D3
CDK4
CDK6
PTEN
PI3K
pAkt
pRb (807)
p53
Cdc-2 (tyr15)
beta actin

Figure 4

A



B



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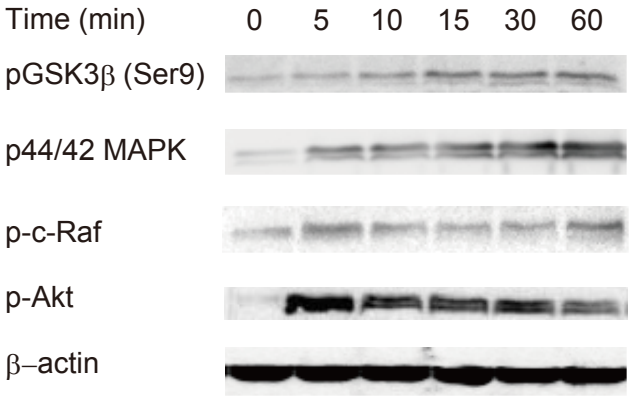
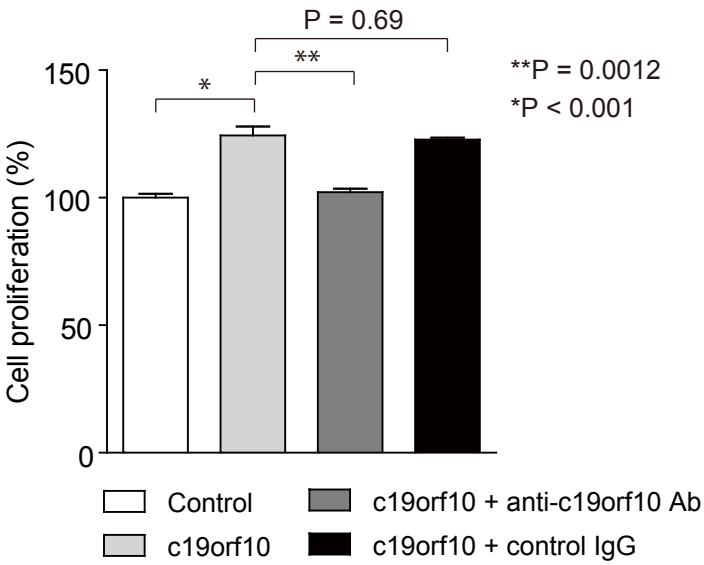


D



Supplemental Table 1 Genes overexpressed in the HCC library compared with the normal liver library

Tag Sequence	P-value	HCC	normal liver	T/N ratio	Name	UniGene ID
CCAAGACTTC	<0.00001	449	3	149.6667	no reliable match	
CTAAGGTAGT	<0.00001	61	0	>61	no reliable match	
TGGGCAGGTG	<0.00001	33	0	>33	Chromosome 5 open reading frame 13	Hs.483067
CTGACTTGTG	<0.00001	24	0	>24	Major histocompatibility complex, class I, B	Hs.77961
CTGCCCTCCC	<0.00001	24	1	24	Iduronidase, alpha-L-	Hs.89560
TACAGTATGT	<0.00001	207	9	23	Glutamate-ammonia ligase (glutamine synthase)	Hs.518525
GCAAAAGAAA	<0.00001	23	1	23	Glutamate-ammonia ligase (glutamine synthase)	Hs.518525
GATTTCTTTG	<0.00001	21	0	>21	glypican 3	Hs.435036
TGATATAAAT	<0.00001	21	0	>21	Cytochrome P450, family 3, subfamily A, polypeptide 4	Hs.442527
TCCCCGCTAC	<0.00001	21	0	>21	no reliable match	
TAAAAAAGGT	<0.00001	19	1	19	multiple match	
GCAAAATATC	<0.00001	31	2	15.5	Liver cancer associated non coding mRNA, partial sequence	Hs.214343
GATCACTGCT	<0.00001	30	2	15	multiple match	
CTTCAGAGAA	<0.00001	15	1	15	Oxoglutarate dehydrogenase-like	Hs.17860
GGGTTTTTAT	<0.00001	15	1	15	multiple match	
GCTTATGTTA	<0.00001	15	0	>15	multiple match	
CTATACTTTG	<0.00001	14	1	14	Aldehyde dehydrogenase 5 family, member A1	Hs.371723
TGCTACTGGT	<0.00001	14	1	14	Surfeit 1	Hs.512464
GGCCCAGGAC	<0.00001	14	0	>14	multiple match	
GCTGCGGTCC	<0.00001	14	1	14	multiple match	
GATCTCCTGC	<0.00001	14	1	14	multiple match	
ATGCTCCCTG	<0.00001	14	0	>14	multiple match	
TTACTTATAC	<0.00001	14	0	>14	multiple match	
TCTTCAACAA	<0.00001	14	0	>14	multiple match	
CCAGGGGAGA	<0.00001	38	3	12.66667	interferon, alpha inducible protein 27	Hs.532634
TCACTTTCAT	<0.00001	12	1	12	Dehydrogenase E1 and transketolase domain containing 1	Hs.104980
AGAGCGCAGC	<0.00001	12	1	12	crystallin, alpha A	Hs.184085
CCCGGCTCTT	<0.00001	12	0	>12	Platelet-activating factor acetylhydrolase, isoform Ib, beta subunit 30kDa	Hs.188501
AAGCTAAGTC	<0.00001	12	1	12	transmembrane protein 7	Hs.196584
GTGATGAGCT	<0.00001	12	1	12	carboxylesterase 2 (intestine, liver)	Hs.282975
GTGCACTGTG	<0.00001	12	0	>12	Testis enhanced gene transcript (BAX inhibitor 1)	Hs.35052
AGCCTGCAGA	<0.00001	12	1	12	Chromosome 19 open reading frame 10	Hs.465645
GAGCTCCACA	<0.00001	12	1	12	Protein kinase (cAMP-dependent, catalytic) inhibitor gamma	Hs.472831
CTGTTATAGG	<0.00001	12	0	>12	YME1 like 1 (S. cerevisiae)	Hs.499145

