

Figure 7. EpCAM blockage inhibits the tumorigenic and invasive capacity of EpCAM⁺ HCC cells. (A) Enrichment of EpCAM⁺ cells after 5-FU treatment. HuH1 cells refer as control or without treatment (green) or treated with 2 μ g/mL of 5-FU (orange) for 3 days and analyzed by FACS using anti-EpCAM and anti-CD133 antibodies. (B) Spheroid formation of HuH1 cells treated with 2 μ g/mL of 5-FU for 3 days. (C) FACS analysis of HuH1 cells treated with a control siRNA (orange) or EpCAM-specific siRNA (green) at day 3 after transfection. (D) Spheroid formation or (E) invasive capacity of EpCAM⁺ HuH1 cells transfected with a control siRNA or EpCAM-specific siRNA. Experiments were performed in triplicate and the data are shown as mean \pm SD. (D) siRNAs. (F) Inhibition of tumor formation in vivo by EpCAM gene silencing. EpCAM⁺ HuH1 cells were transfected with siRNA oligos and 1000 cells were injected 24 hours after transfection.

BASIC-LIVER, PANCREAS, AND BILIARY TRACT

comprehensively investigate the expression patterns of stem cell markers to characterize the population of CSCs that may correlate with the activation of their distinct molecular pathways.

CSCs may be more resistant to chemotherapeutic agents than differentiated tumor cells possibly owing to an increased expression of adenosine triphosphate-binding cassette transporters and anti-apoptotic proteins.⁴ Thus, the development of an effective strategy to target CSC pools together with conventional chemotherapies is essential to eradicate a tumor mass.¹⁴ By blocking the programs that activate self-renewal and/or inhibit asymmetric division, CSC features could be destemmed.^{46,47} Consistently, EpCAM blockage could inhibit cellular invasion and tumorigenicity of EpCAM⁺ HCC cells, revealing the feasibility of targeting a CSC marker to destem CSC features. EpCAM may induce c-Myc,⁴⁸ a common molecular node activated in HpSC-HCC.²⁷ c-Myc, together with Oct3/4, Sox2, and Klf4, can induce pluripotent stem cells from adult fibroblasts.⁴⁹ It is possible that EpCAM blockage to inhibit hepatic CSCs may

result in a suppression of c-Myc signaling. Encouragingly, EpCAM-specific antibodies are currently in phase II clinical trials.⁵⁰ Furthermore, a recent study indicated that EpCAM⁺ circulating tumor cells identified by a unique microfluidic platform can be used to monitor outcomes of patients undergoing systemic treatment.⁵¹ Therefore, it may be useful to combine EpCAM antibodies with conventional chemotherapy to target both CSCs and non-CSCs for the treatment of HCC.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.12.004.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

FACS and MACS Analyses

Cultured cells were trypsinized, washed, and re-suspended in Hank's balanced salt solutions (Lonza, Basel, Switzerland) supplemented with 1% HEPES and 2% fetal bovine serum. Cells then were incubated with FITC-conjugated anti-EpCAM monoclonal antibody Clone Ber-EP4 (DAKO, Carpinteria, CA) on ice for 30 minutes, and EpCAM⁺ and EpCAM⁻ cells were isolated by a BD FACSAria cell sorting system (BD Biosciences). For magnetic separation, cells were labeled 24 hours after enzymatic dissociation with primary EpCAM antibody (mouse IgG1; Dako), subsequently magnetically labeled with rat anti-mouse IgG1 Microbeads, and separated on a MACS LS column (Miltenyi Biotec, Inc, Auburn, CA). All the procedures were performed according to the manufacturer's instructions. The purity of sorted cells was evaluated by FACS. Fixed cells also were analyzed by FACS using a FACSCalibur (BD Biosciences). Anti-EpCAM antibody VU-1D9, anti-CD133/2 clone 293C3 (Miltenyi Biotec Inc), and anti-CD90 clone 5E10 (Stem-Cell Technologies Inc, Vancouver, British Columbia, Canada) were used to detect EpCAM⁺, CD133⁺, or CD90⁺ cells. Intracellular AFP levels were examined by a BD Cytofix/Cytoperm Fixation/Permeabilization Kit (San

Jose, CA) and anti-AFP rabbit polyclonal antibody (DAKO).

Quantitative Reverse Transcription–Polymerase Chain Reaction and IHC Analyses

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The expression of selected genes was determined in triplicate using the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described.¹ Genes expressed in embryonic stem cells were determined in quadruplicate using TaqMan Human Stem Cell Pluripotency Array (Applied Biosystems). IHC analyses with specific antibodies were performed essentially as previously described.¹ Confocal fluorescence microscopic analysis was performed essentially as previously described.²

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Supplementary Table 1. Clinicopathologic Characteristics of HpSC-HCC and MH-HCC Cases Used for Oligonucleotide Microarray Analyses

Parameters	HpSC-HCC (n = 60)	MH-HCC (n = 96)	P value ^a
Mean age, y (SD)	46.0 ± 10.7	52.9 ± 10.5	.0004
Sex: male/female	50/10	87/9	.18
Cirrhosis: yes/no/no data	56/4	88/7/1	.72
Median AFP level, ng/mL (25%–75%)	1706 (865–5915)	11.8 (4.0–48.6)	<.0001
Histologic grade ^b			
I–II	14	41	
II–III	44	48	
III–IV	2	5	
No data	0	2	.031
Mean tumor size, cm (SD)	5.1 ± 3.0	4.4 ± 3.0	.088
Multinodular: yes/no	16/44	15/81	.09
Portal vein invasion, yes/no ^c	11/49	9/87	.10
TNM classification			
I	24	46	
II	22	42	
III	14	8	.03
Virus status: HBV/HBV + HCV/unknown	56/4/0	95/0/1	.43

^aMann–Whitney *U* test or χ^2 test.^bEdmondson–Steiner.^cMacroscopic portal vein invasion.**Supplementary Table 2.** Clinicopathologic Characteristics of HpSC-HCC and MH-HCC Cases Used for IHC

Parameters	HpSC-HCC (n = 24)	MH-HCC (n = 55)	P value ^a
Mean age, y (SD)	46.4 ± 9.4	58.4 ± 11.9	<.0001
Sex: male/female	20/4	48/7	.64
Cirrhosis: yes/no	23/1	46/9	.14
Median AFP level, ng/mL (25%–75%)	1620 (887–3166)	12 (9.3–219)	<.0001
Histologic grade ^b			
I–II	12	32	
II–III	8	21	
III–IV	4	2	.13
Mean tumor size, cm (SD)	7.1 ± 3.6	5.2 ± 3.6	.014
Multinodular: yes/no	4/20	16/39	.24
Portal vein invasion: yes/no ^c	12/12	12/43	.012
TNM classification			
I	4	19	
II	8	20	
III	12	16	.14
Virus status: HBV/HCV/unknown	21/2/1	32/21/2	.026

^aMann–Whitney *U* test or χ^2 test.^bEdmondson–Steiner.^cMacroscopic portal vein invasion.

