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## Inhibition of tumor–stromal interaction through HGF/Met signaling by valproic acid

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### Abstract

Hepatocyte growth factor (HGF), which is produced by surrounding stromal cells, including fibroblasts and endothelial cells, has been shown to be a significant factor responsible for cancer cell invasion mediated by tumor–stromal interactions. We found in this study that the anti-tumor agent valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, strongly inhibited tumor–stromal interaction. VPA inhibited HGF production in fibroblasts induced by epidermal growth factor (EGF), platelet-derived growth factor, basic fibroblast growth factor, phorbol 12-myristate 13-acetate (PMA) and prostaglandin E<sub>2</sub> without any appreciable cytotoxic effect. Other HDAC inhibitors, including butyric acid and trichostatin A (TSA), showed similar inhibitory effects on HGF production stimulated by various inducers. Up-regulations of HGF gene expression induced by PMA and EGF were also suppressed by VPA and TSA. Furthermore, VPA significantly inhibited HGF-induced invasion of HepG2 hepatocellular carcinoma cells. VPA, however, did not affect the increases in phosphorylation of MAPK and Akt in HGF-treated HepG2 cells. These results demonstrated that VPA inhibited two critical processes of tumor–stromal interaction, induction of fibroblastic HGF production and HGF-induced invasion of HepG2 cells, and suggest that those activities serve for other anti-tumor mechanisms of VPA besides causing proliferation arrest, differentiation, and/or apoptosis of tumor cells.

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**Keywords:** Hepatocyte growth factor; Valproic acid; Histone deacetylase inhibitor; Butyric acid; Trichostatin A; Induction; Tumor invasion; Dermal fibroblast

Hepatocyte growth factor (HGF), also known as scatter factor, was originally discovered as a mitogenic factor of rat hepatocytes in primary culture [1–5]. HGF is now recognized as a pleiotropic factor that functions as a mitogen, motogen, morphogen, and anti-apoptotic factor acting on various types of cells [6,7]. Based on these actions, HGF has been shown to play critical roles in

developmental and regenerative events of the liver and other tissues [8–11]. In addition to regulation of normal cell functions, many studies have shown that HGF is involved in malignant cell transformation and growth, invasion and metastasis in tumor cells [12,13]. HGF is mainly produced by surrounding stromal cells such as fibroblasts and endothelial cells and stimulates growth, metastasis, and/or invasiveness of cancer cells expressing the HGF receptor Met in a paracrine manner [14]. Thus, the HGF and Met pathway is one of the most commonly cited soluble factor signaling pathways in the

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tumor–stromal interaction [14]. Clinical studies on hepatocellular carcinoma (HCC) have also suggested the involvement of interaction of HGF and c-Met in human tumor invasion and metastasis. High levels of serum HGF in patients with HCC are associated with tumor metastasis [15]. Therefore, inhibition of fibroblastic HGF production and HGF-induced aggressive behavior of tumor cells is expected to suppress proliferation, metastasis, and invasiveness of malignant tumor cells, including HCC cells.

Acetylation and deacetylation of nucleosomal core histones play an important role in the modulation of chromatin structure and the regulation of gene expression. The disruption of balance between histone acetyltransferases and histone deacetylases (HDACs) has been suggested to be associated with cancer development. HDAC activity is increased in cancer cells and has been linked to carcinogenesis [16]. Indeed, it has recently been shown that global hypo-acetylation of histone H4 is a common feature of human tumor cells [17]. Valproic acid (VPA), an effective anticonvulsant in the treatment of epilepsy, as well as butyric acid (BA) inhibits the activity of zinc-dependent class I and class II HDACs [18]. HDAC inhibitors induce proliferation arrest, differentiation, and/or apoptosis of tumor cells but not of normal cells [17]. Based on these activities, HDAC inhibitors have exhibited anti-tumor effects in clinical trials [17,19]. However, the exact mechanisms by which HDAC inhibitors exert an anti-tumor effect and modulate gene expression are not completely understood and remain a subject of intense investigation.

In the present study, we investigated whether VPA affects the HGF and Met pathway in the tumor–stromal interaction and we found that VPA potently inhibited two processes of the interaction, induction of fibroblastic HGF production and HGF-induced invasion of HepG2 HCC cells. Our results suggest that VPA exerts anti-tumor effects at least partly through the inhibition of tumor–stromal interaction which may constitute a new class of targets for chemoprevention of tumor invasion.

## Materials and methods

**Cell culture.** Human dermal fibroblasts derived from 200 individual neonatal donors (Cell Systems, Kirkland, WA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air as described previously [20]. HepG2 cells were obtained from Tohoku University (Sendai, Japan) and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

**Determination of HGF production.** The medium of confluent fibroblasts cultured in 96-well plates (Nunc, Roskilde, Denmark) was replaced with the fresh medium described in the previous section or that containing HDAC inhibitors, and the cells were preincubated for 1 h. HGF inducers were then added, and the conditioned medium was collected after being incubated for various periods. The sandwich ELISA for human HGF was performed at room temperature as described previously [21], with slight modification [22].

**MTT assay.** Confluent fibroblasts were incubated with HDAC inhibitors and HGF inducers as described in the previous section. HepG2 cells ( $5 \times 10^4$  cells/0.2 ml/well) seeded in 96-well plates (Nunc) were preincubated for 1 h with or without VPA and incubated for 24 h with or without HGF in the presence or absence of VPA. The medium was then replaced with 100 µl of the same fresh medium, and the cultures were incubated for 1 h. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed as described previously [23].

**Northern blot analysis.** The medium of confluent fibroblasts grown in 90-mm dishes (Nunc) was replaced with the same fresh medium, and the cells were incubated for about 15 h. VPA or trichostatin A (TSA) was added without a medium change, and the cells were preincubated for 1 h. The HGF inducer was then added. After being incubated for 15 or 40 h, total RNA was isolated from the cells using RNA Bee (TEL-TEST, Friendswood, TX). Northern blotting was performed as described previously [20].

**Real-time PCR analysis.** The medium of confluent fibroblasts cultured in 6-well plates (Nunc) was replaced with the same fresh medium, and the cells were incubated for 24 h. After treatment with or without cycloheximide for 1 h, the cells were incubated for 1 h with or without VPA and then for an additional 8 h with or without phorbol 12-myristate 13-acetate (PMA). Total cellular RNA was isolated as described above. After treatment with DNase, first-strand cDNA synthesis from 0.5 µg RNA was performed using reverse transcriptase with random and oligo-dT primers. Real-time PCR was performed with a Light-Cycler (Roche, Indianapolis, IN) using SYBR<sup>®</sup> Green Real-time PCR Master Mix (Toyobo Co., Osaka, Japan) according to the manufacturer's protocol. The nucleotide sequences of primers for HGF were as follows: forward, 5'-CAATAGCATGTCAAGTGGAG-3'; reverse, 5'-CTGTGTCGTGTGGTATCAT-3' (amplicon size: 180 bp). The nucleotide sequences of primers for 28S rRNA used as an internal control were: forward, 5'-GTTCACCCACTAATAGGGAACG-3'; reverse, 5'-GGATTCTGACTTAGAGGCGTTC-3' (amplicon size: 213 bp). The PCR conditions were as follows: HGF, 1 cycle of 95 °C for 30 s followed by 60 cycles of 95 °C for 5 s, 57 °C for 0 s, and 72 °C for 25 s; 28S rRNA, 1 cycle of 95 °C for 30 s followed by 55 cycles of 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 15 s. Relative cDNA copy numbers were computed on the basis of data with a serial dilution of a representative sample for each target gene.

**Western blot analysis.** The medium of subconfluent HepG2 cells grown in 24-well plates (Nunc) was replaced with the same fresh medium, and the cells were incubated for about 15 h. VPA was added without a medium change, and the cells were preincubated for 1 h. Then, HGF was added. After being incubated for an appropriate period, the cells were harvested, and Western blotting was performed as described previously [24]. In some experiments, cytosolic and nuclear extracts of the cells in 6-well plates (Nunc) were prepared according to the manufacturer's instructions (Active Motif, Carlsbad, CA).

**Cell invasion assay.** The *in vitro* invasion activities were examined as reported previously [25]. Polycarbonate membranes with 8-µm pores of Transwell<sup>®</sup> inserts (Corning, New York), upper culture chambers, were coated with 50 µl of growth factor-reduced Matrigel<sup>™</sup> (BD Biosciences, San Jose, CA) in cold RPMI 1640 medium (0.25 mg/ml) and dried overnight. HepG2 cells suspended in RPMI 1640 medium supplemented with 2% FBS were seeded onto the upper culture chambers at a density of  $1.5 \times 10^5$  cells/cm<sup>2</sup> (0.2 ml/well), whereas the lower culture chambers of 24-well plates were each filled with 0.8 ml of serum-free RPMI 1640 medium containing HGF, VPA or the combination of HGF and VPA. After the incubation for 24 h, the cells on the upper surface of the membrane were wiped off with a cotton swab. The cells that had invaded the lower surface of membranes were fixed for 10 min with methanol, stained with Giemsa solution overnight, and counted under a microscope.

**Statistical analysis.** All results were expressed as means and SEM of several independent experiments. The data were analyzed by Dunnett's *t*-test, Dunnett's T3, Tukey's test or Student's *t*-test. *P* values less than 0.05 were regarded as significant.

