

## Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma<sup>☆</sup>

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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.

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**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](http://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; CAGTGGCACTGACTCTTCC, *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  $1 \times 10^4$  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% CO<sub>2</sub> 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 2000™ reagent (Invitrogen), as described by the manufacturer. A total of  $2 \times 10^3$  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 $\beta$  (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  $\beta$ -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+™ 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 MetaCore™ software, we found that the candidate transcription factors activated were distinct in each HCC library (Table 1). Several of these transcription factors, including NF- $\kappa$ B, c-Myc, c-Jun, and HNF4- $\alpha$ , 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- $\kappa$ B	Antigen presentation	0.004	
		Antigen processing		
		Defense response		
	SREBF1	Immune response		
		Cholesterol biosynthesis		0.05
		Lipid biosynthesis		
		$\beta$ -Glucoside transport		
	Negative regulation of lipoprotein metabolism			
	SP1	Electron transport; drug metabolism		0.05
		Oxygen and reactive oxygen species metabolism		
IRF1	Cell-substrate junction assembly; wound healing	0.05		
	Immune response			
	Antigen presentation; antigen processing			
HCC–HBV	HNF4- $\alpha$	Defense response; positive regulation of cell	0.002	
		Lipid transport		
	HNF1	Fatty acid metabolism		
		Smooth muscle cell proliferation		0.01
		Acute-phase response; lipid transport		
	Negative regulation of lipid catabolism			
	SP1	$\beta$ -Glucoside transport		0.01
		Negative regulation of lipoprotein metabolism		
	c-Jun	Zinc ion homeostasis; response to biotic stimulus		0.03
		Nitric oxide mediated signal transduction		
	C/EBP- $\alpha$	Copper ion homeostasis; fatty acid biosynthesis		0.03
		Progesterone catabolism; progesterone metabolism		
	SREBF1	Regulation of lipid metabolism;		0.03
		Prostaglandin metabolism		
		Lipid transport; negative regulation of lipid catabolism		
	c-Myc	Negative regulation of lipoprotein metabolism		0.03
		Fatty acid biosynthesis; fatty acid metabolism		
		Fatty acid desaturation;		
	USF1	Activation of pro-apoptotic gene products		0.03
		Release of cytochrome c from mitochondria		
PPAR- $\alpha$	Fatty acid metabolism	0.03		
	Smooth muscle cell proliferation			
COUP-TFI	Fatty acid metabolism	0.03		
	Smooth muscle cell proliferation			
C/EBP- $\beta$	Lipid transport	0.03		
	Smooth muscle cell proliferation			
	Acute-phase response			
	Regulation of interleukin-6 biosynthesis			
	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- $\kappa$ 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 MetaCore<sup>TM</sup>, 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 $\beta$ , 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*

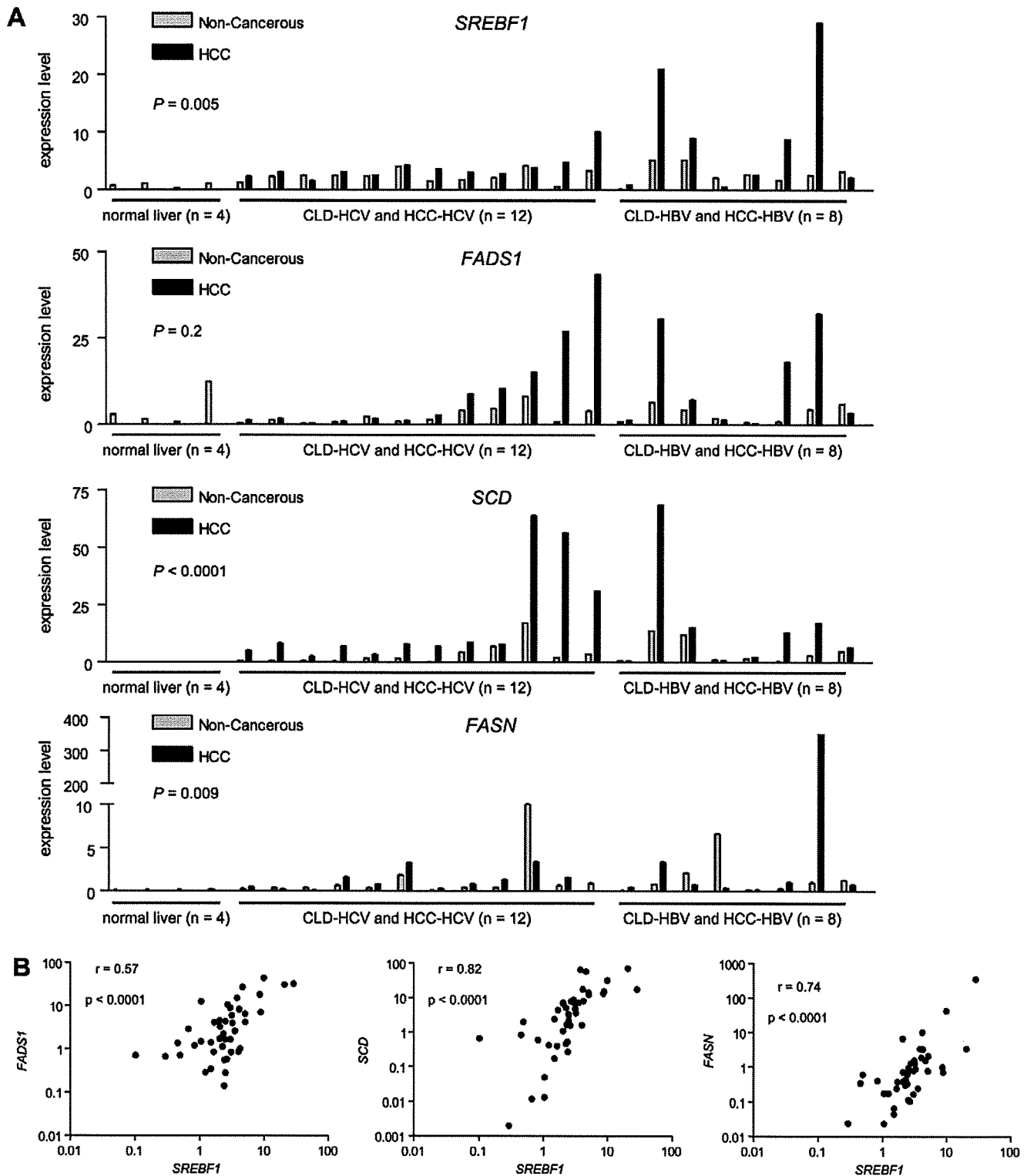


Fig. 1. (A) Real-time quantitative RT-PCR analysis. RNA was isolated from 44 tissue samples: 20 HCC, 20 corresponding CLD, and four normal liver samples. Differential expression of each gene among normal liver tissues, CLD tissues, and HCC tissues was examined by Kruskal–Wallis tests. (B) Scatter plot 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

