

Table 9  
Sequences of *cII* mutations in the lung of control Muta<sup>TM</sup> Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
a1	146	C to T	ATT	CCA	AAG	Pro to Leu
a2	196	G to A	GAC	GAC	ATG	Asp to Asn
a3	113	C to T	AAG	TCG	CAG	Ser to Leu
a4	217	C to T	CGA	CAA	GTT	Gln to Stop
a5	180	G to A	GAA	TGG	GGG	Trp to Stop
a6	73	G to A	CTT	GGA	ACT	Gly to Arg
a7	132	G to T	TGG	AAG	AGG	Lys to Asn
a8	58–61	–A	AAC	AAA	ATC	Frameshift
a9	241–246	–A	AAA	AAA	CGC	Frameshift
a10	149–164	–AGTTCTCAATGCTGCT				Deletion
a11	25	G to T	AAC	GAG	GCT	Glu to Stop
a12	214	C to T	GCG	CGA	CAA	Arg to Stop
a13	197	A to G	GAC	GAC	ATG	Asp to Gly
a14	111	G to T	GAT	AAG	TCG	Lys to Asn
a15	206	G to C	GCT	CGA	TTG	Arg to Pro
a16	212	C to T	TTG	GCG	CGA	Ala to Val
a17	38	T to G	CGA	ATC	GAG	Ile to Ser
a18	40	G to A	ATC	GAG	AGT	Glu to Lys
a19 <sup>a</sup>	40	G to A	ATC	GAG	AGT	Glu to Lys
a20	34	C to T	CTA	CGA	ATC	Arg to Stop
a21	90–91	GG to T	GCG	GAA	GCT	Frameshift
a22	113	C to T	AAG	TCG	CAG	Ser to Leu
a23	140	G to A	GAC	TGG	ATT	Trp to Stop
a24	110	A to T	GAT	AAG	TCG	Lys to Met
a25	175	G to A	CTT	GAA	TGG	Glu to Lys
a26	172	C to T	GTT	CTT	GAA	Leu to Phe
a27	89	C to T	ACA	GCG	GAA	Ala to Val
a28 <sup>a</sup>	34	C to T	CTA	CGA	ATC	Arg to Stop
a29	221	T to G	CAA	GTT	GCT	Val to Gly
a30	273–275	ACA to CGATGCACG				Insertion
a31	212	C to T	TTG	GCG	CGA	Ala to Val
a32	193	G to A	GAC	GAC	GAC	Asp to Asn
a33	34	C to T	CTA	CGA	ATC	Arg to Stop
a34	31	C to G	GCT	CTA	CGA	Leu to Val
a35	155	C to A	TTC	TCA	ATG	Ser to Stop
a36	128	G to A	AGG	TGG	AAG	Trp to Stop
a37	88	G to T	ACA	GCG	GAA	Ala to Ser
a38 <sup>a</sup>	34	C to T	CTA	CGA	ATC	Arg to Stop

<sup>a</sup> Ascribable to the same mutation obtained in an identical mouse.

highest *lacZ* mutant frequency in the lung. Chrysene also significantly induced mutation in all of the six organs examined, although chrysene showed similar mutant frequencies in all the six organs. These results suggest that nitrogen substitution in the chrysene skeleton may enhance the inducibility of mutation in the mouse lung.

4,10-DAC showed the highest mutagenic activity, followed in order by: quinoline > 1,7-phenanthroline ≈ 1,10-DAC > benzo[*h*]quinoline ≈ 10-azaBaP = ben-

zo[*f*]quinoline. The number of mutagenic activity of chrysene is the same as that of benzo[*f*]quinoline, which shows the lowest mutagenic activity among the series of azaPAHs tested. The 4,10-DAC gave mutant frequencies higher than 1,10-DAC in all the test organs and the increase in *lacZ* MF obtained by 4,10-DAC is more than twice that by 1,10-DAC in most of the organs tested. Therefore, these results suggest that the nitrogen-substituted position affects the strength of mutagenicity of the DAC structure.

Table 10  
Summary of *cII* mutation spectra in the liver of Muta<sup>TM</sup>Mouse

Mutation class	Control (%)	1,10-DAC (%)	4,10-DAC (%)	Chrysene (%)
Total	30 (100)	38 (100)	38 (100)	36 (100)
Base substitution	26 (87)	35 (92)	35 (92)	32 (89)
Transitions				
GC to AT	14 (47)	7 (18)	8 (21)	9 (25)
AT to GC	1 (3)	2 (5)	0 (0)	2 (6)
Transversions				
AT to TA	1 (3)	3 (8)	1 (3)	2 (6)
AT to CG	2 (7)	1 (3)	1 (3)	1 (3)
GC to TA	7 (23)	17 (45)	18 (47)	13 (36)
GC to CG	1 (3)	5 (13)	7 (18)	5 (14)
–1 Frameshifts	2 (7)	1 (3)	1 (3)	3 (8)
+1 Frameshifts	0 (0)	0 (0)	0 (0)	0 (0)
Deletion	2 (7)	1 (3)	0 (0)	1 (3)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	0 (0)	1 (3)	2 (5)	0 (0)

The same mutations from an identical mouse were counted as a single event.