## Results

### *Inhibition by valproic acid and other HDAC inhibitors of HGF induction in human dermal fibroblasts*

HGF expression has been demonstrated to be up-regulated in stromal cells by tumor cell-secreted soluble growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) [26]. Therefore, human dermal fibroblasts were incubated for 72 h with VPA in the presence and absence of suboptimal doses of those growth factors. HGF secreted from the cells was then determined by an HGF ELISA. The effect of VPA on basal HGF production could hardly be determined because of the small amount of HGF produced (data not shown). VPA significantly inhibited EGF-, PDGF- and bFGF-induced HGF production with  $IC_{50}$  values of 0.15, 0.19, and 0.17 mM, respectively (Table 1). HGF production stimulated by other inducers, PMA, 8-bromo-cAMP, and prostaglandin  $E_2$  ( $PGE_2$ ), was also inhibited by VPA with  $IC_{50}$  values of 0.86, 0.48, and 0.12 mM, respectively (Table 1). The number of viable cells in the cultures treated with or not treated with PMA and 8-bromo-cAMP was not affected by any concentration of VPA as determined by the MTT method (Fig. 1A and B). On the other hand, VPA inhibited the EGF-induced increase in number of viable cells with  $IC_{50}$  of 0.42 mM and up to the level in control cultures at 2 mM (Fig. 1C), whereas cell viability, determined by the Trypan blue-exclusion test, in the cultures treated or not treated with EGF was not decreased by VPA (data not shown).

Next, we examined whether other HDAC inhibitors modulate HGF induction in human dermal fibroblasts. While BA as well as VPA inhibits class I and class IIa members of the HDAC family, TSA inhibits broad-spectrum HDACs [18]. Both BA (2 mM) and TSA (1  $\mu$ M) also significantly inhibited HGF production induced by EGF, PMA, 8-bromo-cAMP and  $PGE_2$  (data not shown).

EGF- and PMA-induced HGF production is accompanied by up-regulation of HGF gene expression [26,27]. Effects of VPA and TSA on HGF gene expression up-reg-

ulated by PMA or EGF are shown in Fig. 2A and B. VPA significantly inhibited PMA- and EGF-induced HGF mRNA expression by 32% and 81%, respectively. TSA inhibited PMA- and EGF-induced HGF mRNA expression by 90% and 61%, respectively.

### *Inhibition of HGF gene up-regulation by VPA is not blocked by cycloheximide treatment*

HDAC inhibitors generally activate expression of many genes, but the expression of some genes, including estrogen receptor  $\alpha$ , is down-regulated by histone acetylation induced by HDAC inhibitors [28]. Since the down-regulation in most cases is prevented by concomitant treatment with cycloheximide, reduction of gene expression by HDAC inhibitors is presumably dependent on the synthesis of transcriptional repressors [28]. Thus, we tested whether VPA-caused repression of HGF gene up-regulation induced by PMA is such a case. As shown in Fig. 2C, treatment with cycloheximide did not prevent VPA-caused reduction of HGF gene up-regulation.

### *Inhibition of HGF-induced invasion of HepG2 cells by valproic acid*

Tumor–stromal interaction through HGF/c-Met signaling plays an important role in growth, invasion, and metastasis of tumor cells. It has been shown that HGF stimulates the invasiveness of the human hepatoblastoma cell line HepG2 in a Matrigel cell invasion assay [29,30]. To investigate whether VPA affects the cell invasion induced by HGF, HepG2 cells were incubated with HGF, VPA or the combination of HGF and VPA for 24 h. The number of cells that invaded the filters coated with Matrigel basement membrane matrix was then counted. HGF strongly induced the invasion of HepG2 cells, as reported previously [29,30] (Fig. 3A and B). Importantly VPA significantly suppressed HGF-induced invasion of HepG2 cells (Fig. 3A and B), whereas the number of viable cells, determined by the MTT assay, in the cultures treated with HGF was not decreased by VPA (Fig. 3C). BA (2 mM) and TSA

Table 1  
Inhibition of HGF induction by VPA

HGF inducer	Secreted HGF (ng/mg cellular protein)						$IC_{50}$ (mM)
	VPA (mM)						
	0	0.1	0.2	0.5	1	2	
EGF	6.9 ± 0.4	4.3 ± 0.5**	2.4 ± 0.3***	1.1 ± 0.2***	1.3 ± 0.4***	0.9 ± 0.5***	0.15
PDGF	8.9 ± 1.0	6.3 ± 1.2	4.1 ± 0.5***	1.5 ± 0.1***	0.7 ± 0.2***	0.9 ± 0.9***	0.19
bFGF	7.3 ± 0.9	4.8 ± 0.1**	3.2 ± 0.4***	1.5 ± 0.4***	0.4 ± 0.4***	1.4 ± 0.4***	0.17
PMA	31.2 ± 3.3	33.0 ± 2.2	32.4 ± 3.3	25.0 ± 3.3	11.9 ± 0.6***	3.5 ± 0.6***	0.86
8-Bromo-cAMP	27.5 ± 1.9	22.0 ± 1.8	18.5 ± 2.2**	13.4 ± 2.1***	8.6 ± 0.9***	6.1 ± 0.8***	0.48
$PGE_2$	11.7 ± 1.5	6.6 ± 1.6*	3.0 ± 0.9*	ND	ND	ND	0.12

Confluent fibroblasts were preincubated for 1 h with the indicated concentrations of VPA and then incubated for 24 h with or without 3 nM PMA, for 72 h with or without 3 ng/ml of EGF, 10 ng/ml of PDGF, 3 ng/ml of bFGF or 0.3 mM 8-bromo-cAMP and for 120 h with or without 30  $\mu$ M  $PGE_2$  in the presence or absence of VPA. The data are means ± SEM of three independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, as compared with the values of respective inducer alone. ND, not detectable.

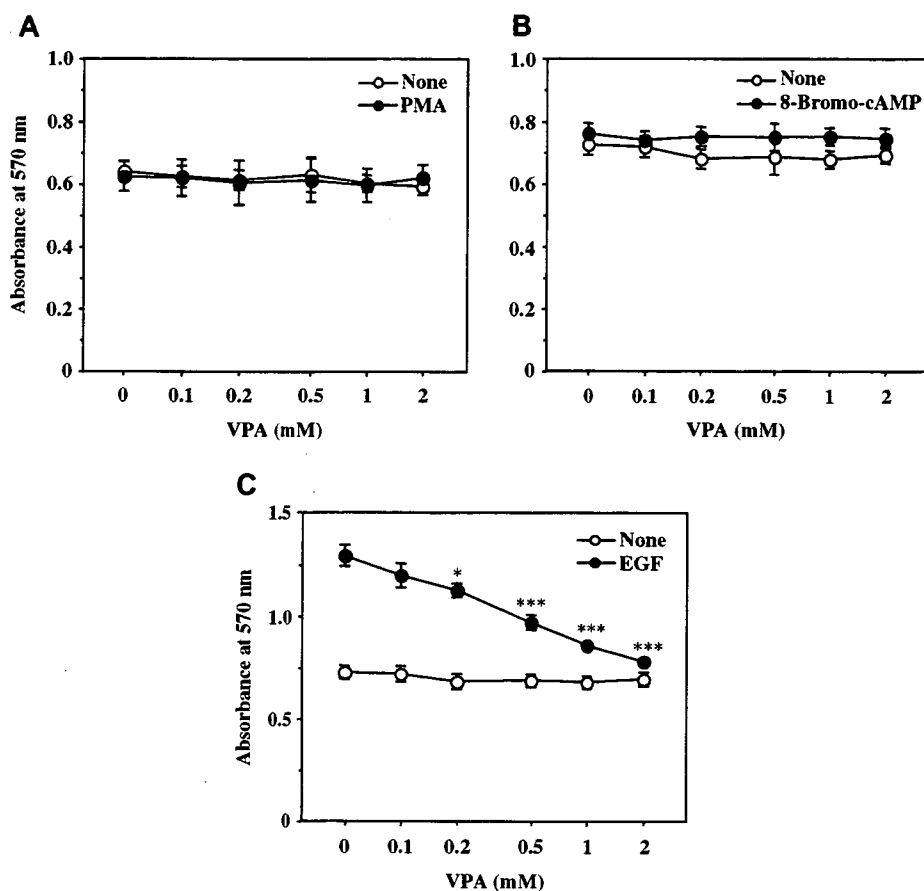


Fig. 1. VPA has no cytotoxic effects on fibroblasts treated with or without PMA and 8-bromo-cAMP and inhibits EGF-induced cell proliferation. Confluent cells were preincubated for 1 h with the indicated concentrations of VPA and then incubated for 24 h with or without 3 nM PMA and for 72 h with or without 0.3 mM 8-bromo-cAMP or 3 ng/ml of EGF in the presence or absence of VPA. The number of viable cells was measured by the MTT method. The data are means  $\pm$  SEM of three independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$ , as compared with the values of medium alone or respective inducer alone.

(1  $\mu$ M) also significantly inhibited HGF-induced invasion of HepG2 cells (data not shown).

It has been suggested that HGF induces cell invasion of HepG2 cells via the ERK pathway and phosphatidylinositol 3-kinase (PI3K)-Akt pathway [30,31]. VPA (2 mM), however, did not show any significant effect on phosphorylation of ERK and Akt or nuclear translocation of phosphorylated ERK in the cells treated for 15, 30, and 60 min with HGF (10 ng/ml) (data not shown).