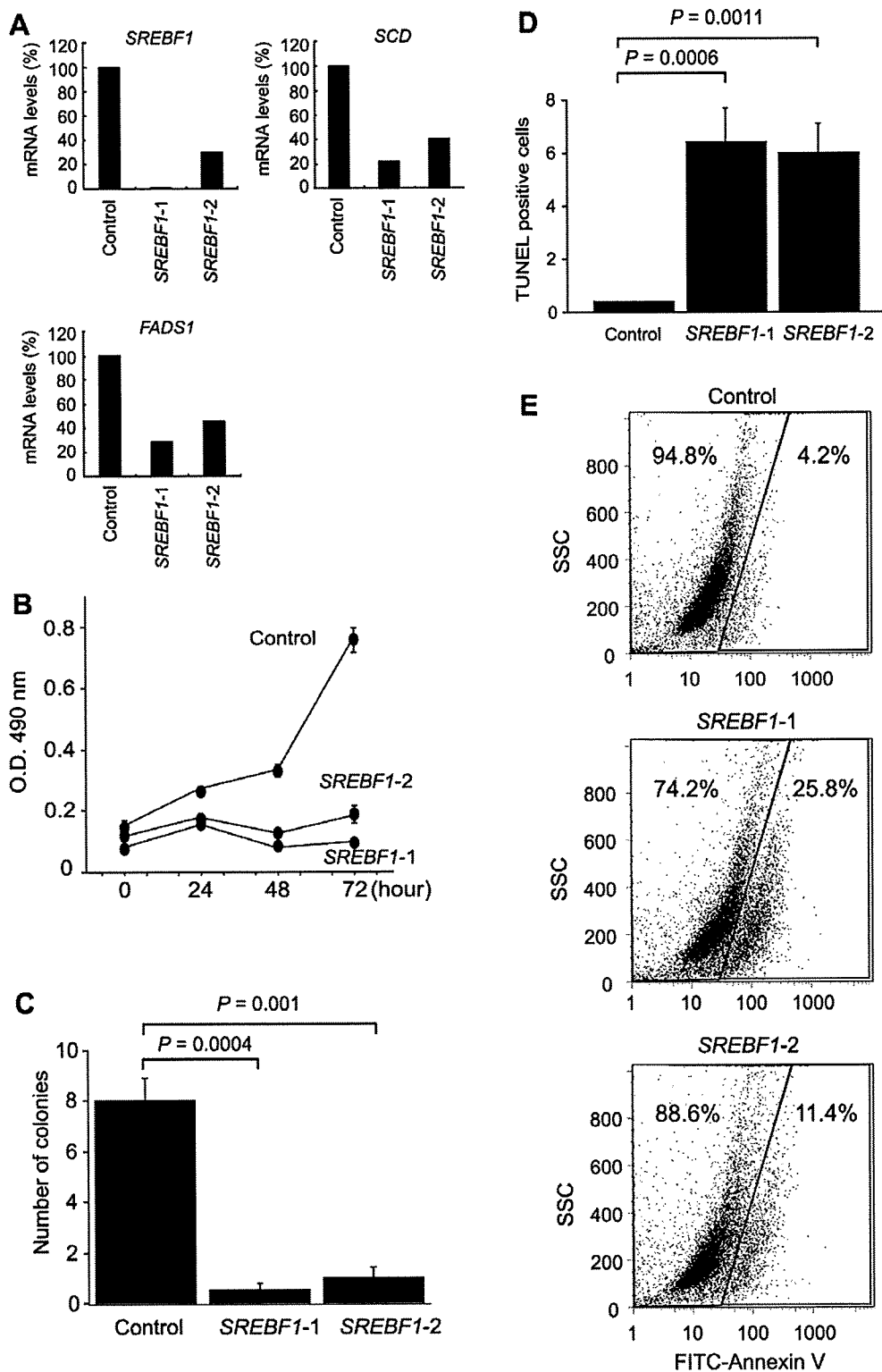


Fig. 2. (A) Effect of RNA interference targeting *SREBF1* in HuH7 cells. Expression levels of *SREBF1* mRNA were reduced by si-RNAs targeting different exons in *SREBF1*. Transcripts of *FADS1* and *SCD* were also down-regulated, showing transcriptional deactivation of the lipogenesis pathway. (B) Cell 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.

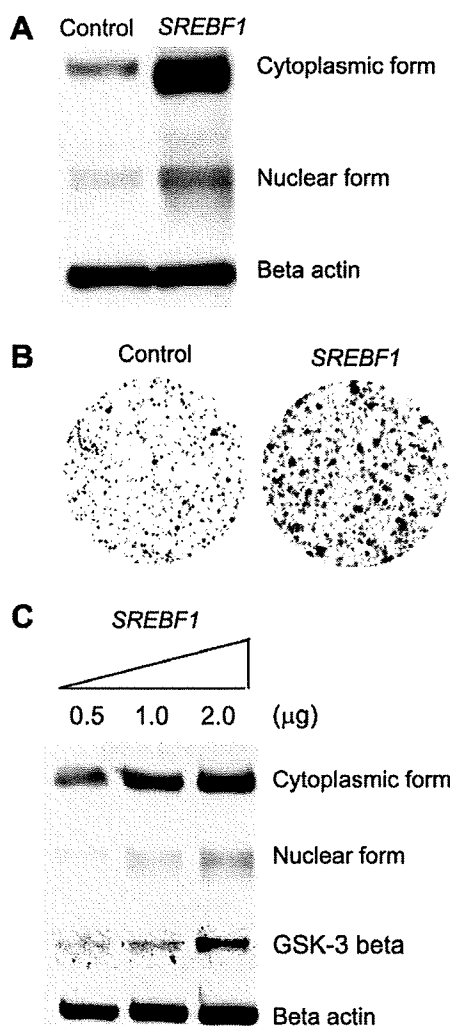


Fig. 3. (A) Western blot analysis of *SREBF1* protein expression in HuH7 cells transfected with control pCMV7 plasmids or pCMV7-*SREBF1c* plasmids. Both cytoplasmic and nuclear forms of *SREBF1* protein expression were increased by pCMV7-*SREBF1c* overexpression. (B) Focus assay of HuH7 cells transfected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