Supplementary Table 3. Top 10 List of Canonical Pathways Activated in HpSC-HCC From Ingenuity Pathway Analysis

Pathways	Genes included in cluster A
Axonal guidance signaling	
Up	ROBO2, ARPC5L (includes EG:81873), SEMA4G, PDGFRB, PLCB1, PRKCD, FGFR3, FZD5, MERTK, DDR1, LINGO1, SEMA3C
Down	PIK3C3, IGF1, PIK3C2G, MAP2K2, ARHGEF15
Transforming growth factor- β signaling	
Up	PDGFRB, FGFR3, MERTK, UBD, DDR1, SMAD5
Down	MAP2K2, HNF4A
Integrin signaling	
Up	ARPC5L (includes EG:81873), PDGFRB, FGFR3, GRB7, MERTK, ITGB5, DDR1, DDEF1
Down	PIK3C3, MYLK, PIK3C2G, MAP2K2
Apoptosis signaling	
Up	PDGFRB, BAK1, CYCS, FGFR3, MERTK, DDR1
Down	MAP3K5, MAP2K2
G2/M DNA damage checkpoint regulation	
Up	YWHAZ, CCNB2, UBD, WEE1
Down	CDKN2A, GADD45A
ERK/MAPK signaling	
Up	ELF3, PDGFRB, YWHAZ, PRKCD, FGFR3, MERTK, DDR1
Down	PIK3C3, DUSP1, PIK3C2G, ESR1, MAP2K2
Wnt/ β -catenin signaling	
Up	DKK1, SOX9, FZD5, UBD, TCF7L2, CSNK1E
Down	CDKN2A, RARG
PI3K/AKT signaling	
Up	PDGFRB, YWHAZ, FGFR3, MERTK, DDR1
Down	MAP3K5, MAP2K2, GYS2
Amyloid processing	
Up	BACE2, CSNK1E, MAPK13
Down	
Leukocyte extravasation signaling	
Up	PRKCD, CLDN4, CLDN1, MMP11, MAPK13
Down	PIK3C3, CLDN2, PIK3C2G, MAP2K2

NOTE. The top 10 pathways were selected based on the significance for the enrichment of the genes with a particular canonical signaling pathway determined by the one-sided Fisher exact test ($P < .01$).

Supplementary Table 4. Top 10 List of Canonical Pathways Activated in MH-HCC From Ingenuity Pathway Analysis

Pathways	Genes included in cluster B
Lipopolysaccharide/interleukin-1-mediated inhibition of RXR function	
Up	SULT1C2, ACSL4, ACSL3, FABP5, GSTP1
Down	NR1I2, NR1I3, CYP7A1, ALDH1L1, ABCB1, SLC10A1, SLC27A2, CD14, GSTM1, ALDH6A1, GSTM4, ACSL5, CES2 (includes EG:8824), FMO3, SULT2A1 (includes EG:6822), GSTA1, CYP2C8, LC27A5, CYP3A7, ABCG5, ALDH8A1, APOC4 (includes EG:346), CYP3A4, ACSL1, ABCB11, FMO4, MAOA
Xenobiotic metabolism signaling	
Up	SULT1C2, PRKCD, GSTP1, MAPK13
Down	NR1I2, NR1I3, ALDH1L1, ABCB1, UGT2B15, MAP2K2, UGT2B7, PPARGC1A, GSTM1, PIK3C3, ALDH6A1, GSTM4, CES2 (includes EG:8824), MAP3K5, FMO3, PIK3C2G, SULT2A1 (includes EG:6822), CYP1A2, GSTA1, CYP2C8, CYP3A7, NQO2, ALDH8A1, CYP3A4, CES1 (includes EG:1066), FMO4, MAOA
Hepatic cholestasis	
Up	ADCY3, PRKCD
Down	CD14, ABCG5, NR1I2, CYP7A1, CYP7B, CYP8B1, ABCB1, ESR1, SLC10A1, ABCB11, ABCB4, HNF4A
Aryl hydrocarbon receptor signaling	
Up	GSTP1
Down	CDKN2A, NQO2, GSTM1, ALDH8A1, ALDH6A1, ALDH1L1, GSTM4, ESR1, CYP1A2, GSTA1, RARG
NRF2-mediated oxidative stress response	
Up	DNAJA4, PRKCD, GSTP1
Down	NQO2, GSTM1, AOX1, PIK3C3, GSTM4, MAP3K5, SOD1, PIK3C2G, MAP2K2, FKBP5, GSTA1
Complement system	
Up	
Down	C8A, C1R, MASP1, C6, C8B, MASP2
Coagulation system	
Up	
Down	SERPINC1, KLKB1, F9, KNG1 (includes EG:3827), F11
Acute-phase response signaling	
Up	MAPK13
Down	APCS, RBP5, C1R, MAP3K5, HRG, MAP2K2, KLKB1, SAA4
p53 signaling	
Up	THBS1
Down	CDKN2A, PIK3C3, SNAI2, GADD45A, PIK3C2G, GADD45B
LXR/RXR activation	
Up	HMGCR
Down	CD14, ABCG5, APOA5, CYP7A1, APOC4 (includes EG:346)

LXR/RXR, liver X receptor/retinoid X receptor; NRF2, NF-E2-related factor 2.

NOTE. The top 10 pathways were selected based on the significance for the enrichment of the genes with a particular canonical signaling pathway determined by the one-sided Fisher exact test ($P < .01$).

Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma[☆]

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Background/Aims: Metabolic dysregulation is one of the risk factors for the development of hepatocellular carcinoma (HCC). We investigated the activated metabolic pathway in HCC to identify its role in HCC growth and mortality.

Methods: Gene expression profiles of HCC tissues and non-cancerous liver tissues were obtained by serial analysis of gene expression. Pathway analysis was performed to characterize the metabolic pathway activated in HCC. Suppression of the activated pathway by RNA interference was used to evaluate its role in HCC *in vitro*. Relation of the pathway activation and prognosis was statistically examined.

Results: A total of 289 transcripts were up- or down-regulated in HCC compared with non-cancerous liver ($P < 0.005$). Pathway analysis revealed that the lipogenic pathway regulated by sterol regulatory element binding factor 1 (*SREBF1*) was activated in HCC, which was validated by real-time RT-PCR. Suppression of *SREBF1* induced growth arrest and apoptosis whereas overexpression of *SREBF1* enhanced cell proliferation in human HCC cell lines. *SREBF1* protein expression was evaluated in 54 HCC samples by immunohistochemistry, and Kaplan–Meier survival analysis indicated that *SREBF1*-high HCC correlated with high mortality.

Conclusions: The lipogenic pathway is activated in a subset of HCC and contributes to cell proliferation and prognosis. © 2008 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Keywords: Hepatocellular carcinoma; Serial analysis of gene expression; Lipogenesis; Gene expression profiling; Sterol regulatory element binding factor 1

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Abbreviations: HCC, hepatocellular carcinoma; *SREBF1*, sterol regulatory element binding factor 1; HBV, hepatitis B virus; HCV, hepatitis C virus; SAGE, serial analysis of gene expression; RT-PCR, reverse transcription-polymerase chain reaction; IHC, immunohistochemistry; FADS1, fatty acid desaturase 1; SCD, stearoyl CoA desaturase; FASN, fatty acid synthase; si-RNA, short interfering-RNA; CLD, chronic liver disease; PCNA, proliferating cell nuclear antigen; IGF, insulin-like growth factor.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently occurring malignancies in the world [1]. The major risk factors associated with HCC include chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol abuse, and exposure to aflatoxin B1 [2]. HCC usually develops from liver cirrhosis, which involves continuous inflammation and hepatocyte regeneration, suggesting that reactive oxygen species and DNA damage are involved in the process of hepatocarcinogenesis [3].

The development of gene expression profiling technologies including DNA microarrays and serial analysis

of gene expression (SAGE) have enhanced our ability to identify inventory transcripts and global genetic alterations in HCC [4–10]. In general, these methods have demonstrated that transcripts associated with cell growth are up-regulated, whereas those related to inhibition of cell growth are down-regulated, in HCC [11]. It is difficult, however, to decipher molecular pathways activated during hepatocarcinogenesis.

Epidemiological studies suggest that metabolic dysregulation in the liver increases the risk of HCC development. For example, diabetes is associated with a 2-fold increase in the risk of HCC [12]. Obesity and hepatic steatosis also increase the risk of HCC [13–15]. Furthermore, recent studies indicate that HCV infection provokes hepatic steatosis, which may be a vulnerable factor for liver inflammation and HCC development [16,17]. Thus, dysregulation of a metabolic pathway may play a crucial role to promote HCC growth, but the molecular mechanism is still obscure. In this study, we have utilized SAGE [18,19], which enables us to monitor the differential expression of all genes, to determine the global changes in gene expression that occur during hepatocarcinogenesis.