## Discussion

Enhancement of cancer cell invasion by stromal fibroblasts has been demonstrated in a variety of cancers, including breast, gallbladder, esophageal, and prostate cancers [32]. HGF, which is mainly secreted by surrounding stromal cells, including fibroblasts and endothelial cells, has been shown to be a significant factor responsible for cancer cell invasion mediated by tumor-stromal interactions [14]. Elevated levels of tumor and plasma HGF have

been observed in a number of cancers such as lung, breast, prostate, and hepatocellular cancers [33]. We demonstrated in this study that VPA and other HDAC inhibitors blocked tumor-stromal interactions at two steps: inhibition of HGF induction in human dermal fibroblasts and suppression of HGF-induced HepG2 cell invasion. Thus, HDAC inhibitors have other anti-tumor mechanisms besides causing proliferation arrest, differentiation, and/or apoptosis of tumor cells.

HGF expression has been demonstrated to become up-regulated in stromal cells by tumor cell-secreted soluble cytokines and growth factors, including IL-1 $\alpha$ , IL-1 $\beta$ , EGF, PDGF, and bFGF [26,27]. PGE<sub>2</sub>, which is implicated in the proliferation and invasiveness of tumor cells [30], also induces HGF production in fibroblasts [20]. Induction of HGF by EGF, other growth factors and PGE<sub>2</sub> was more sensitive to VPA than was HGF induction by other inducers, the IC<sub>50</sub> being approximately 0.15 mM. This concentration is clinically achieved in plasma of patients treated with VPA for epilepsy, e.g., a mean trough

plasma level of 0.44 mM after 3-month treatment with an average daily dose of 720 mg [34]. The values in the MTT assay of cultures of human dermal fibroblasts and HepG2 cancer cells were not decreased by treatment with VPA except for those in EGF-stimulated human dermal fibroblasts. In accordance with the known antiproliferative

activity of the short-chain fatty acid, VPA decreased the values in the MTT assay induced by EGF up to those of control cultures not treated with EGF. In addition, VPA did not decrease cell viability, determined by the Trypan blue-exclusion test, in cultures treated or not treated with EGF. These findings collectively indicate that VPA is not cytotoxic to the cells.

Histone acetylation causes alteration of nucleosomal conformation and thus increases the accessibility of transcriptional regulatory proteins to chromatin templates and subsequent transcription [35]. These changes are thought to be one mechanism by which HDAC inhibitors generally activate expression of many genes. Nevertheless, the expression of some genes, including estrogen receptor  $\alpha$  gene, the oncogene MYC, and tumor suppressor gene p53, is down-regulated by histone acetylation induced by HDAC inhibitors [28]. Since VPA- and TSA-caused reduction in expression of most of those genes is blocked by concomitant treatment with cycloheximide, down-regulation of those genes by HDAC inhibitors is presumably dependent on the synthesis of transcriptional repressors [28]. Inhibition of up-regulated HGF gene expression by VPA, however, was not prevented by cycloheximide and was thus not such a case.

VPA potently inhibited HepG2 cell invasion induced by HGF without suppressing cell proliferation. Cell invasion is a major event involved in the complex multistep process of tumor metastasis. Invasion of tumor cells requires destruction of basement membranes, proteolysis of extracellular matrix (ECM), pseudopodial extension, and cell migration [36]. After modifying the ECM barrier, tumor cells migrate through the barrier and proliferate at a secondary site. HGF is one of the potent motility factors and contributes to metastasis by stimulation of motility [12]. Activation of PI3K-Akt and ERK pathways has been suggested to be critical for cell motility stimulated by HGF [30,31,37], but phosphorylation of neither Akt nor ERK in

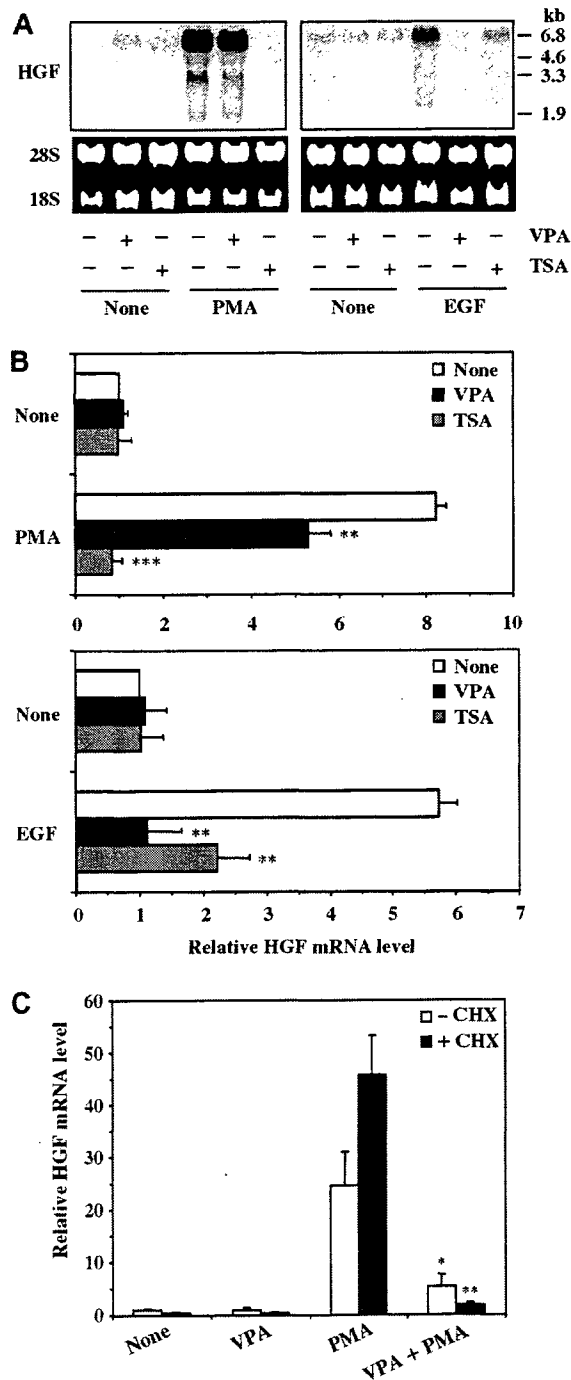


Fig. 2. VPA- and TSA-caused inhibition of HGF gene expression up-regulated by PMA or EGF and effects of cycloheximide on VPA-caused inhibition. (A, B) Confluent fibroblasts were preincubated for 1 h with or without 2 mM VPA or 1  $\mu$ M TSA and then incubated for 15 h with or without 3 nM PMA or for 40 h with or without 3 ng/ml of EGF in the presence or absence of VPA or TSA. The signal intensity of the 6.4-kb HGF mRNA band in the autoradiograms was normalized to the fluorescence intensity of the 28S rRNA band, and results are expressed as relative levels to the value of untreated cells. The data are means of three independent experiments. Bars indicate SEM. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , as compared with the values of medium alone or respective inducer alone. (C) Confluent fibroblasts were preincubated for 1 h with or without 1  $\mu$ g/ml of cycloheximide (CHX) and then for 1 h with or without 2 mM VPA followed by incubation for an additional 8 h with or without 3 nM PMA. The expression levels of HGF mRNA and 28S rRNA were measured by real-time PCR. Results are expressed as relative levels to the value of untreated cells after being normalized to the 28S rRNA levels. The data are means of three independent experiments. Bars indicate SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , as compared with the values of the respective PMA alone.