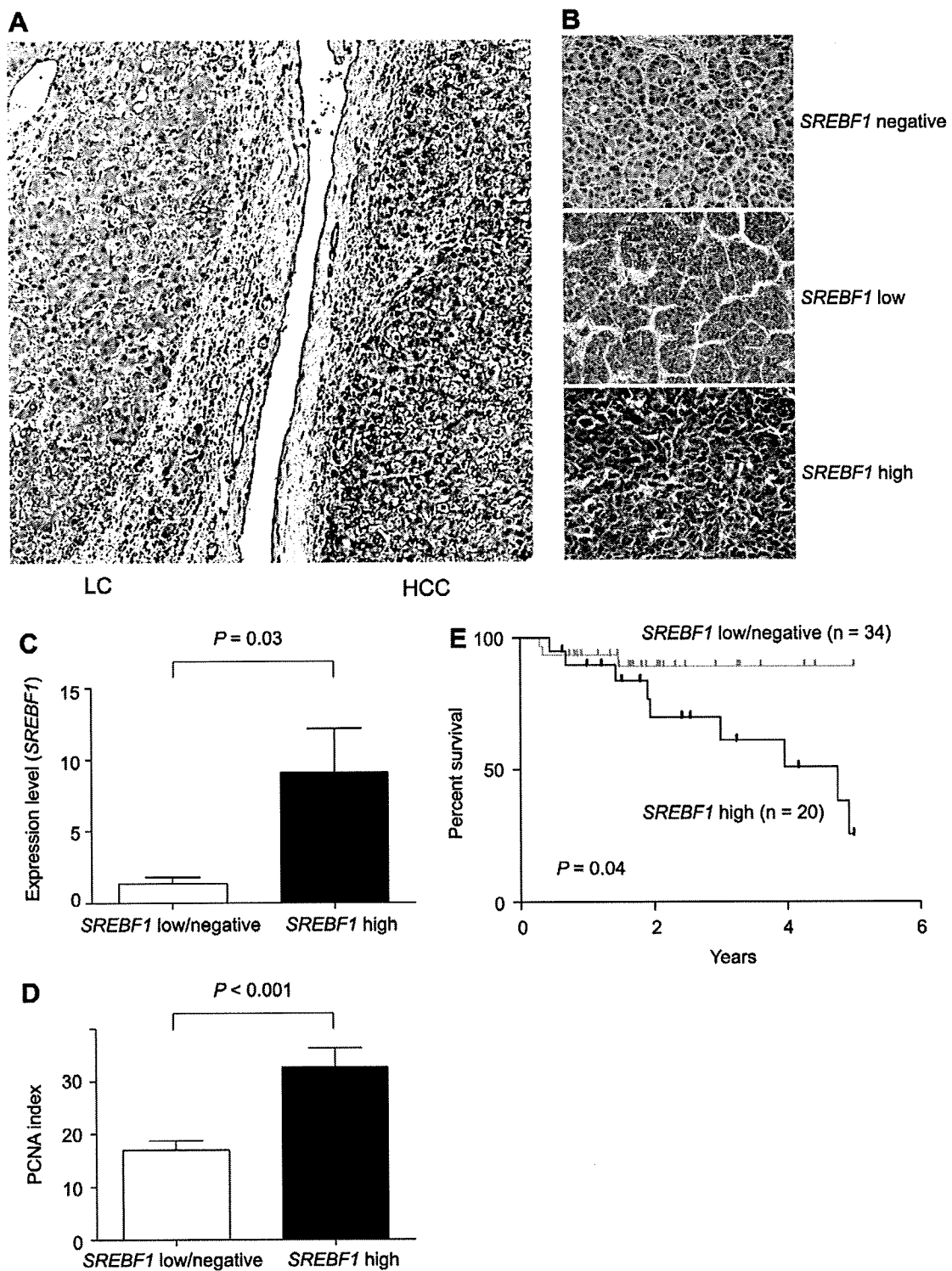


Fig. 4. (A) A photomicrograph of an HCC with adjacent non-cancerous cirrhotic liver stained with anti-*SREBF1* antibodies. (B) Representative photomicrographs of *SREBF1*-negative-, *SREBF1*-low-, and *SREBF1*-high-HCC tissues stained with anti-*SREBF1* antibodies. (C) *SREBF1* gene expression by real-time 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.

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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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# Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis

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**SUMMARY.** In hepatitis C virus (HCV) infection, the Th1-type immune response is involved in liver injury. A predominance of immunosuppressive regulatory T cells (Treg) is hypothesized in patients with persistently normal alanine aminotransferase (PNALT). Our aim was to clarify the role of Treg in the pathogenesis of PNALT. Fifteen chronically HCV-infected patients with PNALT, 21 with elevated ALT (CH) and 19 healthy subjects (HS) were enrolled. We determined naturally-occurring Treg (N-Treg) as CD4+CD25<sup>high</sup>+FOXP3+ T cells. The expression of FOXP3 and CTLA4 in CD4+CD25<sup>high</sup>+ cells was quantified by real-time reverse transcriptase-polymerase chain reaction. Bulk or CD25-depleted CD4+ T cells cultured with HCV-NS5 loaded dendritic cells were assayed for their proliferation and

cytokine release. We examined CD127–CD25–FOXP3+ cells as distinct subsets other than CD25+ N-Treg. The frequencies of N-Treg in patients were significantly higher than those in HS. The FOXP3 and CTLA4 transcripts were higher in PNALT than those in CH. The depletion of CD25+ cells enhanced HCV-specific T cell responses, showing that co-existing CD25+ cells are suppressive. Such inhibitory capacity was more potent in PNALT. The frequency of CD4+CD127–CD25–FOXP3+ cells was higher in CH than those in PNALT. Treg are more abundant in HCV-infected patients, and their suppressor ability is more potent in patients with PNALT than in those with active hepatitis.

**Keywords:** HCV, PNALT, regulatory T cell.

## INTRODUCTION

Hepatitis C virus (HCV) causes a wide range of chronic liver diseases in infected hosts, including chronic hepatitis (CH), liver cirrhosis and hepatocellular carcinoma (HCC).

Abbreviations: ALT, alanine aminotransferase; CH, chronic hepatitis; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HS, healthy subjects; IFN, interferon; IL, interleukin; IU, international units; MoDC, monocyte-derived dendritic cell; N-Treg, naturally occurring regulatory T cell; PNALT, persistently normal ALT; RT-PCR, reverse transcriptase-polymerase chain reaction; SLE, systemic lupus erythematosus; TGF, transforming growth factor; Treg, regulatory T cell.