2. Materials and methods

2.1. Tissue samples

All HCC tissues, adjacent non-cancerous liver tissues, and normal liver tissues were obtained from 69 patients who underwent hepatectomy from 1997 to 2005 in Kanazawa University Hospital. Normal liver tissue samples were obtained from patients undergoing surgical resection of the liver for treatment of metastatic colon cancer. HCC and surrounding non-cancerous liver samples were obtained from patients undergoing surgical resection of the liver for the treatment of HCC. The samples used for SAGE, real-time reverse-transcription (RT)-PCR analysis, and immunohistochemistry (IHC) are listed in Supplemental Table 1. All samples used for SAGE and real-time RT-PCR analysis were snap-frozen in liquid nitrogen. Four normal liver tissues and 20 HCCs and their corresponding non-cancerous liver tissues were used for real-time RT-PCR analysis; seven of these HCC samples, along with 47 additional HCC samples, were formalin-fixed paraffin-embedded and used for IHC. HCC and adjacent non-cancerous liver were histologically characterized as described [20].

All strategies used for gene expression analysis as well as tissue acquisition processes were approved by the Ethics Committee and the Institutional Review Board of Kanazawa University Hospital. All procedures and risks were explained verbally, and each patient provided written informed consent.

2.2. SAGE

Total RNA was purified from each homogenized tissue sample using a ToTally RNA extraction kit (Ambion, Inc., Austin, TX), and polyadenylated RNA was isolated using a MicroPoly (A) Pure kit (Ambion). A total of 2.5 µg mRNA per sample was analyzed by SAGE [18]. SAGE libraries were randomly sequenced at the Genomic Research Center (Shimadzu-Biotechnology, Kyoto, Japan), and the sequence files were analyzed with SAGE 2000 software. The size of each SAGE library was normalized to 300,000 transcripts per library, and the abundance of transcripts was compared by SAGE 2000 soft-

ware. Monte Carlo simulation was used to select genes with significant differences in expression between two libraries without multiple hypothesis testing correction ($P < 0.005$) [21]. Each SAGE tag was annotated using a gene-mapping web site (<http://www.ncbi.nlm.nih.gov/SAGE/index.cgi>).

2.3. Analysis of signaling networks

Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com) was used to investigate the molecular pathways activated in an HCC SAGE library compared with an adjacent non-cancerous liver SAGE library. All reliable transcripts statistically up-regulated in HCC were investigated and annotated with biological processes, protein-protein interactions, and gene regulatory networks, using a reference-based data file with statistical significance. All identified pathways were screened individually. MetaCore™ software (GeneGo Inc., St. Joseph, MI) was used to evaluate candidate transcription factors responsible for up-regulation of transcripts in HCC.

2.4. RT-PCR

A 1-µg aliquot of each total RNA was reverse-transcribed using SuperScript II reverse-transcriptase (Invitrogen, Carlsbad, CA). Real-time RT-PCR analysis was performed using ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Using the standard curve method, quantitative PCR was performed in triplicate for each sample-primer set. Each sample was normalized relative to β-actin. The assay IDs used were Hs00231674_m1 for sterol regulatory element binding factor 1 (*SREBF1*); Hs00203685_m1 for fatty acid desaturase 1 (*FADS1*); Hs00748952_s1 for stearoyl CoA desaturase (*SCD*); Hs00188012_m1 for fatty acid synthase (*FASN*); and Hs99999_m1 for β-actin. *SREBF1a* and *SREBF1c* mRNA levels were assayed by semi-quantitative RT-PCR [22].

2.5. RNA Interference targeting *SREBF1*

Si-RNAs targeting *SREBF1* were constructed using a Silencer™ SiRNA Construction kit (Ambion) according to the manufacturer's protocol. We constructed two different si-RNAs, targeting different sites of *SREBF1* (*SREBF1*-1; CAGTGGCACTGACTCTCC, *SREBF1*-2; TCTACGACCAGTGGGACTG). Control si-RNA duplexes targeting scramble sequences were also synthesized (Dharmacon Research, Inc., Lafayette, CO). Lipofectamine 2000™ reagent (Invitrogen) was used for transfection according to the manufacturer's instructions.

2.6. Cell proliferation assay

Cell proliferation assays were performed using a Cell Titer96 Aqueous kit (Promega, Madison, WI). Results are expressed as the mean optical density (OD) of each five-well set. All experiments were repeated at least twice.

2.7. Soft agar assay

To each well of a six-well plate, containing a base layer of 0.72% agar in growth medium, was added 1×10^4 cells, suspended in 2 ml of 0.36% agar with growth medium (DMEM supplemented with 10% FBS), and the plates were incubated at 37 °C in a 5% CO₂ incubator for 2 weeks. The numbers of colonies in each well were counted as previously described [23].

2.8. TUNEL assay

A DeadEnd™ Colorimetric TUNEL System (Promega) was used to measure nuclear DNA fragmentation as described previously [24].

2.9. Annexin V staining

To evaluate apoptotic cell death, Annexin V binding to cell membranes was evaluated using Annexin V-FITC antibodies and FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ), as described by the manufacturer.

2.10. Focus assay

HuH7 cells and Hep3B cells were transiently transfected with pCMV7 or pCMV7-*SREBF1c* vectors (kindly provided by Dr. Hitoshi Shimano) using Lipofectamine 2000TM reagent (Invitrogen), as described by the manufacturer. A total of 2×10^3 cells were seeded on six-well plates 48 h after transfection, and cultured in usual media with 400 ng/ml of Geneticin for 9 days. The foci were fixed with ice-cold 100% methanol and stained with 0.5% crystal violet solution. All experiments were performed in triplicates.

2.11. Western blotting

Whole cell lysates were prepared using RIPA lysis buffer. Antibodies used were rabbit polyclonal antibodies to phospho-GSK-3 β (ser9) (Cell Signaling Technology Inc., Danvers, MA), rabbit anti-sterol regulatory element binding protein-1 (encoded by *SREBF1*) polyclonal antibody H-160 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and β -actin (Sigma-Aldrich Japan K.K., Tokyo, Japan). Immune complexes were visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ) as described in the manufacturer's protocol.

2.12. Immunohistochemistry

Rabbit anti-*SREBF1* polyclonal antibody H-160 (Santa Cruz Biotechnology, Inc.) and mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody PC10 (Calbiochem, San Diego, CA) were used to evaluate the immunoreactivity of HCC samples, using a DAKO EnVision+TM Kit, as described by the manufacturer. The signal intensity of *SREBF1* was scored as negative, low, or high determined by the representative staining of the normal liver tissue and cirrhotic liver tissue (Supplemental Fig. 1). HCC was referred as *SREBF1*-high if *SREBF1* expression in the tumor was higher than that in the cirrhotic liver tissue. PCNA index was evaluated as previously described [25].

2.13. Statistical analysis

Kruskal–Wallis test was used to compare the differentially expressed genes, as shown by real-time PCR, among normal liver, CLD, and HCC tissues. Mann–Whitney U test was also used to evaluate the statistical significance of differences of gene expression between CLD and HCC tissues. Spearman's correlation coefficient was used to assess correlations between the expression levels of *SREBF1*, *FADS1*, *SCD*, and *FASN*. Univariate Cox proportional hazards regression analysis was used to evaluate the association of gene expression and clinicopathologic parameters with patient outcomes. All statistical analyses were performed using SPSS software (SPSS software package; SPSS Inc., Chicago, IL) and GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Gene expression profiling of HCC

We constructed two SAGE libraries from a HCC–HBV tissue and a corresponding non-cancerous tissue (chronic liver disease (CLD)–HBV). We also used two

previously described SAGE libraries, from an HCC–HCV sample and a corresponding non-cancerous tissue sample (CLD–HCV) [4]. After excluding tags detected only once in each library, to avoid the contamination of tags derived from sequence errors, we selected 105,288 tags corresponding to the 9731 genes in all libraries. Using Monte Carlo simulation, we compared the differentially expressed transcripts in HCC and corresponding CLD libraries. Compared with their corresponding CLD libraries, there were statistically significant increases or decreases in 140 transcripts in the HCC–HBV library and in 197 transcripts in the HCC–HCV library ($P < 0.005$).