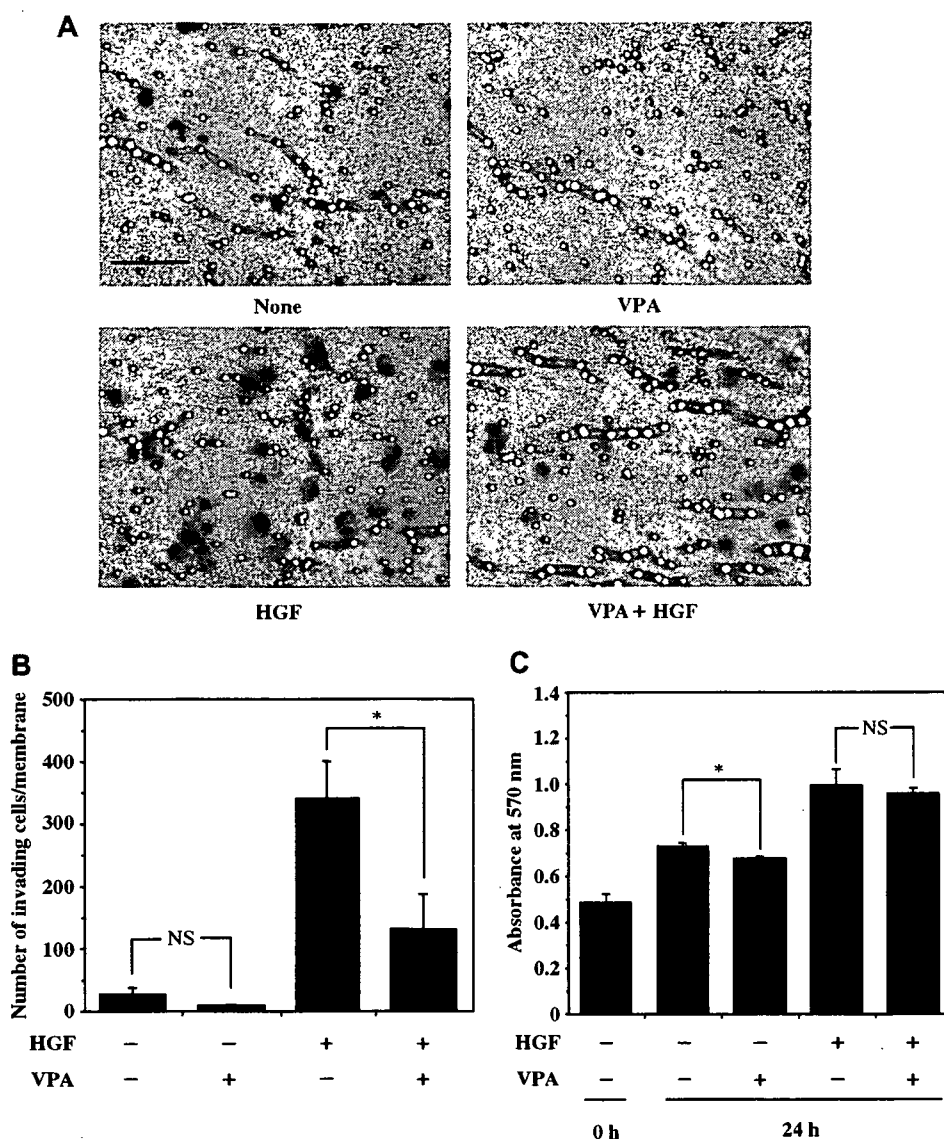


Fig. 3. Inhibition of HGF-induced invasion of HepG2 cells by VPA. (A, B) HepG2 cells, seeded onto the upper chamber consisting of filters coated with Matrigel™, were preincubated for 1 h with or without of 2 mM VPA added to the lower chamber and then incubated for 24 h with or without 10 ng/ml of HGF added to the lower chamber. Cells that invaded the filter were visualized and counted microscopically at  $\times 200$  magnifications. Scale bar = 100  $\mu\text{m}$ . (C) HepG2 cells seeded in 96-well plates were preincubated for 1 h with or without 2 mM VPA and incubated for 24 h with or without 10 ng/ml of HGF in the presence or absence of VPA. The number of viable cells was then measured by the MTT method. The data are means of four (invasion) or three (MTT) independent experiments. Bars indicate SEM. \* $P < 0.05$ , as compared with the values of medium alone or HGF alone. NS, not significant.

HepG2 cells stimulated with HGF was reduced by treatment with VPA. Although its mechanism remains to be investigated, inhibition of tumor cell invasion by HDAC inhibitors may constitute a new class of strategies of chemoprevention of tumor metastasis.

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## Alanine aminotransferase flare-up in hepatitis C virus carriers with persistently normal alanine aminotransferase levels in a hyperendemic area of Japan

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**Background.** The clinical features of hepatitis C virus (HCV) carriers with persistently normal alanine aminotransferase (PNALT) levels (ALT  $\leq$  34 IU/l) have not been fully elucidated. We investigated clinical factors associated with ALT flare-up in PNALT individuals in a HCV hyperendemic area of Japan. **Methods.** We analyzed 101 HCV carriers who had PNALT between 1993 and 2000. The first occurrence of ALT flare-up (ALT  $\geq$  35 IU/l) between 2001 and 2005 was evaluated by the Kaplan-Meier method. Multivariate analysis of factors predicting ALT flare-up were conducted using Cox proportional hazards models. **Results.** The mean follow-up period was 2.8 years, and the 5-year cumulative incidence of ALT flare-up was estimated to be 31.8%. In multivariate analysis, an ALT level of 20–34 IU/l and a high serum ferritin level ( $\geq$ 90 ng/ml) in the most recently available data up to the year 2000, as well as H63D heterozygosity in the *HFE* gene, were independently and strongly associated with the incidence of ALT flare-up (Hazard ratios = 5.6, 3.1, and 4.8, respectively). In addition, *HFE* H63D heterozygosity was significantly associated with higher serum ferritin levels in subjects with PNALT (153.8  $\pm$  73.3 ng/ml in subjects with the 63HD genotype vs. 89.4  $\pm$  51.3 ng/ml in subjects with the 63HH genotype,  $P = 0.043$ ). **Conclusions.** HCV carriers with PNALT in this population were at risk for ALT flare-up. Basal ALT levels, serum ferritin levels, and *HFE* polymorphism are potentially important predictors of ALT flare-up.

**Key words:** hepatitis C virus, persistent normal ALT, community-based population, ferritin, ALT flare-up

### Introduction

Persistent hepatitis C virus (HCV) infection is one of the most common causes of chronic liver disease, liver cirrhosis, and hepatocellular carcinoma (HCC).<sup>1–3</sup> The progression of HCV infection to hepatic fibrosis and HCC is associated with several factors, including elevated levels of alanine aminotransferase (ALT), duration of infection, age, and sex.<sup>4–7</sup> Short-term studies have shown that 20%–30% of patients with persistent HCV infection have persistently normal serum ALT levels and minimal necroinflammatory changes in the liver. Liver damage in these HCV carriers does not appear to progress to severe hepatitis or HCC.<sup>8–10</sup> For this reason, HCV-infected patients with persistently normal ALT (PNALT) are typically not treated for infection or examined by liver biopsy.<sup>11,12</sup> However, there have also been reports indicating that hepatic fibrosis can progress slowly even when serum ALT levels remain normal,<sup>13,14</sup> suggesting that PNALT patients should be treated and biopsied.<sup>15</sup> Recently, Tanaka et al.<sup>6</sup> reported that individuals with normal ALT levels are still at risk for developing HCC. These contrasting findings may result from differing clinical definitions of ALT abnormality, the time frame for defining persistence, and patient age at the time of infection or liver biopsy. Because of these ambiguities, the clinical features and disease progression in HCV carriers with PNALT remain unclear and warrant investigation.

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ALT reactivation can occur many years after infection in some PNALT patients,<sup>10,16,17</sup> potentially leading to progressive liver damage. Although the efficacy of combination therapy with interferon and ribavirin or interferon monotherapy in PNALT patients may be similar to that in patients with abnormal ALT levels,<sup>18,19</sup> these therapies are expensive, effective in only 50% of patients, poorly tolerated, and unsuitable for some patient populations, especially older individuals. Because of this variability, it is important to define the clinical features of HCV carriers with PNALT, especially in older patients. This information will help identify HCV carriers at risk for fibrosis and HCC and help determine the best treatment options.

Since 1993, we have been following HCV-seropositive residents in a hyperendemic area of Japan. Our previous studies of this community-based population showed that abnormal ALT levels ( $\geq 35$  IU/l) were associated with a fourfold increased risk of HCC.<sup>20</sup> Because of reports that HCV patients with normal ALT levels are also at risk for HCC, we decided to elucidate the clinical and virological features of HCV carriers with PNALT. The present analysis focuses on ALT flare-up in HCV carriers with PNALT. In addition, the average age of subjects in this study was 71.4 years, which is older than the average age of HCV carriers in the United States. Because it is estimated that the age of HCV carriers in the United States and Europe will increase over the next two or three decades, becoming more similar to the situation in Japan,<sup>21</sup> this seminal study provides important clinical information applicable to other HCV patient populations.

## Methods

### *Study population*

Between 1993 and 1995, we examined 1151 residents who tested positive for anti-HCV antibodies in a hyperendemic area (Town C) of Japan.<sup>22</sup> The overall prevalence of anti-HCV antibodies was higher (20.6%) in this region than in the surrounding area. As part of a collaborative effort between the University of Miyazaki, the local government, and the public health service, an ultrasonography screening program was started in 1994 to detect HCC in HCV seropositive residents of Town C. In 2001, a clinical research study was initiated in conjunction with the liver disease screening program.

Of these residents, 440 HCV carriers with at least four annual ALT measurements between 1993 and 2000 were included in the present analysis. These subjects tested positive for HCV core antigen (HCVcAg) or HCV RNA at least 6 months after their initial anti-HCV screening and were diagnosed as having persistent

HCV infection (HCV carriers) in 1995. Although these subjects included HCV carriers who had taken oral or intravenous medical herbs or other palliative therapies, we excluded those subjects who had received interferon therapy or were diagnosed with HCC before 2000. Subjects with normal ALT levels between 1993 and 2000 were considered to have PNALT in 2000.

### *Serological studies and viral markers*

Between 1993 and 1995, HCV-specific antibodies were detected using a second-generation enzyme immunoassay kit (Immunocheck F-HCV Ab, International Reagents, Kobe, Japan). Biochemical tests were also performed to measure levels of ALT (normal value,  $< 35$  IU/l), aspartate aminotransferase (normal value,  $< 40$  IU/l), and  $\gamma$ -glutamyl transpeptidase (normal values: males,  $< 70$  IU/l; females,  $< 30$  IU/l) annually from 1993 to 2000. ALT levels in HCV-infected patients can be affected by the progression of liver fibrosis, and platelet counts correlate with the progression of liver fibrosis. However, platelet counts were not obtained before 2001 and could not be included in this study. Serum levels of HCVcAg were determined by a fluorescence enzyme immunoassay (Immunocheck F-HCV Ag Core, International Reagents),<sup>23</sup> with a detection threshold of 8 pg/ml of HCVcAg. For anti-HCV antibody-positive residents with HCVcAg levels below 8 pg/ml, HCV RNA was examined in 1995 by a qualitative reverse transcription polymerase chain reaction (PCR) assay (Amplicore HCV, Roche Diagnostics, Tokyo, Japan). The serologically defined genotype (serotype) of HCV was determined using a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents). We also examined patient ferritin levels (normal values: males,  $\geq 24$  and  $\leq 286$  ng/ml; females,  $\geq 7$  and  $\leq 110$  ng/ml) using serum stored from 1996 to 2000.