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One of the critical determinants promoting the development of HCV-induced liver disease is sustained liver inflammation, explaining the therapeutic rationale of alleviating this condition to help prevent liver cancer [1]. Among chronically infected individuals, approximately 20–30% display persistently normal serum alanine aminotransferase levels [2,3]. Although it is reported that 40–50% of them progress to the active stage of liver inflammation within 5 years of observation [4], the incidence of HCC in the remaining patients continues to be lower than in those with elevated serum ALT levels [5]. Cumulative studies have revealed that HCV is not directly cytopathic to hepatocytes. It has been demonstrated that a Th1-type or cytotoxic T lymphocyte (CTL) response is critically involved in HCV-mediated liver injury [6,7]. Therefore, it is conceivable that some suppressor mechanisms exist against Th1-type immune responses in patients with persistently normal ALT levels (PNALT), which may be distinct from those in patients with active liver inflammation.

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Regulatory T cells (Treg) are a unique subset of T cells with inhibitory capacity against auto-reactive T cells [8]. Substantial data have been reported about the involvement of Treg in the pathogenesis of various diseases, including autoimmune, cancer or infectious diseases [9–13]. Currently, the existence of several types of Treg has been reported [14]. Naturally occurring Treg (N-Treg) are derived from the thymic stromal environment from progenitor cells and suppress auto-reactive T cells in antigen-specific and antigen-nonspecific manner. Forkhead/winged helix transcription factor (FOXP3) is one of the specific markers of N-Treg, the expression of which is well correlated with the gain of a suppressor function [15,16]. As cells with high expression of CD25 also display FOXP3, it is generally accepted that CD25+FOXP3+ is the most reliable marker for Treg. In HCV infection, several reports have described a higher frequency of N-Treg in the periphery and the liver [17–20], suggesting their active role in HCV persistence. It has also been demonstrated that CD25+FOXP3+ regulatory cells are inducible in the periphery [21]. Owing to the lack of a specific phenotypic marker of these induced regulatory cells, referred to as adaptive Treg, their role in the pathogenesis of HCV infection has not been clearly understood. A recent study has demonstrated that the expression of interleukin (IL)-7 receptor (CD127) is downregulated in Treg to a degree that is inversely correlated with FOXP3 expression [22]. These findings offer the possibility that adaptive Treg are traceable, not all but in part, by the combination of CD127 and FOXP3 independent of CD25 expression.

In this study, our aim was to elucidate whether or not Treg are involved in the pathogenesis of PNALT patients, by comparing the frequency and function of these cell subsets with those in active hepatitis patients or healthy subjects. A

distinct equilibrium was found between N-Treg and CD127–CD25–FOXP3+ T cells according to differences in liver inflammation.

## MATERIALS AND METHODS

### Subjects

Among chronically HCV-infected patients who had been followed at Osaka University Hospital, 15 patients with PNALT levels and 21 patients with elevated or fluctuating ALT levels (the CH group) were enrolled in this study. As controls, 19 healthy subjects (HS) who were negative for HCV and hepatitis B virus (HBV) markers were examined. The study protocol was approved by the ethical committee of Osaka University Graduate School of Medicine. At enrolment, written informed consent was obtained from each subject. In this study, PNALT patients were defined as those whose ALT levels remained within the normal range (<30 IU/mL) without any medications for more than 1 year. At enrolment, the patients were confirmed to be positive for both serum anti-HCV and HCV RNA, but were negative for other viral infections, including HBV and human immunodeficiency virus. The presence of other causes of liver disease, such as autoimmune, alcoholic and metabolic disorders was excluded by the use of laboratory and imaging analyses. Liver biopsy was carried out in some of the patients. Histological examination was performed according to the METAVIR scoring system. In all patients, a combination of repetitive biochemical tests, ultrasonography or computed tomography scans ruled out the presence of cirrhosis and liver tumours. The clinical background of the subjects are shown in Table 1.

**Table 1** Baseline clinical characteristics of the patients

	Chronic hepatitis patients	Patients with PNALT	Healthy subjects*	
<i>n</i>	21	15	19	
Sex (M/F)	8/13	5/10	ND	NS
Age	50.6 ± 11.6	47.8 ± 12.7	ND	NS
ALT (IU/L)	88.3 ± 41.4	20.9 ± 6.9	ND	<i>P</i> < 0.0001 <sup>†</sup>
Plt (10 <sup>4</sup> /μL)	13.5 ± 5.4	20.0 ± 3.9	ND	<i>P</i> < 0.01 <sup>†</sup>
HCV RNA (Meq/mL)	8.6 ± 11.3	9.7 ± 7.8	ND	NS

\*The background data of healthy subjects (blood donors) were not accessible owing to the confidentiality regulations of the blood centre, but their serum ALT levels were confirmed to be within the normal range. <sup>†</sup>Statistical significance was analysed by Mann–Whitney *U* test between chronic hepatitis patients and patients with PNALT. The values are expressed as mean ± SD. PNALT, persistently normal alanine aminotransferase level; ND, not determined; NS, not significant; plt, platelet count.

### Frequency analyses of Treg cells

For the numerical analyses of Treg cells, heparinized venous blood was obtained from all subjects. Peripheral blood mononuclear cells were collected by density-gradient centrifugation on a Ficoll-Hypaque cushion. The cells were subsequently stained with a combination of various fluorescence-labelled anti-human mouse monoclonal antibodies for phenotypic markers. The antibodies for CD25 (clone B1.49.9) and CD4 (clone 13B8.2) were purchased from Beckman Coulter (Fullerton, CA, USA), that for CD127 (clone 40131) from R&D Systems (Minneapolis, MN, USA) and that for FOXP3-PE (clone PCH101) from eBioscience (San Diego, CA, USA), respectively. The cells were stained in phosphate-buffered saline containing 1% fetal bovine serum (FBS) with various antibodies or isotype controls for 15 min at room temperature. Intracellular staining of FOXP3 was performed using a human FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. The cells were analysed by FACSCalibur (BD Biosciences, San Jose, CA, USA) and CellQuest software.

### Functional analysis of CD4+CD25+ T cells in HCV-specific CD4+ T cell response

We first examined the HCV-specific CD4+ T cell response in the presence or absence of CD4+CD25+ T cells. Monocyte-derived dendritic cells (MoDC) were generated from CD14+ cells as reported previously. In brief, CD14+ cells were cultured in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS, 50 IU/mL of penicillin, 50 mg/mL of streptomycin, 2 mM of L-glutamine, 10 mM of Hepes buffer, 10 mM of nonessential amino acids in the presence of 50 ng/mL of granulocyte/macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 10 ng/mL of IL-4 (PeproTech) for 7 days at 37 °C and 5% CO<sub>2</sub>. On day 6 of the culture, MoDC were pulsed with 10 µg/mL of recombinant HCV NS5 (amino acid position: NS5B 1-544; kindly provided by Japan Tobacco, Inc., Tokyo, Japan) and cultured for 24 h. The antigen-pulsed MoDC were then cultured with autologous bulk CD4+ T cells or CD4+CD25- T cells in 96-well flat-bottom plates (Corning, NY, USA) for 5 days. Enrichment of CD4+ T cells or CD4+CD25- T cells was performed using a CD4+CD25+ Regulatory T cell Isolation kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. On day 6 of the co-culture, the cells were pulsed with 1 µCi of [3H]-thymidine during the last 16 h of incubation. The supernatants were collected before pulsing with [3H]-thymidine and subjected to cytokine enzyme-linked immunosorbent assay (ELISA). The incorporation of [3H]-thymidine in CD4+ T cells was measured using a β-counter (Wallac-Perkin-Elmer, Wallac, Finland).