The HCC–HBV library contained one SAGE tag encoding the HBV–X region, which was increased more than 35-fold compared with its expression in the corresponding CLD–HBV library (Supplemental Table 2). We identified two additional SAGE tags, encoding unknown genes (GTTCTAAAGG, GCATTATGAT), which were expressed more than 10-fold in the HCC–HBV library than in the corresponding CLD–HBV library. The HCC–HBV library also contained tags associated with lipogenesis, at greater than 10-fold abundance, in the HCC–HBV library; these including tags for steroyl-CoA desaturase, fatty acid synthase, and fatty acid desaturase 1.

In contrast, SAGE tags associated with the immune response were up-regulated in the HCC–HCV library. These included tags for Th1-type chemokines, including chemokine ligand 10 (C–X–C motif), chemokine ligand 9 (C–X–C motif), and major histocompatibility complex classes IA and IB (Supplemental Table 3). In addition, tags associated with lipogenesis were increased in the HCC–HCV library, including tags for 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 and cytochrome P450, family 51, subfamily A, polypeptide 1. Taken together, the differential gene expression patterns may exist in HCC–HBV and HCC–HCV. HBV–X and lipogenesis-related genes are activated in HCC–HBV, whereas genes associated with inflammation as well as lipogenesis are activated in HCC–HCV.

3.2. Analysis of molecular pathways activated in HCC

To further characterize the gene expression patterns of HCC–HBV and HCC–HCV, we performed pathway analysis on SAGE data. Using MetaCoreTM software, we found that the candidate transcription factors activated were distinct in each HCC library (Table 1). Several of these transcription factors, including NF- κ B, c-Myc, c-Jun, and HNF4- α , have been reported to be activated in HCC [26–29]. In addition, our findings indicated that the transcription factor *SREBF1* may be activated in both HCC–HBV and HCC–HCV (to avoid a confusion, we use HUGO symbol *SREBF1* to indicate both gene/protein name).

Table 1
Candidate transcription factors that regulate molecular pathways activated in HCC.

SAGE library	Transcription factor	Molecular processes	P-value	
HCC–HCV	NF- κ B	Antigen presentation	0.004	
		Antigen processing		
		Defense response		
		Immune response		
	<i>SREBF1</i>	Cholesterol biosynthesis		0.05
		Lipid biosynthesis		
		β -Glucoside transport		
	SP1	Negative regulation of lipoprotein metabolism		0.05
		Electron transport; drug metabolism		
	IRF1	Oxygen and reactive oxygen species metabolism		0.05
Cell-substrate junction assembly; wound healing				
Immune response				
HCC–HBV	HNF4- α	Antigen presentation; antigen processing	0.002	
		Defense response; positive regulation of cell		
	HNF1	Lipid transport		0.01
		Fatty acid metabolism		
		Smooth muscle cell proliferation		
	SP1	Acute-phase response; lipid transport		0.01
		Negative regulation of lipid catabolism		
		β -Glucoside transport		
	c-Jun	Negative regulation of lipoprotein metabolism		0.03
		Zinc ion homeostasis; response to biotic stimulus		
	C/EBP- α	Nitric oxide mediated signal transduction		0.03
		Copper ion homeostasis; fatty acid biosynthesis		
	<i>SREBF1</i>	Progesterone catabolism; progesterone metabolism		0.03
		Regulation of lipid metabolism;		
		Prostaglandin metabolism		
	c-Myc	Lipid transport; negative regulation of lipid catabolism		0.03
		Negative regulation of lipoprotein metabolism		
		β -Glucoside transport		
	USF1	Positive regulation of interleukin-8 biosynthesis		0.03
Lipid biosynthesis; fatty acid biosynthesis				
Fatty acid metabolism				
PPAR- α	Negative regulation of lipid catabolism	0.03		
	Negative regulation of lipoprotein metabolism			
COUP-TFI	Fatty acid biosynthesis; fatty acid metabolism	0.03		
	Fatty acid desaturation;			
C/EBP- β	Activation of pro-apoptotic gene products	0.03		
	Release of cytochrome c from mitochondria			
	Fatty acid metabolism			
C/EBP- β	Smooth muscle cell proliferation	0.03		
	Fatty acid metabolism			
	Smooth muscle cell proliferation			
C/EBP- β	Lipid transport	0.03		
	Smooth muscle cell proliferation			
	Acute-phase response			
C/EBP- β	Regulation of interleukin-6 biosynthesis	0.03		
	Fat cell differentiation			
	Inflammatory response			

These findings were evaluated by other pathway analysis software, Ingenuity Pathways Analysis (IPA). We applied the signaling network analysis to the transcripts up-regulated in the HCC libraries ($P < 0.005$). We found that the top signaling network activated in HCC–HBV contained several pathways involved in ERK/MAPK signaling, PPAR signaling, linoleic acid metabolism, and fatty acid metabolism (Supplemental Fig. 2A). Similarly, pathways involved in interferon signaling, NF- κ B signaling, antigen presentation, PPAR signaling, linoleic

acid metabolism, and fatty acid metabolism were included in the top signaling network activated in HCC–HCV (Supplemental Fig. 2B). Consistent with the results of transcription factor analysis by MetaCoreTM, pathway analysis indicated that *SREBF1* participates in the lipogenesis pathway in both HCC–HBV and HCC–HCV (blue nodes in Supplemental Fig. 2A and B). *SREBF1*, a major regulator of the lipogenesis pathway, binds to sterol regulatory elements on the genome [30], but less is known about its role in

HCC [31]. We therefore focused on the role of *SREBF1* signaling in HCC.

3.3. Validation of SAGE and signaling network analysis

We performed real-time RT-PCR analysis of *SREBF1* and three representative target genes (*SCD*, *FADS1*, and *FASN*) [20] on 44 samples not used for SAGE. We found that the levels of *SREBF1*, *SCD*, and *FASN* mRNAs were higher in HCC tissues and CLD tissues compared with normal liver, and that these differences were statistically significant (Fig. 1A). We further compared the expression of *SREBF1*, *FADS1*, and *FASN* between HCC and non-cancerous liver tissues, and identified the overexpression of *SREBF1* in HCC with statistical significance (Supplemental Fig. 3). Scatter plot analysis showed that the expression levels of *SREBF1* were correlated with those of *FADS1* ($R = 0.57$, $P < 0.0001$), *SCD* ($R = 0.82$, $P < 0.0001$), and *FASN* ($R = 0.74$, $P < 0.0001$) (Fig. 1B).

Since the mammalian genome encodes two *SREBF1* isoforms, *SREBF1a* and *SREBF1c* [22], we performed semi-quantitative RT-PCR with isoform specific primers to determine which of these isoforms was up-regulated in HCC. We found that *SREBF1c* mRNA, but not *SREBF1a* mRNA, was up-regulated in HCC compared with adjacent non-cancerous liver and normal liver tissues (Supplemental Fig. 4A).

3.4. Functional assay of the lipogenesis pathway in cell lines

Although genome-wide expression profiling showed that the lipogenesis pathway was activated in HCC possibly through up-regulation of *SREBF1*, it was not clear that this pathway played a role in HCC growth. To investigate the role of lipogenesis in HCC cell proliferation, we transfected two short interfering (si)-RNAs (*SREBF1-1* and *SREBF1-2*) targeting *SREBF1* into the HuH7 and Hep3B cells. These cell lines have no chromosome amplification or deletion on 17p11, on which *SREBF1* is located [32]. Transfection of the si-RNA constructs for *SREBF1-1* or *SREBF1-2* decreased expression of *SREBF1* 90% and 70%, respectively, and the expression of both *SCD* and *FADS1* 70% and 60%, respectively (Fig. 2A). Because differences in *SREBF1c* and *SREBF1a* sequence alignments are very small, we could not design si-RNAs specifically targeting *SREBF1c*. We therefore checked the effect of si-RNAs on the expression of the *SREBF1* isoforms. We found that the expression of *SREBF1c* was relatively more suppressed than that of *SREBF1a* (Supplemental Fig. 4B), which may have been associated with the higher expression of *SREBF1a* than *SREBF1c* in cultured cell lines [25].