### *Mutational analysis of the HFE gene*

Mild to moderate iron overload is associated with liver injury in patients with chronic hepatitis C. *HFE* mutations could be associated with excess iron loading in patients with chronic hepatitis C. We determined whether *HFE* mutations were associated with ALT flare-up in subjects with PNALT. The following three major point mutations in *HFE* have been associated with hereditary hemochromatosis: cysteine to tyrosine at amino acid 282 (C282Y), serine to cysteine at amino acid 65 (S65C), and histidine to aspartic acid at amino acid 63 (H63D). To test for these mutations in PNALT HCV carriers, genomic DNA was extracted using a MagExtractor System MFX-2000 (Toyobo, Osaka, Japan), according to the manufacturer's protocols.

Real-time PCR allelic discrimination assays were designed using TaqMan single nucleotide polymorphism (SNP) genotyping assays (Applied Biosystems, Foster City, CA, USA). Typing reagents for the *HFE* gene SNPs G845A (dbSNP ID: rs1800562; TaqMan SNP genotyping assay ID, C\_1085595\_10), which confers a C282Y mutation, and C187G (dbSNP ID: rs1799945; TaqMan SNP genotyping assay ID, C\_10856009\_10), which confers a H63D mutation, were purchased from Applied Biosystems. Genotyping of the A193T SNP (dbSNP ID: rs28934888) in the *HFE* gene, which confers a S65C mutation, was performed using the following primers and probes: primer F (GACCAGCTGTTCTGTTCTATGAT), primer R (CCACATCTGGCTTGAAATTCTACTG), probe F (ACGGCGACTCTCAT, labeled with the dye VIC), and probe R (CGGCGACACTCAT, labeled with the dye FAM), with a custom TaqMan genomic assay. Briefly, 5 ng of DNA were mixed with the Allelic Discrimination Assay Mix (900 nM of each forward and reverse primer and 200 nM of each reporter dye (FAM or VIC)-labeled probe) and TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR conditions were 50°C for 2 min with AmpErase uracil *N*-glycosylase and 95°C for 10 min, followed by 40 cycles of 92°C for 15 s, and 60°C for 1 min. Genotypes were assessed by the TaqMan allele-specific assay method using the ABI Prism 7000 Sequence Detection System, according to the manufacturer's protocols (Applied Biosystems). All genotypes were scored using the allelic discrimination program of ABI software.

#### Follow-up of subjects

At the beginning of 2001, ALT measurements were obtained an average of twice a year until September 2005. An increase in ALT levels to greater than 35 IU/l was considered an ALT flare-up. For those subjects in whom all prior ALT measurements had been normal, the follow-up period for ALT flare-up spanned from 2001 until (1) the date of the initial ALT flare-up, (2) the last sequential ALT measurement, or (3) the conclusion of the study in September 2005, whichever occurred first.

#### Statistical analysis

All statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA, USA) or SPSS software (SPSS, Chicago, IL, USA). The cumulative incidence of ALT flare-up was analyzed by the Kaplan-Meier method; the differences in the curves were evaluated by a log-rank test. Multivariate analysis was performed by Cox proportional hazards models. Fisher's exact test or the Mann-Whitney *U* test was also

used, where appropriate. A *P* value of less than 0.05 was considered statistically significant.

## Results

In 2000, 159 of the 440 (35.7%) HCV carriers were considered to have PNALT. Of the 159 subjects with PNALT, 58 subjects did not have ALT measurements beginning in 2001 and were excluded from the present analyses. Table 1 shows the characteristics of the remaining 101 subjects with PNALT who were included in this study to analyze the incidence of ALT flare-up.

The mean follow-up period was 2.8 years in the 101 subjects with PNALT. Over this 2.8-year period, 21 subjects experienced an ALT flare-up, with an estimated five-year cumulative flare-up incidence of 31.8%, determined using the Kaplan-Meier method (Fig. 1). The cumulative incidence of ALT flare-up after 2001 was similar between men and women (data not shown).

Based on univariate analysis, age in 2000, HCVcAg level in 1995, sex, and HCV serotype were not associated with an increased rate of ALT flare-up in subjects with PNALT (Table 2). An ALT level of 20–34 IU/l [hazard ratio (HR) = 4.72] and a serum ferritin level  $\geq 90$  ng/ml (HR = 2.96) in the most recently available data up to 2000 were associated with a significantly increased rate of ALT flare-up. In addition, although

**Table 1.** Demographic and virologic data for 101 subjects positive for HCV core antigen or HCV RNA that had at least four annual ALT measurements available between 1993 and 2000

Characteristics	<i>n</i> = 101
Age <sup>a</sup>	71.4 ± 7.9 (101)
Sex, male / female	22 / 79
Body mass index	22.2 ± 2.8 (101)
Alcohol intake (none / occasional or daily)	68 / 33
HCV core antigen (pg/ml) <sup>b</sup>	194.8 ± 196.4 (90)
HCV serotype (I/II) <sup>c</sup>	57 / 34
AST (IU/l) <sup>d</sup>	28.2 ± 7.2 (101)
ALT (IU/l) <sup>d</sup>	19.6 ± 5.0 (101)
$\gamma$ -GTP (IU/l) <sup>d</sup>	16.6 ± 9.5 (101)
HbA1c (%) <sup>d</sup>	5.6 ± 0.5 (78)
Total cholesterol (mg/dl) <sup>d</sup>	179 ± 35.1 (101)
Triglyceride (mg/dl) <sup>d</sup>	122.4 ± 74.3 (101)
Hemoglobin (g/dl) <sup>d</sup>	13.2 ± 1.0 (79)
Serum ferritin (ng/ml) <sup>d</sup>	94.6 ± 54.7 (88)

Data are shown as means ± SD (number of subjects examined)  
HCV, hepatitis C virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase;  $\gamma$ -GTP,  $\gamma$ -glutamyl transpeptidase; HbA1c, hemoglobin A1c

<sup>a</sup>In 2000

<sup>b</sup>Excluding subjects with HCV core antigen levels below 8 pg/ml

<sup>c</sup>Excluding subjects whose HCV serotype was undetermined.

<sup>d</sup>Most recently available data between 1996 and 2000

**Table 2.** Results of univariate analysis for ALT flare-up in subjects with persistently normal ALT

Variable	Number of patients	Hazard ratio	95% CI	P value
Age (years) <sup>a</sup>				
<65	15	1.0		
≥65	86	0.63	0.21–1.87	0.40
Sex				
Female	79	1.0		
Male	22	1.19	0.40–3.55	0.76
Body mass index				
<25	82	1.0		
≥25	19	1.36	0.526–3.51	0.53
Alcohol intake				
none	68	1.0		
occasional or daily	33	1.10	0.45–2.70	0.83
HCVcAg <sup>b</sup> (pg/ml)				
<100	46	1.0		
≥100	55	2.34	0.91–6.04	0.71
HCV serotype <sup>c</sup>				
Type 1	57	1.0		
Type 2	34	1.18	0.49–2.85	0.08
AST (IU/l) <sup>d</sup>				
<30	60	1.0		
≥30	41	1.70	0.72–4.00	0.23
ALT (IU/l) <sup>d</sup>				
<20	51	1.00		
20–34	50	4.72	1.59–14.03	<0.01
γ-GTP (IU/l) <sup>d</sup>				
<20	76	1.0		
≥20	25	1.08	0.42–2.80	0.87
HbA1c (%) <sup>d</sup>				
<5.9	51	1.0		
≥5.9	27	1.22	0.44–3.44	0.70
Total cholesterol (mg/dl) <sup>d</sup>				
<180	54	1.0		
≥180	47	1.16	0.49–2.73	0.74
Triglyceride (mg/dl) <sup>d</sup>				
<120	66	1.0		
≥120	35	1.90	0.81–4.48	0.14
Hemoglobin (g/dl) <sup>d</sup>				
<14	61	1.0		
≥14	18	1.88	0.71–4.96	0.20
Serum ferritin (ng/ml) <sup>d</sup>				
<90	45	1.0		
≥90	43	2.96	1.17–7.49	0.02
<i>HFE</i> H63D				
HH (wild)	87	1.0		
HD (mutation)	7	3.52	1.18–10.49	0.02

CI, confidence interval

<sup>a</sup>In 2000<sup>b</sup>HCV core antigen<sup>c</sup>Excluding subjects whose HCV serotype was undetermined<sup>d</sup>Most recently available data between 1996 and 2000.