### Enzyme-linked immunosorbent assay

The concentrations of IL-10, TGF-β1 and interferon (IFN)-γ in the culture supernatants were determined by ELISA. We used matched pairs of relevant monoclonal antibodies (Endogen, Woburn, MA, USA) for IL-10 and IFN-γ, and the DuoSet ELISA development system (R&D Systems) for TGF-β1, according to the manufacturer's instructions. The detection thresholds of IL-10, TGF-β1 and IFN-γ were 10, 10 and 16 pg/mL, respectively.

### Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

In order to analyse the expression of FOXP3 and CTLA-4 in N-Treg, we collected CD4+CD25<sup>high</sup> T cells by using FACSaria. The purity of the isolated cells was more than 95% as determined by FACS. Total RNA was extracted from sorted CD4+CD25<sup>high</sup> T cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using the SuperScript III First-Strand synthesis system (Invitrogen, Carlsbad, CA, USA). Assays-on-demand primers and probes (PE Applied Biosystems, Foster City, CA, USA) were used to quantify FOXP3 and CTLA4 expression. The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The thermal cycling conditions for all genes were as follows: the reaction was started with a 10-min denaturing cycle at 95 °C, followed by 40 cycles of PCR performed with 15 s of denaturing at 95 °C, then 1 minute at 60 °C for annealing and extension. We identified a calibrator sample from the healthy volunteers. The expressions of molecules were given as the relative values to the calibrator samples. To standardize the amount of total RNA added to each reaction mixture, we quantified β-actin mRNA from each sample as a control of internal RNA and corrected all values with this.

### Statistical analysis

Statistical analyses were performed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). Mann-Whitney *U*-test was used to compare differences in unpaired samples. For all analyses, a *P*-value of less than 0.05 was considered to be statistically significant.

## RESULTS

### Peripheral N-Treg are increased in HCV-infected patients

We compared the frequency of Treg between HCV-infected patients and healthy donors. In HCV-positive individuals, they were further categorized into PNALT and CH groups according to the difference in their serum ALT levels. The clinical backgrounds of these groups were not different except for

serum ALT levels and platelet counts (Table 1). N-Treg were defined as the cells with CD4+CD25<sup>high</sup>+FOXP3+ cells. As the cut-off value between CD25<sup>high</sup>+ and CD25<sup>intermediate</sup>+ cells is a critical determinant for Treg analyses, we defined CD4+CD25<sup>high</sup>+ as the cells with CD25 levels higher than those of CD4-CD25+ cells (Fig. 1a). We first compared the frequency of CD4+FOXP3+ T cells. The frequency of FOXP3+ cells in the CD4+ T cell population in HCV-infected patients was significantly higher than those in the HS (Fig. 1b). However, no difference was observed in FOXP3+ cells between the PNALT and CH patients (Fig. 1b). The frequency of CD4+CD25<sup>high</sup>+FOXP3+ T cells in CH or PNALT patients were significantly higher than those in HS, whereas those in HCV-positive patients did not differ regardless of their ALT levels (Fig. 1c). Similar results were obtained for the frequency of CD4+CD25-FOXP3+ T cells (Fig. 1d).

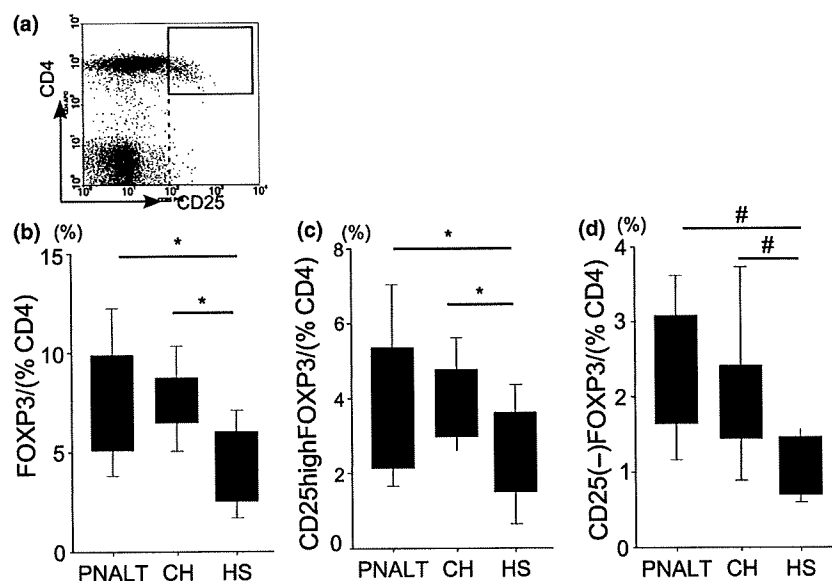
Next, we examined whether or not the frequency of N-Treg is correlated with clinical parameters. Among all HCV-infected patients, no correlation was observed between the frequency of N-Treg (CD4+CD25<sup>high</sup>+FOXP3+ T cells) and serum ALT, HCV RNA levels, age or platelet counts (data not shown). In the analyses of patients who had undergone liver biopsy, the frequency of N-Treg was not correlated with METAVIR grade/stage scores (data not shown).

#### The expressions of FOXP3 and CTLA4 are higher in N-Treg from PNALT patients compared with those from the CH group

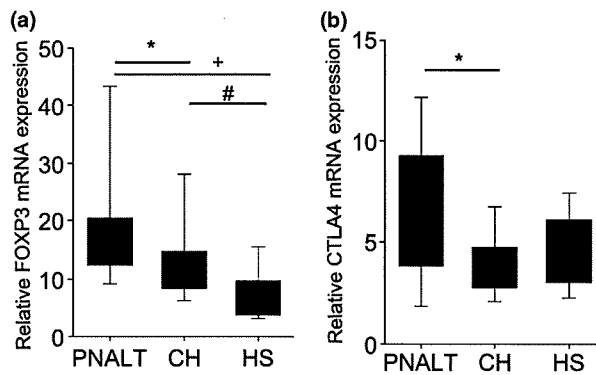
FOXP3 is the master gene of Treg in the development and gaining of suppressor functions. Alternatively, CTLA4 is one of the key molecules of Treg in exerting inhibitory function. We thus evaluated FOXP3 and CTLA4 mRNA expression in sorted N-Treg (CD4+CD25<sup>high</sup>+ T cells) by means of real-time RT-PCR. The expression of FOXP3 in PNALT or CH patients was significantly higher than those in HS (Fig. 2a). Of note is the higher expression of FOXP3 in N-Treg from the PNALT group than in those from the CH group (Fig. 2a). In contrast, the expression of CTLA4 in N-Treg from the PNALT was higher than those in the CH, while it did not differ between the CH and HS groups (Fig. 2b).