We found that the growth of these transfected cells was significantly inhibited at 72 h compared with mock transfected cells (Fig. 2B and Supplemental Fig. 5A). Examination of anchorage independent cell growth showed strong suppression by deactivation of the lipogenesis pathway (Fig. 2C). Because insulin-like growth factor (IGF) is known to induce cancer cell proliferation through activation of PI3-kinase signaling followed by *SREBF1* induction, we investigated the effect of *SREBF1* knockdown on IGF2 mediated cell proliferation. Interestingly, *SREBF1* knockdown abrogated the IGF2 dependent cell proliferation (Supplemental Fig. 5B). Moreover, both the TUNEL assay and annexin V staining showed that transfection of *SREBF1* si-RNAs increased apoptosis compared with mock transfected cells (Fig. 2D and E).

We further investigated the role of *SREBF1* overexpression on cell growth *in vitro*. We transiently transfected control pCMV7 plasmids or pCMV7-*SREBF1c* plasmids (Fig. 3A), and cell proliferation was enhanced in *SREBF1* overexpressing cells compared with the control in both HuH7 and Hep3B cells evaluated by focus assay (Fig. 3B and supplemental Fig. 6). Furthermore, overexpression of *SREBF1* intensified the phosphorylation of GSK-3 β , one of the major kinase phosphorylated by the activation of IGF signaling, in a dose-dependent manner (Fig. 3C).

3.5. SREBF1 Expression and prognosis

Since the above results indicated that *SREBF1* signaling may play an important role on tumor cell growth, we investigated the relationship between *SREBF1* expression and mortality in 54 HCC patients by IHC. When we examined the expression of *SREBF1* in HCC tissues and adjacent non-cancerous liver tissues, we identified the increase of the cytoplasmic *SREBF1* staining in a subset of HCC (Fig. 4A). We evaluated the expression of *SREBF1* in HCC and classified 4, 30, and 20 HCCs as *SREBF1*-negative, *SREBF1*-low, and *SREBF1*-high HCC, respectively (Fig. 4B and Supplemental Fig. 1). We could not detect any differences of clinico-pathological characteristics between *SREBF1*-high HCC and *SREBF1*-low/-negative HCC including histological steatosis (Supplemental Table 4). Since the seven of these HCC samples were also used for real-time RT-PCR analysis, we investigated the relation of *SREBF1* RNA and protein expression (Fig. 4C). *SREBF1* RNA expression was significantly higher in *SREBF1*-high HCC than in *SREBF1*-low/-negative HCC with statistical significance ($P = 0.03$). Then we examined the cell proliferation of these HCC samples by PCNA staining. Notably, PCNA indexes were significantly higher in *SREBF1*-high HCC than *SREBF1*-low/-negative HCC with statistical significance ($P < 0.001$) (Fig. 4D). We further investigated the relationship between *SREBF1*

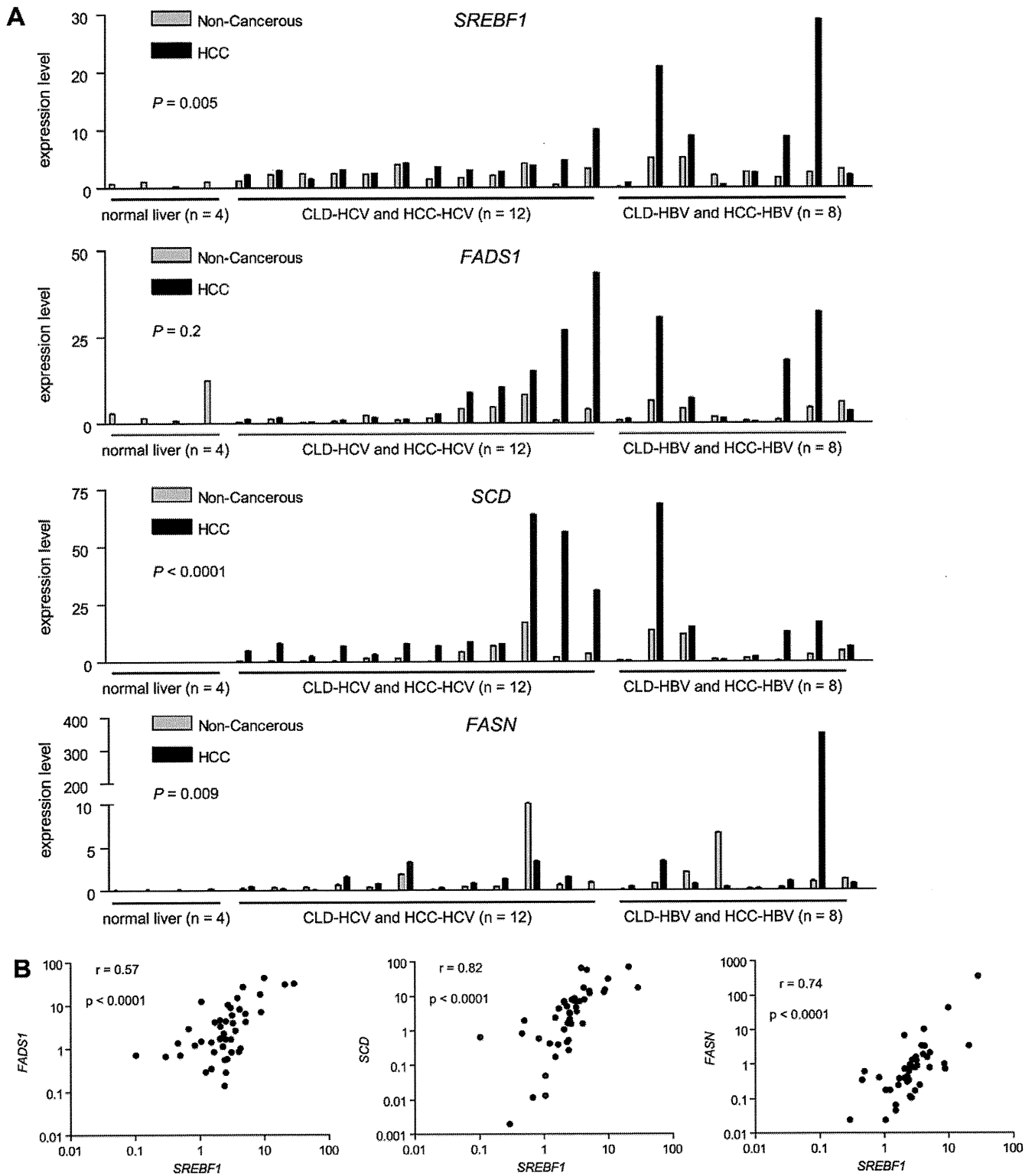


Fig. 1. (A) Real-time quantitative RT-PCR analysis. RNA was isolated from 44 tissue samples: 20 HCC, 20 corresponding CLD, and four normal liver samples. Differential expression of each gene among normal liver tissues, CLD tissues, and HCC tissues was examined by Kruskal–Wallis tests. (B) Scatter plot analysis. Gene expression levels of *FADS1*, *SCD* and *FASN* were well-correlated with those of *SREBF1*, as shown by Spearman’s correlation coefficients.

protein expression and prognosis. Kaplan–Meier survival analysis showed a significant relationship between poor survival and high *SREBF1* protein expression

($P = 0.04$; Fig. 4E). Univariate Cox regression analysis showed a correlation between high *SREBF1* protein expression and high risk of mortality with statistical