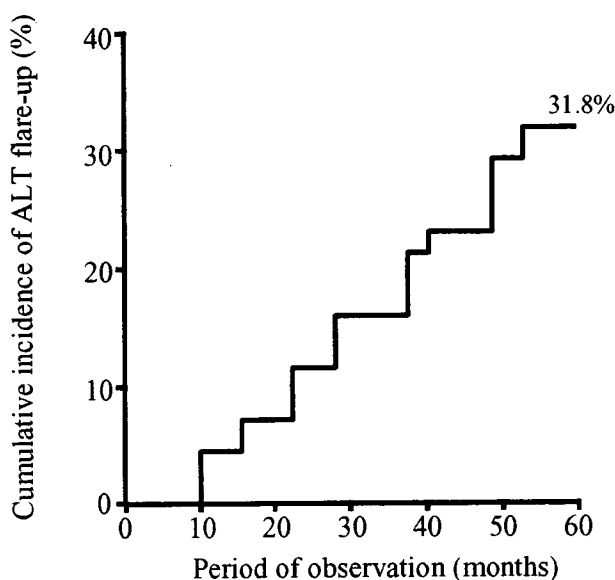
none of the subjects carried the C282Y or S65C *HFE* mutation or were homozygous for 63D/D in the *HFE* gene, we observed an association between the H63D *HFE* mutation and ALT flare-up in PNALT subjects (HR = 3.52). In a multivariate regression analysis includ-

ing ALT, serum ferritin level, and presence of the *HFE* H63D mutation as variables, ALT (HR = 5.59), serum ferritin levels (HR = 3.10), and *HFE* H63D mutation (HR = 4.75) remained significant independent factors associated with the incidence of ALT flare-up in sub-

**Table 3.** Results of multivariate analysis for ALT flare-up in subjects with persistently normal ALT

Variable		Hazard Ratio (95% CI)	P value
ALT <sup>a</sup>	20–34 IU/l	5.59 (1.78–17.55)	0.003
Serum ferritin <sup>a</sup>	≥90 ng/ml	3.10 (1.21–8.01)	0.019
<i>HFE</i> H63D	Mutation, HD	4.75 (1.51–14.90)	0.008

<sup>a</sup>Most recently available data between 1996 and 2000



**Fig. 1.** Cumulative incidence of alanine aminotransferase (ALT) flare-up in subjects with persistently normal ALT levels, based on the Kaplan-Meier method

jects with PNALT (Table 3). In addition, serum levels of ferritin were significantly higher in subjects with the H63D mutation than in subjects without the mutation ( $153.8 \pm 73.3$  ng/ml in subjects with the 63HD genotype vs.  $89.4 \pm 51.3$  ng/ml in subjects with the 63HH genotype,  $P = 0.043$ ). The incidence of ALT flare-up was also significantly higher in subjects with the 63HD genotype than those with the 63HH genotype (57.1% vs. 19.5%,  $P = 0.042$ ).

## Discussion

This study examined a HCV hyperendemic area, where the prevalence of anti-HCV antibodies and persistent HCV infection are 20.6% and 70.7%, respectively.<sup>22,24</sup> In the present analysis, we focused on ALT flare-up in subjects with PNALT, and demonstrated that subjects with PNALT were at risk for ALT flare-up. Specifically, basal ALT levels and serum ferritin levels before ALT-

flare-up and an *HFE* mutation (H63D) were correlated with ALT flare-up in PNALT subjects.

Previously, there has not been a clear definition of PNALT, either in terms of normal ALT levels or the time period of observation.<sup>11,25–28</sup> One report defined PNALT as three consecutive measurements within the normal ALT range during a 6-month period.<sup>11</sup> Puoti et al.<sup>16</sup> suggested that HCV carriers with normal ALT levels need to be observed for at least 18 months before they can be categorized as PNALT patients. Recently, Alberti<sup>29</sup> reported that the prevalence of cases with significant fibrosis was higher in studies in which PNALT status was determined from shorter observation periods or fewer ALT measurements than in studies in which PNALT classification was based on a longer observation period or more ALT tests. For this reason, we chose to define PNALT as having ALT levels within the normal range (<35 IU/l) over at least four independent, annual measurements. In addition, the PNALT guideline recommends that patients with ALT levels greater than 30 IU/l be treated the same as patients with chronic hepatitis C in Japan. We also analyzed the association between ALT flare-up and other factors if normal ALT levels were defined as values less than 30 IU/l. Eighty-five subjects with low PNALT (all ALT values  $\leq 30$  IU/l) were included to analyze the incidence of ALT flare-up (ALT > 30 IU/l). In a multivariate regression analysis including ALT levels (20–30 IU/l), serum ferritin levels, and the presence of the *HFE* H63D mutation as variables, ALT [HR = 3.02; 95% confidence interval (CI), 1.25–7.30] remained an independent factor associated with the incidence of ALT flare-up in subjects with PNALT (all ALT levels  $\leq 30$  IU/l). These results suggest that ALT levels are the most important factor for ALT flare-up in our study.

Okanoue et al.<sup>30</sup> previously showed that ALT levels increased in 86% of PNALT patients over 5 years. In our study, the estimated 5-year cumulative flare-up incidence was 31.8%, lower than the incidence rate reported by Okanoue et al. Only 33 of 80 (45%) PNALT patients without flare-up were followed until September 2005, and the mean follow-up period without flare-up was 2.9 years. It is possible that the ALT flare-up rate is lower in our study owing to the short follow-up period or the small number of subjects.

Our study suggests that ALT flare-up may be associated with higher serum ferritin levels, as previously reported.<sup>31</sup> Serum ferritin levels correlate with iron loading, a factor linked to HCV-associated fibrosis progression.<sup>32</sup> Serum iron, serum ferritin, and transferrin saturation are commonly elevated in patients with HCV.<sup>33,34</sup> The levels of these serum iron markers are indicators of hepatic iron stores, which may affect hepatic stellate cell activation and fibrosis progression.<sup>32,35</sup> Despite the links between HCV infection and hepatic iron load, the association between serum iron levels and ALT flare-up in patients with PNALT is not well understood. Vendemiale et al.<sup>31</sup> reported that an impaired redox state confers an increased risk of ALT flare-up in HCV carriers with PNALT (ALT levels  $\leq$  40 IU/l, measured every 2 months for at least 6 months); thus, an altered hepatic oxidative balance may have prognostic significance with respect to disease activity.<sup>31</sup> Serum ferritin levels correlate with hepatic iron. Excess hepatic iron storage may induce an altered hepatic redox state,<sup>31,36</sup> with high levels of intrahepatic iron accelerating liver injury and ALT flare-up. Thus, the storage of intrahepatic iron may contribute to liver injury, leading to fibrosis and HCC. Recently, Furutani et al.<sup>37</sup> reported that iron overload induces mitochondrial injury and increases the risk of HCC development in transgenic mice expressing the HCV polyprotein. They also showed that HCV transgenic mice fed an excess-iron diet showed significantly higher ALT levels in their serum than did control mice fed the same excess-iron diet (at 6 months after initiation of feeding). These results indicate that a combination of persistent HCV infection and iron overload may influence ALT flare-up in subjects with PNALT.

The presence of heterozygous *HFE* mutations is associated with higher hepatic iron stores and advanced fibrosis stage in patients with chronic hepatitis C.<sup>33,38</sup> Although heterozygous *HFE* mutations are rare in our study population, there was a significant association between an H63D mutation in the *HFE* gene and ALT flare-up in subjects with PNALT. We also observed that HCV carriers with PNALT who had the H63D *HFE* mutation had higher serum ferritin levels. Although individuals heterozygous for this *HFE* mutation are at low risk of iron overload,<sup>39</sup> this mutation in HCV carriers with PNALT may slowly affect hepatic iron levels and contribute to ALT flare-up over an extended period of time.

ALT levels in HCV-infected patients can be influenced by other factors, such as alcohol consumption or serum HCV RNA.<sup>40-43</sup> Although histological examinations were not performed, no association was observed between either alcohol consumption or HCVcAg levels, used as a correlate of HCV RNA, and ALT flare-up in this study. In addition, there was no correlation between

baseline ALT levels and ferritin levels in subjects with PNALT (data not shown). We focused on HCV carriers with PNALT only, and the small number of subjects may be one reason for these results.

Although Shiffman et al.<sup>44</sup> reported that currently no parameters can be used to identify patients at elevated risk for progressive liver disease, the present results suggest that screening for serum ferritin levels and the H63D *HFE* mutation may help identify PNALT subjects at higher risk for ALT flare-up. Furthermore, to make appropriate decisions for interferon therapy, it is important to clarify whether ALT status correlates with liver disease progression. Although it remains unclear whether liver cirrhosis or HCC occurs in individuals with PNALT, recently, both Tanaka et al.<sup>6</sup> and our group<sup>20</sup> reported that elevated serum ALT levels prior to HCC diagnosis were positively associated with an increased risk of HCC. We found that subjects with at least four repeatedly elevated ALT measurements were at increased risk for HCC compared to patients with PNALT.<sup>20</sup> Subjects with fluctuating ALT levels experienced an age- and sex-adjusted HCC rate threefold that of subjects with normal ALT, although this association was not statistically significant. These results suggest that ALT elevation and, possibly, ALT flare-up are associated with an increased risk of HCC. Therefore, HCV carriers with PNALT exhibiting high serum ferritin levels and *HFE* H63D mutation should be considered candidates for antiviral therapy. Okanoué et al.<sup>30</sup> also have recommended antiviral treatment for HCV carriers with PNALT, depending on the results of follow-up blood chemistry and liver histology. We suggest that serum ferritin levels and H63D mutation in the *HFE* gene should also be examined for these patients.