TTGTGCACGT	<0.00001	12	1	12	CDNA FLJ45284 fis, clone BRHIP3001964	Hs.514273
CAGCTCCGCT	<0.00001	12	0	>12	DUTP pyrophosphatase	Hs.527980
TTTTTAATGT	<0.00001	12	1	12	H3 histone, family 3A	Hs.533624
GCGTCGGGGA	<0.00001	12	1	12	interferon (alpha, beta and omega) receptor 2	Hs.549042
ACATTCTTGT	<0.00001	12	1	12	Transcribed locus, strongly similar to XP_496055.1 similar to	Hs.76704
CTGCTGTAAT	<0.00001	12	0	>12	multiple match	
TTATCAAAAA	<0.00001	12	1	12	multiple match	
TATCACTCTG	<0.00001	12	0	>12	multiple match	
GTAGGTTGTC	<0.00001	12	1	12	multiple match	
TGTGGTGGTG	<0.00001	12	1	12	multiple match	
CCAGCTGCCA	<0.00001	12	1	12	multiple match	
TAAATACAGT	<0.00001	12	0	>12	multiple match	
CTGTGTCTGT	<0.00001	12	1	12	multiple match	
TGATGGCGCA	<0.00001	12	0	>12	no reliable match	
CACCTAATTA	<0.00001	12	1	12	no reliable match	
TCCACTATTA	<0.00001	12	0	>12	no reliable match	
CTAAATCTCG	<0.00001	12	0	>12	no reliable match	
TGGTGTATGC	<0.00001	35	3	11.66667	Tag matches mitochondrial sequence	
TCCTCTCTGT	<0.00001	23	2	11.5	Solute carrier family 38, member 3	Hs.76460
TTAACCCTCT	0.00016	10	0	>10	H3 histone, family 3B (H3.3B)	Hs.180877
ATGACTTAGG	0.00113	10	1	10	UDP glycosyltransferase 2 family, polypeptide B10	Hs.201634
TGACTACTGA	0.00109	10	1	10	apolipoprotein L, 6	Hs.257352
TTAGGCCCTC	0.00111	10	1	10	Erythrocyte membrane protein band 4.1 like 4B	Hs.269180
TTATTATCAC	0.00112	10	1	10	factor H related protein 5	Hs.282594
CCTGTCCAGC	0.00105	10	1	10	BCL2 like 1	Hs.305890
AGAACTTCCT	0.00102	10	1	10	defensin, beta 1	Hs.32949
GCCAAGTTTG	0.00017	10	0	>10	proteasome (prosome, macropain) 26S subunit, non ATPase, 1	Hs.3887
TAATTACTCT	0.00016	10	0	>10	13kDa differentiation associated protein	Hs.44163
ATTTCTTGCC	0.00103	10	1	10	Mitochondrial ribosomal protein L38	Hs.442609
ACACAGTTTT	0.00014	10	0	>10	FAT tumor suppressor homolog 1 (Drosophila)	Hs.481371
ACAAGTACCC	0.00108	10	1	10	Chromosome 5 open reading frame 13	Hs.483067
TGGAGAGCAA	0.00107	10	1	10	S-adenosylhomocysteine hydrolase-like 1	Hs.485365
GAGGTGAAGG	0.00015	10	0	>10	KIAA1914	Hs.501106
GCTGGAGGAG	0.00016	10	0	>10	Transcribed locus	Hs.520115
ACCAGCAAAT	0.00017	10	0	>10	phosphoinositide 3 kinase, regulatory subunit, polypeptide 1 (p85 alpha)	Hs.6241
CTGCCCGCCT	0.00014	10	0	>10	multiple match	

GCTTGAATAA	0.00015	10	0	>10	multiple match
GTAAGATTTG	0.0011	10	1	10	multiple match
CCCGCCCCCG	0.00106	10	1	10	multiple match
CAAGGATCTA	0.00014	10	0	>10	multiple match
GAAACTGAAG	0.00014	10	0	>10	multiple match
CAGACTATGT	0.00115	10	1	10	multiple match
ATTACACCAC	0.00015	10	0	>10	multiple match
AACAGAAGCA	0.0011	10	1	10	multiple match
GAAATCCAAA	0.00013	10	0	>10	multiple match