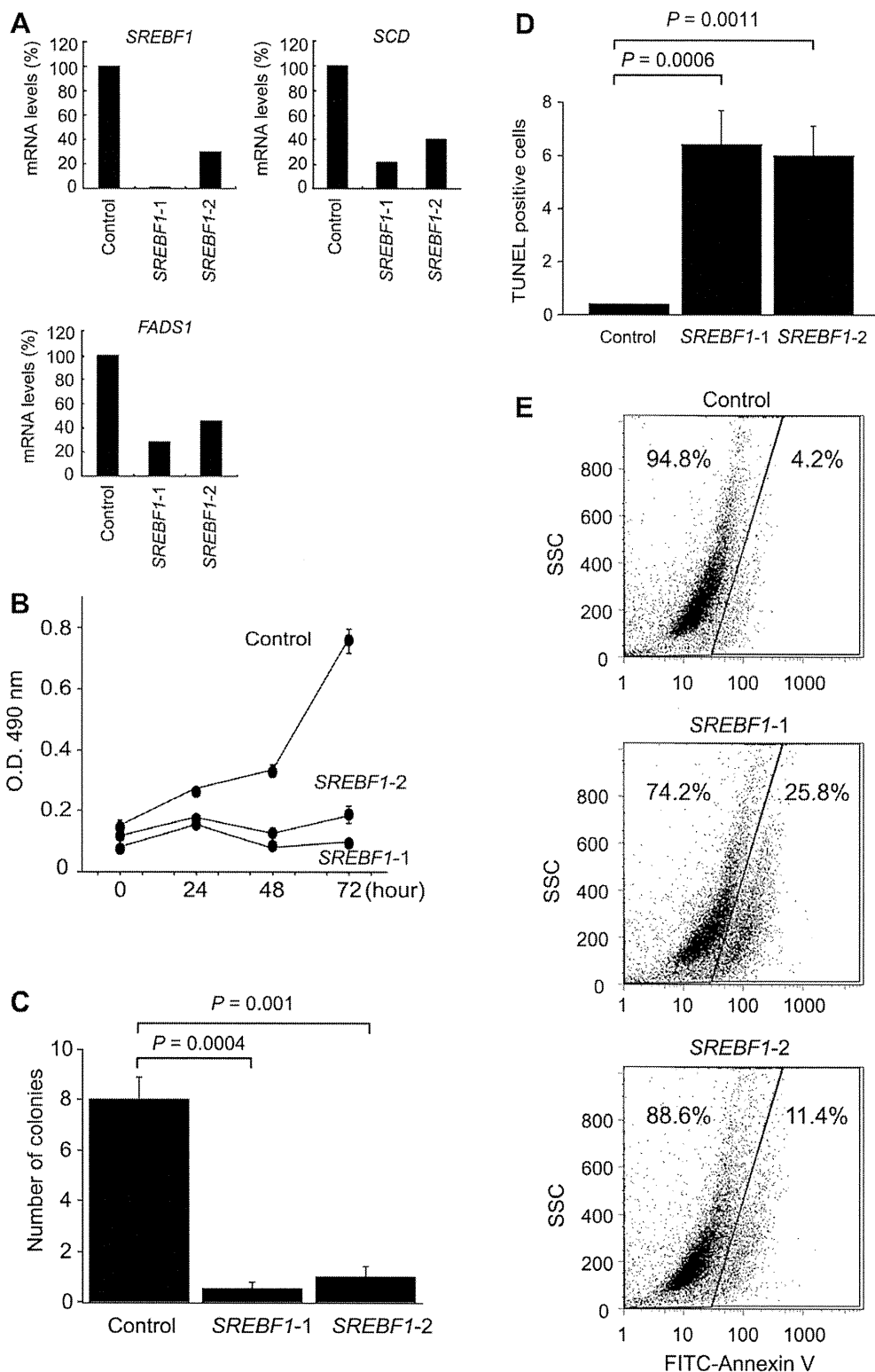


Fig. 2. (A) Effect of RNA interference targeting *SREBF1* in HuH7 cells. Expression levels of *SREBF1* mRNA were reduced by si-RNAs targeting different exons in *SREBF1*. Transcripts of *FADS1* and *SCD* were also down-regulated, showing transcriptional deactivation of the lipogenesis pathway. (B) Cell proliferation assay. Deactivation of the lipogenesis pathway severely reduced cell growth in HuH7 cells. (C) Soft agar assay. Deactivation of the lipogenesis pathway inhibited anchorage independent cell growth in HuH7 cells. (D) TUNEL assay. Deactivation of the lipogenesis pathway significantly increased the number of TUNEL-positive cells in HuH7 cells. (E) Annexin V staining evaluated by flow cytometer. Deactivation of the lipogenesis pathway significantly increased the number of annexin V positive cells in HuH7 cells.

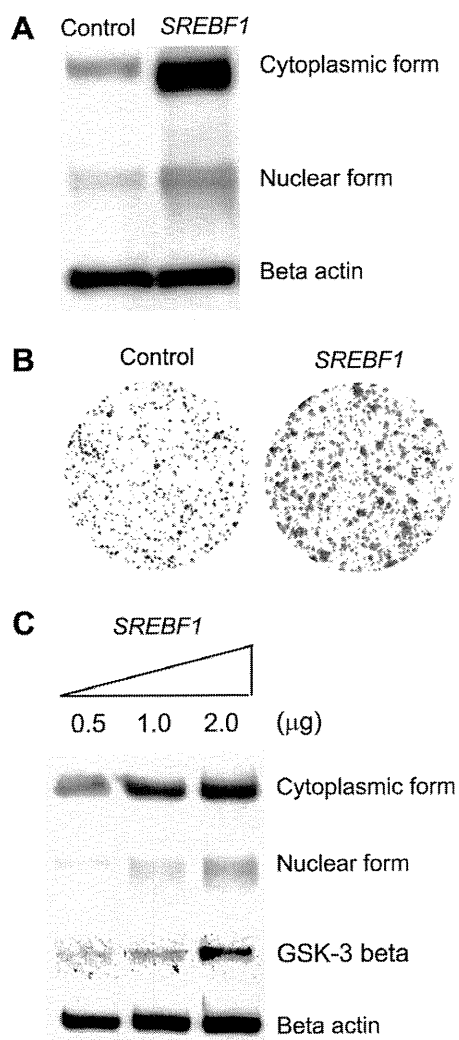


Fig. 3. (A) Western blot analysis of *SREBF1* protein expression in HuH7 cells transfected with control pCMV7 plasmids or pCMV7-*SREBF1c* plasmids. Both cytoplasmic and nuclear forms of *SREBF1* protein expression were increased by pCMV7-*SREBF1c* overexpression. (B) Focus assay of HuH7 cells transfected with control pCMV7 plasmids or pCMV7-*SREBF1c* plasmids. (C) Western blot analysis of *SREBF1* and phospho-GSK-3 β protein expression in HuH7 cells transfected with indicated amounts of pCMV7-*SREBF1c* plasmids.

significance (HR, 3.7; 95% CI, 1.0–13.7; $P = 0.05$; Table 2).

4. Discussion

Using large-scale gene expression profiling, we have shown that the lipogenesis pathway is transcriptionally activated in HCC. Our SAGE profiles will be available on our homepage (<http://www.intmedkanazawa.jp/>) and will be submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>).

We found that the levels of expression of *FADS1*, *SCD*, and *FASN* were each correlated with those of

SREBF1, suggesting that *SREBF1* is one of the main factors involved in the activation of lipogenesis in HCC. Activation of growth signaling pathways, such as the PI 3-kinase and mitogen-activated protein kinase pathways, has been shown to induce up-regulation of *SREBF1* in prostate and breast cancer cells [33,34]. We have observed induction of *SREBF1* protein expression by IGF2 in HuH7 cells (data not shown). Furthermore, we have identified that *SREBF1* overexpression results in the activation of cell proliferation and PI 3-kinase signaling, whereas expression inhibition of *SREBF1* abrogated the IGF2 induced cell proliferation. Although detailed mechanisms should be clarified in future, our results suggest that *SREBF1* is a key component of PI 3-kinase signaling in HCC.

SREBF1 is induced by alcohol [35], insulin, and fat [30,36], and plays a central role in the mechanism of hepatic steatosis [37]. Interestingly, these *SREBF1* inducers are risk factors for HCC [12,13,38,14]. Strikingly, two recent studies have shown that HBV and HCV infection may also induce hepatic steatosis through activation of *SREBF1* [39,40]. Furthermore, a recent report revealed the activation of *SREBF1* signaling in cancer by hypoxia [41]. Thus, these pathologic conditions such as chronic viral hepatitis, alcohol abuse, obesity, diabetes, and local hypoxia may up-regulate the expression of *SREBF1*, which, in turn, may contribute to an increased risk of hepatocarcinogenesis. Transgenic mice overexpressing *SREBF1* in the liver exhibited hepatic steatosis and hepatomegaly, suggesting the role of *SREBF1* on lipid metabolism and cell proliferation. However, it should be noted that no transgenic mice overexpressing *SREBF1* have been reported to have the risk of HCC development thus far. Interestingly, a recent report indicated that HCV core transgenic mice known to develop HCC showed coordinated activation of lipogenic pathway genes and *SREBF1* [42]. Although further studies are clearly required, we speculate that the activation of *SREBF1* may contribute to promote the development of HCC in already-initiated hepatocytes but not in normal hepatocytes.