#### CD4+CD25+ T cells from PNALT patients have more suppressive capacity in the HCV-specific CD4+ T cell response than those from CH patients

In order to compare the ability of N-Treg to inhibit the antigen-specific CD4+ T cell response, we used autologous MoDC pulsed with HCV proteins as antigen-presenting cells. We examined CD4+ T cell proliferation or cytokine



**Fig. 1** Comparison of frequencies of naturally-occurring regulatory T cells (N-Treg) and FOXP3-positive cells among the groups. (a) Gating of CD4+CD25<sup>high</sup>+ T cells under FACS analysis. The cut-off value of CD25<sup>high</sup> expression is set at a level that is more than that of CD4-CD25+ cells (dotted line); CD4+CD25<sup>high</sup>+ T cells are shown in the rectangle drawn in the representative dot plot. (b) Frequencies of FOXP3+ cells, (c) N-Treg (CD25<sup>high</sup>+FOXP3+ cells) and (d) CD25-FOXP3+ cells in CD4+ T cells were compared among the groups. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. \*,  $P < 0.05$ ; #,  $P < 0.0001$  by Mann-Whitney *U*-test. *Abbreviations*: PNALT, hepatitis C virus (HCV)-infected patients with persistently normal alanine aminotransferase (ALT) levels; CH, HCV-infected patients with elevated ALT levels; HS, healthy subjects.

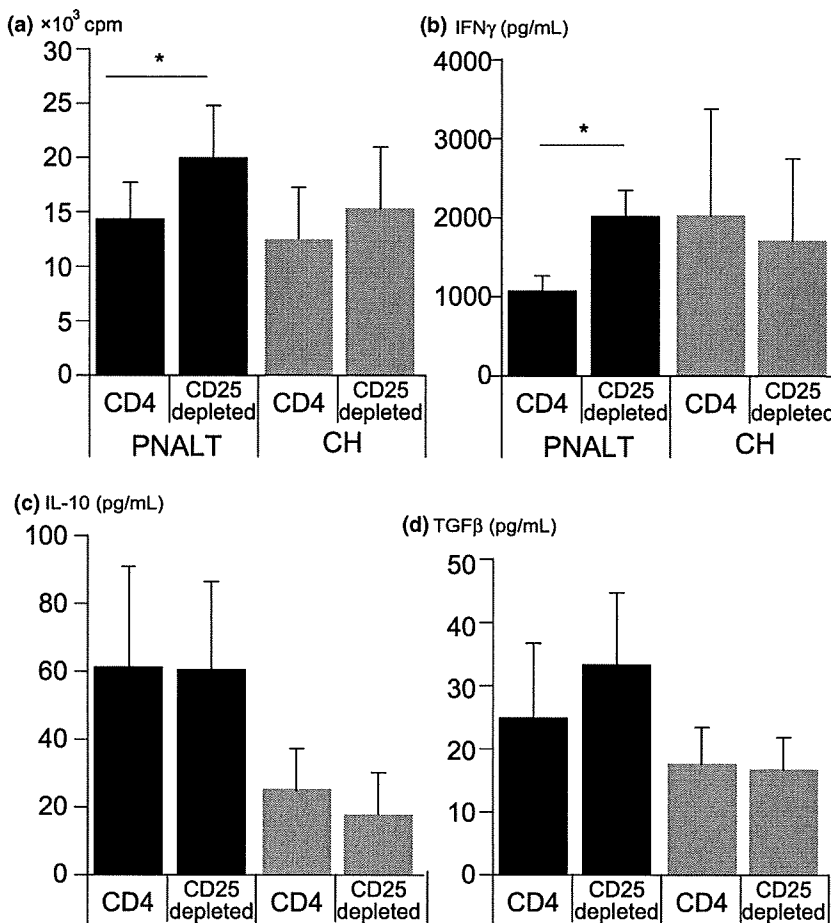


**Fig. 2** Comparison of mRNA expression of FOXP3 and CTLA4 in CD4+CD25<sup>high</sup>+ T cells among the groups. The expression of FOXP3 (a) and CTLA4 (b) in separated CD4+CD25<sup>high</sup>+ T cells were analysed by real-time reverse transcriptase-polymerase chain reaction as described in Materials and methods. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. \*,  $P < 0.05$ ; ,  $P < 0.01$ ; +,  $P < 0.001$ . For definitions of PNALT, CH and HS, see Fig. 1.

production stimulated with antigen-pulsed DC. We compared such responses between samples with or without CD4+CD25+ T cells. In PNALT patients, HCV NS5-specific T cell proliferation or IFN- $\gamma$  production of CD25-depleted CD4+ T cells was significantly higher than those of the bulk CD4+ T cells (Fig. 3a,b). In contrast, in CH patients, such restoration did not occur significantly even when CD4+CD25+ T cells had been depleted (Fig. 3a,b). There was no difference in the production of IL-10 and TGF- $\beta$  between bulk CD4+ T cells and CD25-depleted CD4+ T cells in both CH and PNALT patients (Fig. 3c,d). These results suggest that co-existing CD4+CD25+ T cells play an inhibitory role in the HCV-specific CD4+ T cell response, in which suppression was more potent in the PNALT than in the CH group.