In summary, we identified a high prevalence of HCV carriers with PNALT within a HCV hyperendemic community in Japan. Although HCV carriers are at relatively low risk for HCC, they can experience ALT flare-up, which is associated with an increased incidence of HCC. This study suggests that a subset of HCV carriers exhibit normal, consistently stable ALT levels and do not require liver biopsy or treatment. However, HCV carriers with PNALT whose serum ALT levels are near the upper limit of a normal range and whose serum ferritin levels are higher than 90 ng/ml or who carry the H63D *HFE* mutation are at increased risk for ALT flare-up. Because increases in serum ALT are associated with an elevated risk for HCC, these PNALT HCV patients should be considered for antiviral treatment and liver biopsy.

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# Early Diagnostic Potential for Hepatocellular Carcinoma Using the SELDI ProteinChip System

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**Early detection of HCC increases the potential for curative treatment and improves survival. To facilitate early detection of HCC, this study sought to identify novel diagnostic markers of HCC using surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF/MS) ProteinChip technology. Serum samples were obtained from 153 patients with or without HCC, all of whom had been diagnosed with HCV-associated chronic liver disease. To identify proteins associated with HCC, serum samples were analyzed using SELDI-TOF/MS. We constructed an initial decision tree for the correct diagnosis of HCC using serum samples from patients with (n = 35) and without (n = 44) HCC. Six protein peaks were selected to construct a decision tree using this first group. The efficacy of the decision tree was then assessed using a second group of patients with (n = 29) and without (n = 33) HCC. The sensitivity and specificity of this decision tree for the diagnosis of HCC were 83% and 76%, respectively. For a third group, we analyzed sera from seven patients with HCC obtained before the diagnosis of HCC by ultrasonography (US) and from five patients free of HCC for the past 3 years. Use of these diagnostic markers predicted the diagnosis of HCC in six of these seven patients before HCC was clinically apparent without any false positives. **Conclusion:** Serum profiling using the SELDI ProteinChip system is useful for the early detection and prediction of HCC in patients with chronic HCV infection. (HEPATOLOGY 2007;45:948-956.)**

Approximately 170 million people worldwide are infected with HCV, which when persistent can progress to HCC. The incidence of HCC is rising; in the United States over the past 2 decades, age-specific

incidence has shifted toward younger people.<sup>1</sup> IFN or combined IFN and ribavirin, which are currently the only effective treatments for chronic hepatitis C, reduce the occurrence of HCC.<sup>2,3</sup> Some patients, however, do not receive IFN treatment or fail to clear HCV even with IFN treatment. In addition, a subset of individuals remain unaware that they are infected with HCV; in these patients, HCC may present only in the advanced stage. The prognosis of patients presenting with symptoms related to HCC is extremely poor. In contrast, early detection of HCC before the onset of clinical symptoms can lead to curative treatment, significantly improving prognosis.

Several methods developed for the diagnosis of HCC, including evaluation of serum markers, ultrasonography (US), computed tomography (CT), and magnetic resonance imaging, have been tested clinically. Alpha-fetoprotein (AFP) and des-gamma carboxy prothrombin (DCP), serum proteins that are elevated in HCC, have been the most widely used markers. Although routine screening offers the best chance for early tumor detection and improved survival, the reported sensitivities and specificities of elevated serum AFP and DCP levels vary significantly.<sup>4-9</sup> In addition, AFP levels are elevated in only 30% to 40% of patients with HCC, particularly early in the disease process.<sup>6</sup> Elevated AFP levels are also seen in patients with noncancerous conditions, such as cirrhosis

Abbreviations: AFP, alpha-fetoprotein; AUC, area under the curve; CT, computed tomography; DCP, des-gamma carboxy prothrombin; m/z, mass-to-charge ratio; ROC, receiver operating characteristics; SELDI-TOF/MS, surface-enhanced laser desorption ionization time-of-flight mass spectrometry; US, ultrasonography.

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or exacerbations of chronic hepatitis, which confounds the screening results. Marrero et al.<sup>9</sup> reported that DCP levels were more sensitive and specific than AFP testing for differentiating HCC from nonmalignant chronic liver disease. The usefulness of DCP for the detection of early HCC is limited, however. Wang et al.<sup>8</sup> reported that the number of patients with small HCC (less than 2 cm) demonstrating elevations in DCP was low (56.5%). AFP-L3, the lectin lens culinaris agglutinin-bound fraction and one of the three AFP glycoforms, is the major glycoform of AFP elevated in the serum of HCC patients. At a cutoff level of 15% of total AFP, the reported sensitivities of AFP-L3 as a method of detecting HCC range from 75% to 96.9% with specificities of 90% to 92.0%.<sup>10,11</sup> Because the high percentage of AFP-L3 observed in HCC is closely related to poor differentiation and biologically malignant characteristics, such as portal vein invasion, of neoplastic cells,<sup>11,12</sup> how useful this test is for the early detection of HCC is unclear. In addition, the diagnosis of small mass lesions using US or CT is relatively inaccurate. Thus, additional biochemical markers are necessary for specific detection of early HCC.

The development of proteomic array technology for serum profiling, in which a ProteinChip Array is coupled with surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF/MS; Ciphergen Biosystems Inc., Fremont, CA), has created a powerful tool for the discovery of new biomarkers. This technology has been successfully applied using samples from patients with prostate, ovarian, and gastric cancers. The great advantages of this method are speed, high-throughput capability, and the requirement of only a small amount of sample. Although serum AFP levels and US are the most common examination methods used for HCC surveillance, the classification tree algorithm detailed in this study provided a more accurate classification than these examination methods alone.<sup>13,14</sup>

This study sought to assess and compare protein expression profiles of sera from patients with or without HCC on a background of chronic liver disease attributable to HCV infection. We assessed the ability of SELDI-TOF/MS ProteinChip technology to identify serum markers that could enable early HCC diagnosis.

## Patients and Methods

**Samples.** The 153 male patients with chronic liver disease attributable to HCV infection were selected; serum samples were collected by the Faculty of Medicine of the University of Miyazaki (Miyazaki, Japan). All patients were negative for hepatitis B surface antigen. Seventy-seven of the patients were negative for HCC, which was

confirmed by US or CT of the abdomen. Samples from 64 patients with HCC were obtained before treatment. Patients were randomly divided into two groups; the first analysis group was composed of 35 and 44 patients with and without HCC, respectively, whereas 29 and 33 patients with and without HCC, respectively, made up the second analysis group. The clinical characteristics of the first and second analysis groups were not significantly different except for the average age (Table 1). In conjunction with an ongoing cohort study, we also obtained prediagnostic sera from seven patients determined to have HCC within 1 year of US screening and five patients who have remained free of HCC for the past 3 years.<sup>15</sup> These subjects constitute the third analysis group (Table 2). Twenty-six healthy volunteers without either liver neoplasia or HCV infection served as negative controls. After freezing and thawing once, all samples were separated into 20- to 30- $\mu$ l aliquots and refrozen at  $-80^{\circ}\text{C}$  until analysis.

**SELDI-TOF/MS.** For analysis, we used ProteinChip Arrays (CM10) with anionic surface chemistry. CM10 ProteinChip Arrays incorporate a carboxylate group that acts as a weak cation exchanger. Chips were rinsed with ultra-pure water and put into a bioprocessor (Ciphergen Biosystems, Inc.), a device that holds 12 chips and allows the application of larger volumes of serum to each chip array. Within the bioprocessor, the chips were washed twice with shaking on a platform shaker at a speed of 300 rpm for 5 minutes in 150  $\mu$ l binding/washing buffer (50 mM sodium acetate, pH 4.5) per well. Five-microliter serum samples were denatured in 45  $\mu$ l urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, and 2% ampholites), then diluted 1:9 in binding/washing buffer. After washing the chips extensively in binding/washing buffer, 100  $\mu$ l of the denatured, diluted serum was applied to each chip spot. The bioprocessor was then sealed and shaken on a platform shaker for 40 minutes. Chips were then removed from the bioprocessor. After washing 3 times in binding/washing buffer, we rinsed the chips once in water. Each spot was then treated twice with 0.5  $\mu$ l saturated sinapinic acid (SPA) (Nacalai Tesque Inc, Kyoto, Japan) and allowed to air-dry.