Recently, Yahagi et al. reported the activation of lipogenic enzyme related genes in HCC [31]. In that paper, the authors suggested that *SREBF1* expression was not correlated with the expression of other lipogenic genes by Northern blotting, inconsistent with our current data. One possible explanation of these discrepancies might be the different methods for quantitation of mRNA, and we believe that real-time RT-PCR method used in our study would be more accurate. In addition, we evaluated the expression of *SREBF1* and lipogenic genes using more samples (a total of 44 liver and HCC tissues) than Yahagi et al did (10 HCC tissues). Furthermore, a recent paper indicated the coordinated activation of *SREBF1* and lipogenic genes in HCC

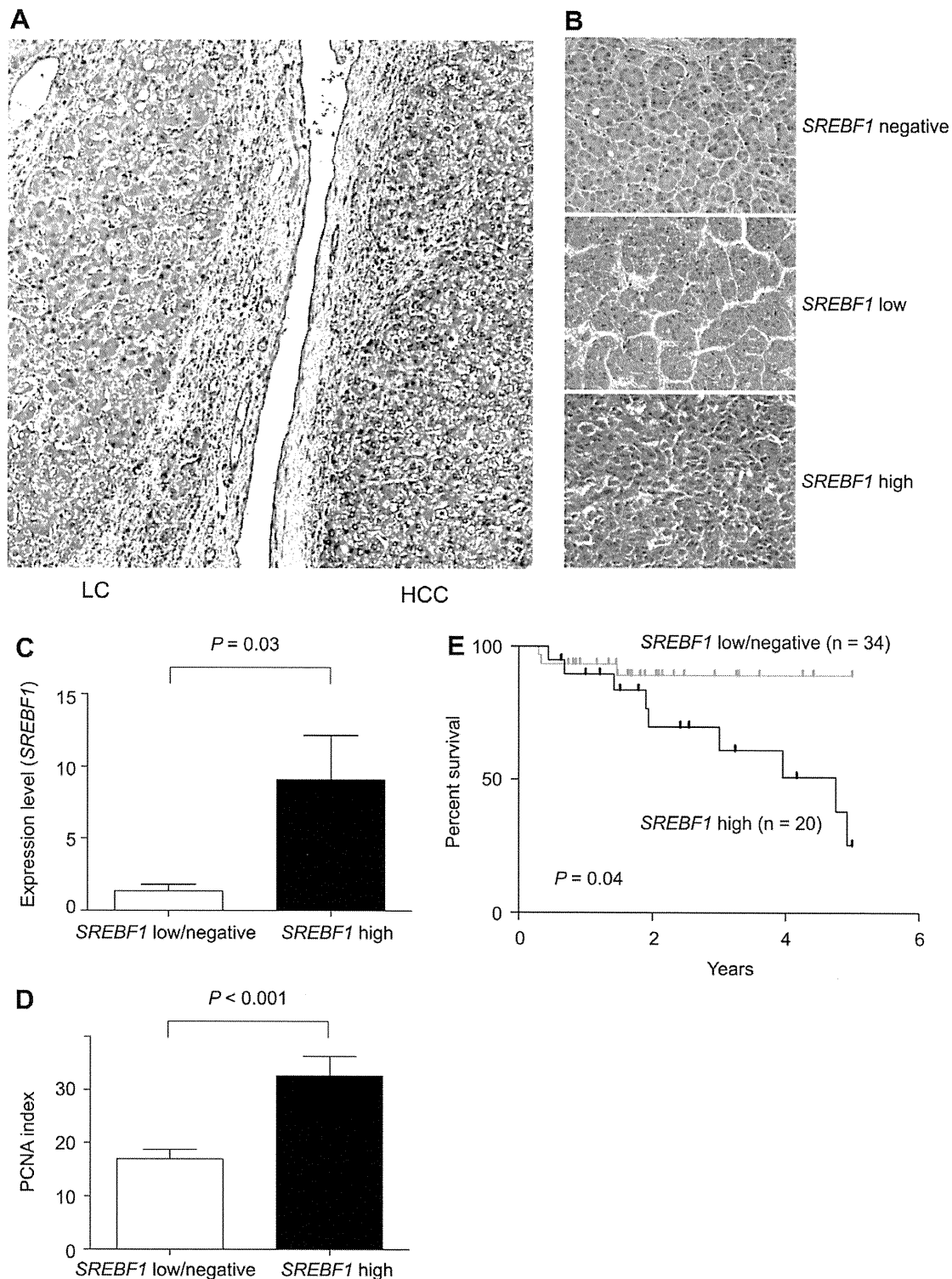


Fig. 4. (A) A photomicrograph of an HCC with adjacent non-cancerous cirrhotic liver stained with anti-*SREBF1* antibodies. (B) Representative photomicrographs of *SREBF1*-negative-, *SREBF1*-low-, and *SREBF1*-high-HCC tissues stained with anti-*SREBF1* antibodies. (C) *SREBF1* gene expression by real-time RT-PCR according to protein expression status assessed by IHC. *SREBF1* was highly expressed in *SREBF1*-high HCC ($P = 0.03$). (D) *SREBF1* expression and cell proliferation in HCC. PCNA indexes in *SREBF1*-high HCC were higher than those in *SREBF1*-low/negative HCC with statistical significance ($P < 0.001$). (E) Kaplan–Meier plots of 54 HCC patients analyzed by immunohistochemistry. The differences between *SREBF1*-high and -low/negative HCC were analyzed by log-rank test.

developed in the liver of HCV core transgenic mice [42], strongly support our data. Although further studies using large numbers of HCC tissues may be required,

these data suggest that the lipogenic gene activation seems to be mediated, at least in part, by *SREBF1* expression in HCC.

Table 2
Univariate Cox regression analysis of survival relative to *SREBF1* protein expression and clinicopathological parameters.

Variables (n)	HR (95% CI)	P-value
<i>SREBF1</i> and mortality (n = 54)		
Tumor size		
<3 cm (n = 37)	1	
≥3 cm (n = 17)	2.2 (0.6–8.3)	0.2
pTNM stage		
I, II (n = 45)	1	
III, IV (n = 9)	2.0 (0.4–9.4)	0.4
Serum AFP		
<20 ng/ml (n = 35)	1	
≥20 ng/ml (n = 19)	1.5 (0.4–5.4)	0.5
<i>SREBF1</i>		
Low (n = 34)	1	
High (n = 20)	3.7 (1.0–13.7)	0.05

Because the majority of our HCC patients analyzed had Child–Pugh class A scores and about 70% had tumors less than 3 cm in diameter, all were expected to have a good prognosis. Indeed, patient survival in this cohort was not segregated by tumor size or pTNM stage (Table 2). Although the sample size was relatively small, we found that enhanced expression of *SREBF1* was a prognostic factor for mortality in HCC possibly due to the highly proliferative nature. Activation of lipogenesis pathways, as shown by overexpression of *FASN*, has been found to correlate with high mortality in breast, prostate, and lung cancer [43], suggesting that activation of lipogenesis may be a fundamental characteristic of cancer with poor prognosis. Thus, *SREBF1* expression may be a good biomarker for HCC classification, a finding that should be validated in a large scale cohort. Because deactivation of the lipogenesis pathway by inhibition of *SREBF1* gene expression could inhibit HCC cell growth *in vitro*, *SREBF1* may be a good target for pharmaceutical intervention in these tumors.

In conclusion, our genome-wide gene expression profiling analyses found that the lipogenesis pathway was activated in a subset of HCC. *SREBF1*, which activates the lipogenesis pathway, may be a good biomarker for HCC prognosis and may be a good target for therapeutic intervention.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.07.036.