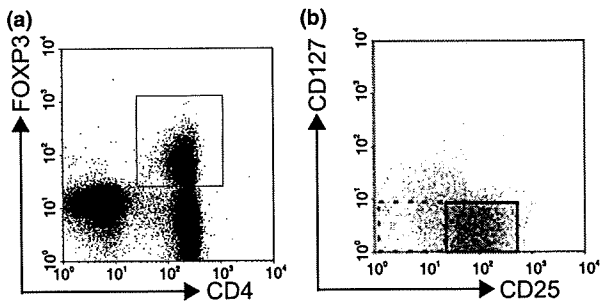
*CD127-FOXP3+ cells, regardless of their CD25 expression, are increased in patients with HCV infection*

In the analyses of N-Treg, the frequency of CD4+CD25-FOXP3+ T cells in HCV-infected patients was higher than those in the healthy donors (Fig. 1d). These results suggest that CD4+FOXP3+ T cells, regardless of the degree of CD25



**Fig. 3** Changes of hepatitis C virus (HCV)-specific CD4+ T cell responses with or without depletion of CD25+ T cells. Bulk CD4+ T cells or those depleted of CD25+ cells were cultured with autologous monocyte-derived dendritic cells in the presence of HCV-NS5 protein for 5 days as described in Materials and methods. (a) On day 4, [<sup>3</sup>H]-thymidine was pulsed and the thymidine incorporation was counted with a  $\beta$ -counter. Before the pulsing, the culture supernatants were harvested and subjected to enzyme-linked immunosorbent assay for interferon- $\gamma$  (b), interleukin-10 (c) and TGF- $\beta$  (d), respectively. \*,  $P < 0.05$  by Mann-Whitney *U*-test. For definitions of PNALT and CH, see Fig. 1.





**Fig. 4** Gating of CD4+CD127-FOXP3+ cells with variable CD25 expression under FACS analysis. After setting the gate on CD4+FOXP3+ cells [rectangle in the dot plot (a)], were displayed on the CD25 and CD127 axis (b). The presence of CD25+ (bold rectangle) and of CD25- cells (dotted rectangle) in CD4+FOXP3+ cells are shown in plot (b). The frequencies of these cells were analysed.

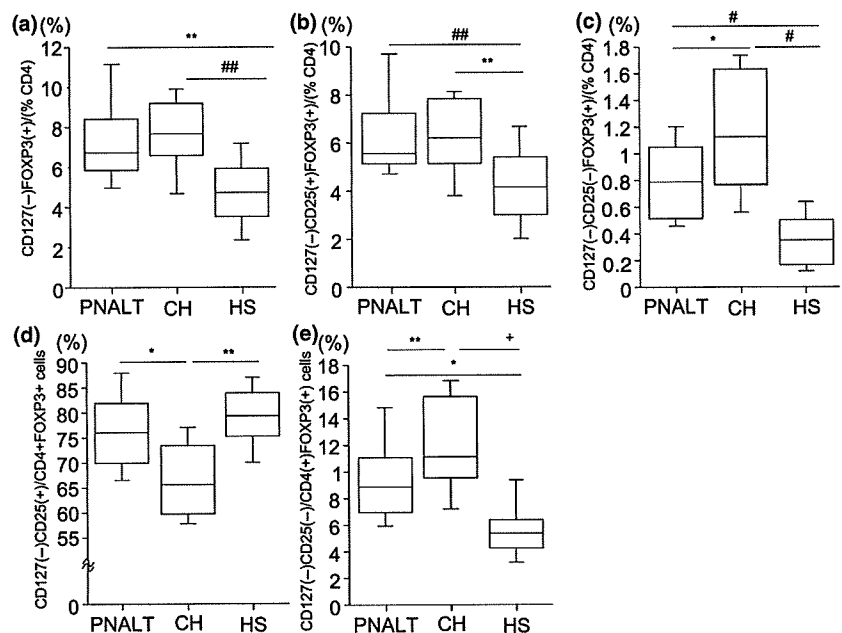
expression, increase in chronic HCV infection. Alternatively, it implies that higher expression of CD25 is not a universal marker for identifying FOXP3+ cells with regulatory activity. It has been reported that CD127 expression on CD4+ T cells is inversely correlated with FOXP3 expression, suggesting that CD127<sup>low</sup>/negative cells consist of those with regulatory activity. In order to analyse regulatory T cell subsets more precisely, we first examined FOXP3 expression on CD127- or CD127+ cells paired with CD25 expression in patients with HCV infection (Fig. 4). As a result, the majority of CD4+FOXP3+ T cells belonged to the CD127- population irrespective of CD25 expression (Fig. 4). Next, we compared the frequency of CD4+CD127-FOXP3+ cells, which consist

of CD25+ and CD25- cells, among the subject groups (Fig. 5a). The frequency of CD4+CD127-FOXP3+ cells was similar in the CH and the PNALT groups, both of which were significantly higher than those in the HS (Fig. 5a). Finally, in order to estimate the profile of CD4+CD127-FOXP3+ cells according to CD25 expression, we compared the percentage of CD25+CD127-FOXP3+ or CD25-CD127-FOXP3+ cells in CD4+ T cells among the groups. The percentage of CD25+CD127-FOXP3+ T cells in CD4+ T cells was comparable for PNALT and CH (Fig. 5b). In clear contrast, the percentage of CD25-CD127-FOXP3+ T cells in the PNALT was lower than those in the CH (Fig. 5c). The frequencies of these cells were higher in the HCV-infected patients than in HS (Fig. 5b,c). When we set the focus on the proportion of CD25+CD127- or CD25-CD127- cells in the FOXP3+ cells in the periphery as a whole, we found that the proportion of CD25+CD127- cells in the PNALT was higher than that in the CH group (Fig. 5d). On the other hand, the proportion of CD25-CD127- cells in FOXP3+ cells was lower in the PNALT than in the CH group (Fig. 5e). Therefore, the phenotypic profiles of FOXP3+ T cells are distinct between PNALT and CH patients, with regard to the expression of CD127 and CD25.

## DISCUSSION

Approximately 30–40% of chronically HCV-infected patients continue to display PNALT for decades. We previously reported the possible contribution of certain human leukocyte antigen haplotypes [23] or DC dysfunction in the maintenance of the PNALT state [24]. However, the precise mechanisms behind this important issue are yet to be

**Fig. 5** Comparison in the frequencies of CD127- regulatory T cell subsets among the groups. Frequencies of CD127-FOXP3+ (a), CD127-CD25+FOXP3 (b) and CD127-CD25-FOXP3+ (c) cells among CD4+ T cells were determined by FACS analysis. The proportion of CD127-CD25+ (d) or CD127-CD25- (e) cells in CD4+FOXP3+ cells were also determined. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. \*,  $P < 0.05$ ; ,  $P < 0.01$ ; \*\*,  $P < 0.005$ ; ##,  $P < 0.001$ ; +,  $P < 0.0001$  by Mann-Whitney *U*-test. For definitions of PNALT, CH and HS, see Fig. 1.



established. Cumulative reports have shown that Th1/Tc1 type responses are instrumental in HCV-induced liver inflammation [7,25,26]. We thus hypothesized that some suppressor mechanisms exist in PNALT patients especially against HCV-specific Th1 and/or CTL reactions.