Arrays were analyzed using a ProteinChip Reader (ProteinChip Biology System II, Ciphergen Biosystems Inc.). Time-of-flight spectra were generated by laser shots collected in positive mode. Laser intensity ranged from 225 to 240, with a detector sensitivity of 6. An average of 65 laser shots per spectrum were performed. For mass accuracy calibration according to the manufacturer's instructions, 500 nl of a mixture of mass standard calibration proteins (All-in-one Peptide Standard; Ciphergen Biosystems) were applied to single spot of the normal phase (NP20) chip array, followed by two applications of 1.0  $\mu$ l

Table 1. Patient Characteristics in First and Second Analysis Groups

1st Analysis Group	Total	HCC <sup>§</sup>	CLD <sup>§§</sup>	P
Patients	79	35	44	
Age	66.7 ± 10.9 <sup>§§§</sup>	72.7 ± 4.7	60.3 ± 13.3	0.007
PLT* (X10 <sup>4</sup> /uL)	9.6 ± 5.3	11.5 ± 5.9	7.6 ± 4.2	0.001
Albumin (g/dL)	3.6 ± 1.2	3.6 ± 0.5	3.5 ± 0.5	NS
ALT** (IU/L)	56.6 ± 31.9	56.6 ± 25.5	56.7 ± 35.6	NS
AFP*** (ng/mL)	209.4 ± 982.3	348 ± 1307	26 ± 25	NS
DCP† (mAU/mL)	191.9 ± 538.5	299 ± 686	42 ± 43	NS
HA†† (ng/mL)	353.6 ± 538.5	412 ± 480	293 ± 265	NS
Diameter of the HCC (mm)	-	23.1 ± 9.8	-	
TMNstage††† (I/II/III/IV)	-	18/14/3/0	-	
2nd Analysis Group	Total	HCC <sup>§</sup>	CLD <sup>§§</sup>	P
Patients	62	29	33	
Age	72.2 ± 8.4 <sup>§§§</sup>	73.6 ± 5.9	71.1 ± 10.2	NS
PLT* (X10 <sup>4</sup> /uL)	10.0 ± 5.2	13.3 ± 5.8	7.6 ± 2.9	0.002
Albumin (g/dL)	4.0 ± 0.7	3.9 ± 1.0	4.0 ± 0.6	NS
ALT** (IU/L)	57.8 ± 33.6	57.2 ± 27.1	58.5 ± 38.7	NS
AFP*** (ng/mL)	61.5 ± 155.8	89.9 ± 189.9	19.2 ± 20.6	NS
DCP† (mAU/mL)	100.5 ± 271.4	163.4 ± 396.6	31.2 ± 26.2	NS
HA†† (ng/mL)	461.6 ± 522.6	286.7 ± 224.1	561.6 ± 615.2	NS
Diameter of the HCC (mm)	-	25.1 ± 10.5	-	
TMNstage††† (I/II/III/IV)	-	15/8/5/1	-	

NOTE. Data are shown as the means ± SD. Gender: male, statistical differences were determined by the Mann-Whitney U test. Values of  $p < 0.05$  were considered to be statistical significant. NS indicates not significant. <sup>§§§</sup>Although age differed between the 1<sup>st</sup> and 2<sup>nd</sup> analysis group, none of the other factors described were not different.

Abbreviation: \*platelet count, \*\*alanine aminotransferase, \*\*\*alpha fetoprotein, †des-γ-carboxy prothrombin, ††hyaluronic acid, ††† TMN; primary tumor/lymph node/distant metastasis, §hepatocellular carcinoma, §§chronic liver disease.

saturated SPA. The mass-to-charge ratio ( $m/z$ ) of each the proteins captured on the array surface was determined according to externally calibrated standards.

**Peak Detection, Data Analysis, and Decision Tree Classification.** Peak detection was performed using Ciphergen ProteinChip Software, version 3.0.2 (Ciphergen Biosystems). Spectra between 1300 and 150,000  $m/z$  were selected for analysis. Smaller masses were not analyzed, because these were determined to be artifacts of energy absorbing molecules. Spectra were normalized to total ion current intensity. In the preliminary examination, we observed significant noise in spectra with ranges

less than 3000  $m/z$ . In addition, no differences were apparent in the peaks of spectra at values greater than 10,500  $m/z$  between 4 serum samples from patients with HCC and 4 samples from patients without HCC. Therefore, after baseline subtraction, we performed automatic peak detection in the optimized range of 3000 to 10,500  $m/z$ , using peak auto-detection set to cluster, a first-pass signal/noise ratio of 5, a minimal peak threshold of 20% for all spectra, and a cluster mass window of 0.3% mass.

Based on the peak intensities of the 55 signal clusters obtained, a decision tree was constructed from the first analysis group. For each sample, the intensity values for

Table 2. Patient Characteristics in the Third Analysis Groups

3rd Analysis Group	HCC††† Occurrence Within 1 Year	No HCC Occurrence Within 3 Years	P
Patients	7	5	
Age	72.8 ± 4.1	75.8 ± 5.6	NS
PLT* (X10 <sup>4</sup> /uL)	14.8 ± 4.8	8.1 ± 1.4	0.04
Albumin (g/dL)	4.1 ± 0.6	4.0 ± 0.4	NS
ALT** (IU/L)	70.0 ± 44.9	59.0 ± 34.3	NS
AFP*** (ng/mL)	195.2 ± 305.7	23.4 ± 22.4	NS
DCP† (mAU/mL)	139.5 ± 226.6	17.2 ± 4.6	0.01
HA†† (ng/mL)	310.6 ± 322.8	459.6 ± 114.8	NS

NOTE. Data are shown as the means ± SD. Gender: male, statistical differences were determined by the Mann-Whitney U test. Values of  $p < 0.05$  were considered to be statistical significant.

Abbreviation: NS, not significant.

\*platelet count, \*\*alanine aminotransferase, \*\*\*alpha fetoprotein, †des-γ-carboxy prothrombin, ††hyaluronic acid, †††hepatocellular carcinoma.

each peak within the 3000-10,500  $m/z$  range were input into Biomarker Patterns Software (Ciphergen Biosystems) and classified according to the tree analysis described.<sup>13,16</sup> Decision trees classify spectrum patterns through sequential questioning, in which the next question asked depends on the answer.<sup>17</sup> With a decision tree, classification of patterns begins at the root node, following the appropriate links based on the answers obtained to the questions posed at each node.

**Peak Reproducibility.** Reproducibility is critical for reliable disease diagnosis and early detection. We examined the reproducibility of our assay system using pooled normal sera from 2 individuals.<sup>13</sup> Four protein peaks randomly selected over the course of the study were used to calculate the coefficient of variance (CV) as described.<sup>18</sup> We then determined the reproducibility of the SELDI spectra, both within and between arrays (intra-assay and interassay, respectively). The intra-assay (spot-to-spot) CV was 10.2% for peak intensity and 0.25% for mass accuracy. The interassay (chip to chip) CV was 15.9% for peak intensity and 0.67% for mass accuracy. We also observed minimal variation of day-to-day instrumentation (data not shown).

**Statistical Analysis.** Values shown are the means  $\pm$  SD. Statistical differences, including laboratory data and individual peaks in SELDI-TOF/MS, were determined by the Mann-Whitney  $U$  test. Values of  $P < 0.05$  were considered statistically significant. The discriminatory power for each putative marker was described via receiver operating characteristics (ROC) area under the curve (AUC). These statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS Inc., Chicago, IL), or Ciphergen ProteinChip Software, version 3.0.2.

Sample numbers for the first group used to develop the decision tree were small. A cross-validation approach using multiple decision trees would be more suitable for the construction of a final decision tree model.<sup>19</sup> In this study, we validated the models using a 10-fold cross-validation approach to construct the final decision tree model as described previously.<sup>16,18</sup> The result of the biomarker patterns software using this approach differed from the classification and regression tree analysis by univariate analysis (Mann-Whitney  $U$  test).<sup>20</sup>

## Results

**Detection of HCC (Data Analysis).** We aimed to identify a single peak protein or pattern of peaks that could distinguish HCC patients from individuals without HCC. Initially, we analyzed serum samples from the first analysis group, a random 35 and 44 patients with and

without HCC, respectively, using the SELDI ProteinChip system. Peaks were detected automatically after baseline subtraction using Ciphergen ProteinChip Software, version 3.0.2.<sup>13</sup> This analysis identified 55 signal peak protein clusters, seen in the spectrum representations of the two groups (HCC and non-HCC) within the 3000 to 10,500  $m/z$  range (Fig. 1). Eight protein peaks were overexpressed, whereas 10 protein peaks were down-regulated significantly in sera from HCC patients in comparison with those from patients without HCC. The mean amplitudes of the peaks for the 2 patient groups are shown in Table 3.

**Structure of the Decision Tree.** Decision trees are flowchart-like tree structures that repeatedly split data sets into subsets in accordance with the given cancer versus noncancer classification task. Each classifier, a simple rule applied to each patient, queries only one mass. Serum samples isolated from 35 HCC patients and 44 chronic liver disease patients without HCC served as the training set. Using the normalized peak intensities of these 55 signal clusters, we constructed and evaluated decision trees using the training set. Peaks with a high discriminatory power were used to create 6 mass classifiers ( $m/z = 3444, 3890, 4067, 4435, 4470, \text{ and } 7770$ ) of differing complexities. Although 2 of these classifiers did not differ significantly between patients with and without HCC ( $m/z = 3444$  and  $3890$ ), the decision tree generated using the combination of these 6 protein peaks correctly classified 97% of HCC samples (Fig. 2, Table 3).

**Testing the Decision Tree.** To determine the accuracy and validity of the algorithm, we reevaluated the decision tree (Fig. 2) that had been constructed using the training set, using the first test set (second analysis group). To evaluate the classification performance, we determined the sensitivity and specificity of the algorithm for the differentiation between patients with and without HCC. The decision tree algorithm correctly diagnosed 83% (24 of 29) patients with HCC and 76% (25 of 33) patients without HCC. Although the ROC AUC of each of the 6 mass classifiers were 0.70, 0.61, 0.71, 0.64, 0.66, and 0.70, which individually were more discriminatory than existing serum marker methods, the decision tree algorithm had highest discriminatory power (Tables 3, 4). Twenty-six healthy volunteers were all correctly identified as free of HCC. The accuracy of the algorithm for HCC diagnosis was higher than that of other known tumor markers (Table 4).

**Decision Tree Predicts HCC Occurrence.** The most fundamental requirement for serum-based marker detection is identification of carcinoma at an early stage when treatment has the greatest impact on prognosis. We investigated the specificity of our classification system using a