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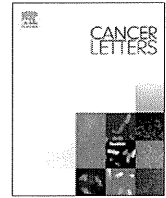
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Frequency of CD45RO⁺ subset in CD4⁺CD25^{high} regulatory T cells associated with progression of hepatocellular carcinoma

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ABSTRACT

The purpose of this study was to assess the properties of CD4⁺CD25^{high/low/negative} T cell subsets and analyze their relation with dendritic cells (DCs) in patients with hepatocellular carcinoma (HCC). In HCC patients, the prevalence of CD45RO⁺ cells in CD4⁺CD25^{high} T cells was increased and associated with higher frequencies of plasmacytoid DCs. Larger proportions of this T cell subset were detected in the patients with larger tumor burdens. These results suggest that increased frequencies of the CD45RO⁺ subset in CD4⁺CD25^{high} Tregs in HCC patients may establish the immunosuppressive environment cooperatively with tolerogenic plasmacytoid DCs to promote disease progression of liver cancer.

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1. Introduction

Hepatocellular carcinoma (HCC) occurs primarily in individuals with cirrhosis related to hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, and alcohol abuse. HCC is the fifth most common cancer, with increasing incidence worldwide. It is characterized by high mortality, frequent postsurgical recurrence and extremely poor prognosis [1–3].

CD4⁺CD25^{high} Foxp3⁺ regulatory T cells (Tregs) have been shown to suppress immune responses by direct interaction with other immune cell types or through immune suppressive cytokines and appear crucial in maintaining immune homeostasis, mediating peripheral tolerance and preventing autoimmunity [4–6]. Increased frequencies of Tregs have been documented in the peripheral blood and in some cases the tumor microenvironment in patients

with several different tumor types [3–12]. It has been reported that, in HCC patients, increased Tregs are correlated with CD8⁺ T-cell impairment [11] and are related to poor prognosis [1].

Tregs are known to consist of heterogeneous subsets and to express various surface markers detectable by flow cytometry, including CD45RO, CTLA-4 (cytotoxic T lymphocyte associated antigen-4), GITR (glucocorticoid-induced TNF receptor-related protein), CD62L, HLA-DR, and CCR7 [8,13–15]. The role of these markers in suppressor functions mediated by human Tregs is currently under discussion [8]. It has been suggested that GITR is associated with T cell activation [16,17] and Treg subset expressing GITR are associated with disease activity in patients with Wegener's granulomatosis [17]. As for HCC, Ormandy et al. demonstrated that Tregs in HCC patients expressed high levels of HLA-DR and GITR [3]. However, there is a paucity of studies presenting the association of Treg subsets with disease progression.

In addition to Tregs, dendritic cells (DCs), a type of professional antigen-presenting cells (APCs), may be

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implicated in the regulation of immune responses. The role of human DCs in modulating Tregs is not clear [18]. It has been suggested that immature and mature myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) may promote Treg cell differentiation, homeostasis and function [19]. It has been shown that lung cancer cells can convert mature DCs into TGF- β 1 producing cells, which demonstrate an increased ability to generate Tregs [20]. Conversely, Tregs can induce the generation of semimature DCs by which they can down-regulate immune responses [21]. These data suggest that there may be a mutual interaction between Tregs and DCs for the maintenance of immunosuppression.

In the present study, we evaluated the frequency and properties of CD4⁺CD25^{high} Foxp3⁺ T cells in HCC patients. Increased numbers of these cells produced more Th2 cytokine than CD4⁺CD25^{low/negative} cells. Furthermore, the proportion of CD45RO⁺ subset was increased in HCC patients. We also analyzed how the subset is related to DC frequencies, and found that some subsets were relevant to disease progression.

2. Materials and methods

2.1. Patients and healthy controls

Sixty-two HCC patients attending Kanazawa University Hospital (Ishikawa, Japan) between September 2006 and July 2008 were enrolled in this study with their informed consent. HCC was radiologically diagnosed by computed tomography (CT), magnetic resonance imaging (MRI), and CT angiography. Blood samples were taken from these HCC patients, as well as from 41 healthy controls, 17 patients with chronic hepatitis (CH) B and C and 16 patients with liver cirrhosis (LC) without a tumor. None of the patients received anticancer nor antiviral therapy at time of blood sample. Patients characteristics and disease classification are shown in Table 1.

2.2. Isolation of PBMC and CD4⁺ T cells

Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained blood by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO). Total cell numbers were counted in the presence of a trypan blue dye to evaluate viability and immediately used for experiments. CD4⁺ T cells were isolated from freshly isolated PBMC by negative magnetic selection using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and QuadroMACS Separation Unit (Miltenyi Biotec) according to the manufacturer's instruction. Isolated CD4⁺ T cells were purified by >90% as measured by flow cytometric analysis using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA).

2.3. Antibodies

The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD4-PerCP, anti-CD25-APC (BD Biosciences, San Jose, CA), anti-CD45RO-FITC (PROIMMUNE, Oxford, UK), anti-CTLA-4-PE, anti-CCR7-PE,

Table 1

Clinical characteristics of hepatocellular carcinoma, liver cirrhosis, chronic hepatitis patients and healthy control.

<i>Hepatocellular carcinoma (n = 62)</i>	
Age (yrs)	68.9 ± 9.5
Gender (M/F)	37/25
Etiology of liver disease	
HBV/HCV/HBV + HCV/NBNC	19/34/2/7
TNM stages I/II/III/IV-A/IV-B	18/12/20/6/6
<i>Largest tumor (mm)</i>	
Child-Pugh A/B/C	37.6 ± 34.4
AFP (ng/mL)	41/8/3
DCP (mAU/mL)	10–35,093 (52)
<i>Liver cirrhosis (n = 16)</i>	
Age (yrs)	10–32,818 (34)
Gender (M/F)	58.3 ± 10.3
Etiology of liver disease	
HBV/HCV/NBNC	11/5 4/7/5
<i>Chronic hepatitis (n = 17)</i>	
Age (yrs)	58.9 ± 10.4
Gender (M/F)	8/9
Etiology of liver disease	
HBV/HCV/NBNC	0/17/0
<i>Healthy controls (n = 41)</i>	
Age (yrs)	46.1 ± 19.1
Gender (M/F)	16/25

Note: Results except for AFP and DCP are expressed as means ± SD. AFP and DCP values are expressed as range (median). The reference range of normal values for the laboratory values: AFP < 10 ng/mL, DCP < 40 mAU/mL. M, Male; F, Female; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B non-C; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

anti-GITR (glucocorticoid-induced TNF receptor-related protein)-PE (R&D Systems, Minneapolis, MN), anti-CD62L-FITC, anti-HLA-DR-FITC, anti-CD45RA-PE (Exalpha Biologicals, Watertown, MA), IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells “Plasmacytoid Subset” and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells “Myeloid Subset” (Beckman Coulter, Miami, FL). Before use, all mAbs were titrated using normal PBMC to establish optimal staining dilutions.

2.4. Surface and intracellular staining

To determine the frequency of CD4⁺CD25^{high} T cells and the surface marker profile, CD4⁺ T cells (at least 2×10^5 cells/tube) were stained with mAbs in the above described panel for 30 min on ice. Appropriate isotype antibody controls were used for each sample. Cells were washed and examined by four-color flow cytometry.

For intracellular Foxp3 and cytokine staining, 2×10^5 CD4⁺ T cells/well in a 96-plate were stimulated with Leucocyte Activation Cocktail containing PMA, ionomycin, and brefeldin A, and then cultured at 37 °C in a humidified CO₂ incubator for 4 h. The activated cells were first incubated with anti-CD4-PerCP for 15 min on ice, followed by fixation and permeabilization of the activated cells for 20 min at room temperature with BD Cytofix/Cytoperm Buffer (BD Biosciences, San Diego, CA). Samples were then stained with anti-CD25-APC, anti-Foxp3-FITC (eBioscience) and PE-labeled anti-cytokine (IL-4, IL-10) antibodies (BD