The involvement of Treg cells in the pathogenesis of various diseases has been reported [9–13]. Most of the studies presented the possibility that N-Treg play substantial roles in the induction of tolerance against aetiological self or nonself antigens, thus leading to alleviation or exacerbation of the disease severity. With regard to HCV infection, several groups have shown that N-Treg are increased both in the periphery and in the liver and are able to inhibit HCV-specific CD4+ or CD8+ T cell responses *in vitro* [17,18,27]. In this study, we showed that the frequency of N-Treg in HCV-infected patients is higher than those in the controls, which is consistent with the previous reports. However, the frequencies of N-Treg are indistinguishable between the patient groups with different disease activities. As for the functional aspect, the deprivation of CD4+CD25+ cells enhanced the HCV NS5-specific CD4+ T cell response in the PNALT than in the CH group, suggesting that co-existing Treg in the PNALT are more suppressive. In addition, the expression of FOXP3 and CTLA4, which are key molecules of the suppressor function, is higher in PNALT than in those with active hepatitis. Venken *et al.* [28] demonstrated that the degree of FOXP3 expression at the single-cell level of N-Treg is well correlated with their suppressive ability, which is supportive of our results. In contrast, Bolacchi *et al.* [29] reported that the frequency of TGF- $\beta$ + N-Treg in the PNALT was higher than in the hepatitis group. Furthermore, their frequency was inversely correlated with the histological inflammatory grade, suggesting that TGF- $\beta$ + Treg play active roles in alleviating hepatitis. The reasons for the lack of correlation between N-Treg and serum ALT or HCV RNA quantity in the present study may be because of the difference in the target of analyses, such as either peripheral or intra-hepatic Treg, or either TGF- $\beta$ + or bulk Treg. Further analyses need to be performed on these important issues, as CD4+FOXP3+ Treg are reported to accumulate more in the portal tract of HCV-infected livers compared with those in the periphery [20].

During the observation period, about 30–40% of PNALT patients began to show elevated or fluctuating ALT abnormalities. What crucial factor triggers HCV-induced liver inflammation remains unknown. One of the plausible explanations is an antigenic shift accompanied by the occurrence of mutations in the HCV genome. In other words, hepatitis may flare up if the mutation raises HCV immunogenicity. Comprehensive analyses of HCV epitopes for CTL using overlapping peptides have shown that the HCV core and NS3 are more immunogenic than the remaining regions; however, the presence of an epitope hierarchy in Treg induction has been controversial. Li *et al.* [30] reported the possibility that Treg are expandable in response to

certain epitopes in HCV proteins. In two patients in whom we observed flare-up of hepatitis in this study, we were able to find that the expression of FOXP3 in N-Treg was high in the PNALT status, but declined in the active hepatitis stage (data not shown). Although it is difficult to state whether such phenotypic changes in N-Treg are the cause or the consequence of disease progression, these results suggest the involvement of N-Treg in the degree of HCV-mediated hepatitis. Further detailed study is needed to examine whether or not such changes in N-Treg are related to the sequence evolution in HCV genomes.

Recent research has disclosed that distinct types of Treg are present in humans. Currently, it is generally accepted that CD25+FOXP3+ is the most reliable marker for Treg, which is induced in parallel with the acquisition of suppressor ability. However, owing to the lack of phenotypic markers for specifically identifying adaptive Treg, their roles in clinical settings have been unclear. In this study, CD4+FOXP3+ cells increased in HCV-infected patients, who were either positive or negative for CD25. In contrast to thymus-derived N-Treg expressing a greater degree of CD25, adaptive Treg are presumed to be induced in the periphery with a lesser degree of CD25 expression. Thus, it is likely that CD4+CD25–FOXP3+ T cells in HCV infection contain some part of adaptive Treg.

Treg have been reported to express low levels of CD127 at their cell surface [31]. Furthermore, the expression of CD127 is inversely correlated with FOXP3 expression and with the suppressive function of CD25<sup>high</sup>+ Treg. Liu *et al.* [22] pointed out the possibility that adaptive Treg are grouped into CD127– cells, which also include FOXP3-negative Tr1 or Th3 cells. Alternatively, You *et al.* [32] reported that murine CD4+CD25<sup>low</sup>FOXP3+ T cells might be adaptive Treg, which exert a TGF- $\beta$ -dependent suppressive function. Taking these reports into consideration, and in order to exclude activated CD25+ T cells, we examined CD4+CD127–CD25–FOXP3+ cells tentatively determined as part of adaptive Treg. In order to confirm that CD4+CD127– cells possess suppressive capacity, we co-cultured sorted CD4+CD127–CD25– or CD4+CD127–CD25+ cells with allogeneic CD4+ T cells stimulated with anti-CD3 and anti-CD28 antibodies. As a result, we found that CD4+CD127– cells, regardless of CD25 expression, significantly suppressed the proliferation of responder CD4+ T cells (manuscript in preparation). Of note is the finding that the frequency of CD127–CD25–FOXP3+ cells is higher in patients with active hepatitis than those in the PNALT group. One of the plausible explanations for such an increase of Treg is the compensatory mechanisms for the aggravation of liver inflammation. In support of this possibility, Bonelli *et al.* [33] reported that CD4+CD127–CD25– cells are increased in patients with systemic lupus erythematosus (SLE), the numbers of which are well correlated with disease activity. With regard to the ability of Treg in SLE patients, CD4+CD127–CD25– cells were potent in the inhibition of T

cell proliferation but not in IFN- $\gamma$  release. Such a defective suppressor capacity may result in the continuation of tissue inflammation regardless of the presence of abundant Treg. The other conceivable role of CD4+CD25-CD127-FOXP3+ cells in active hepatitis may be a peripheral reservoir of CD4+CD25+FOXP3+ cells in case of flare-up of liver inflammation. In mice, it has been reported that CD25-FOXP3+ cells revert to CD25+FOXP3+ cells upon activation signals, thus leading to the expansion of the Treg pool [34]. In order to reach a definite conclusion on the role of CD127-CD25-FOXP3+ cells, further analyses are needed to elucidate whether these cells are inhibitory to either HCV-specific or HCV-nonspecific T cell responses.

Large-scale studies with HCV-infected patients demonstrated that the cumulative incidence of HCC in the PNALT group is extremely low compared with that in patients with apparent hepatitis and liver cirrhosis [35]. The lesser HCC incidence is also evident in patients who attained a lasting biochemical response to IFN-based therapy; even if they had failed to achieve sustained virological response [36]. These results clearly indicate that the maintenance of the PNALT state is one of the surrogate therapeutic goals in chronic HCV infection. Therefore, it is necessary to clarify the mechanisms of Treg induction in HCV infection, whether they are naturally or adaptively introduced, and to establish a feasible modality for controlling Treg. Our study has shown the importance of subset-oriented analyses of Treg for gaining access to that goal.

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#### CONFLICT OF INTEREST

All of the authors do not have any commercial or other association that might pose a conflict of interest.

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