We further attempted *cII* mutant spectrum analysis in the liver and lung, which were shown to give the highest *lacZ* mutant frequencies by chrysene and DACs, respectively. In both organs, the spontaneous mutations consisted mainly of G:C to A:T transitions and the mutant spectrum in the control Muta<sup>TM</sup>Mice showed no difference between the liver and lung. The G:C to A:T transitions in the control mice are likely to be ascribable to the deamination of 5-methylcytosine

at the CpG site as previously reported [28]. DACs predominantly induced the G:C to T:A transversion in both organs. It was especially noteworthy that there was no difference in the *cII* mutant spectrum of 4,10-DAC between the lung and liver in spite of the quantitative difference showed by 4,10-DAC with its 3.9-fold higher *cII* mutant frequency in the lung than in the liver. These results suggest that the high mutagenicity in the lung of 4,10-DAC-treated mice might not arise from

Table 11  
Summary of *cII* mutation spectra in the lung of Muta<sup>TM</sup>Mouse

Mutation class	Control (%)	1,10-DAC (%)	4,10-DAC (%)	Chrysene (%)
Total	35 (100)	41 (100)	39 (100)	35 (100)
Base substitution	30 (86)	34 (83)	35 (90)	32 (91)
Transitions				
GC to AT	19 (54)	3 (7)	6 (15)	10 (29)
AT to GC	1 (3)	1 (2)	0 (0)	3 (9)
Transversions				
AT to TA	1 (3)	3 (7)	0 (0)	1 (3)
AT to CG	2 (6)	3 (7)	0 (0)	1 (3)
GC to TA	5 (14)	16 (39)	25 (64)	11 (31)
GC to CG	2 (6)	8 (20)	4 (10)	6 (17)
–1 Frameshifts	2 (6)	4 (10)	3 (8)	2 (6)
+1 Frameshifts	0 (0)	0 (0)	0 (0)	0 (0)
Deletion	1 (3)	0 (0)	0 (0)	1 (3)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	2 (6)	3 (7)	1 (3)	0 (0)

The same mutations from an identical mouse were counted as a single event.

Table 12  
In vivo mutagenicity of a series of aza-PAHs

Chemical	Total dose (mg/kg)	Tested organ <sup>a</sup>		kidney	bm	testis	Mutagenic activity <sup>b</sup>	Sequence analysis	Ref.
Quinoline	250	LIVER	spleen	lung	kidney	bm	1.76	Increase of GC to CG	[8–10]
Benzofluoranthrene	400	LIVER	spleen	lung	kidney	bm	0.37	Featureless	[15]
Benzofluoranthrene	400	liver	spleen	LUNG	kidney	bm	0.45	Featureless	[15]
1,7-Phenanthroline	200	LIVER	spleen	lung	kidney	bm	1.14	Increase of GC to CG	[15]
10-azaBaP	625	LIVER	spleen	lung	kidney	bm	0.39	Featureless	[5]
1,10-DAC	400	LIVER	SPLEEN	LUNG	KIDNEY	BM	1.08	Increase of GC to TA	From this study
4,10-DAC	800	LIVER	SPLEEN	LUNG	KIDNEY	BM	2.32	Increase of GC to TA	From this study
Chrysene	800	LIVER	SPLEEN	LUNG	KIDNEY	BM	0.37	Increase of GC to TA	From this study

Superscript (c) in footnote (b) stands for fold-increase in *lacZ* MF (%) = *lacZ* MF obtained by test chemical/spontaneous *lacZ* MF × 100.

<sup>a</sup> The organs in capital letters are those that showed significant induction of mutation and those in small letters indicate negative organs. The underlined organ showed the highest increase in *lacZ* MF. BM(bm), bone marrow; gs, glandular stomach; fs, forestomach.

<sup>b</sup> Mutagenic activity = fold-increase in *lacZ* MF<sup>c</sup>/total dose of test chemical.

the increase of organ-specific adduct(s). In our previous studies, quinoline and 1,7-phenanthroline induced the G:C to C:G transversion [9]. On the other hand, 1,10-DAC and 4,10-DAC induced the G:C to T:A transversion. These results suggest that the enlargement of the molecular size in azaPAHs might change the major mutation pattern from the G:C to C:G transversion to the G:C to T:A transversion. Chrysene also increased the G:C to T:A transversion like DACs. Therefore, it was suggested that the nitrogen substitution in the chrysene skeleton may give no influence on the mutation spectrum.

We have previously reported that quinoline, a partial structure of DACs and one of simplest aza-PAHs, showed mutagenicity in both in vitro [14,29] and in vivo [8] and that metabolic activation of quinoline might take place in the pyridine moiety to form the ultimate genotoxic form, an enamine epoxide (1,4-hydrated 2,3-epoxide) [10–14]. Moreover, benzo[*h*]quinoline and 1,7-phenanthroline, tricyclic azaPAHs with the quinoline moiety as a partial structure, may be activated in the pyridine moiety to show the mutagenicity as reported in our previous paper [16]. DACs, which consist of two quinoline moieties as a partial structure, might be also converted to the enamine epoxide structure in the pyridine moiety to be able to induce mutation. We are trying to investigate further to clarify the metabolic activation pathway of DACs.

In conclusion, it is suggested that the two types of nitrogen substitution in the chrysene structure enhances the mutagenicity in the mouse lung, although they have no influence on the organ specificity and mutation spectrum.

## Acknowledgements

We thank Dr. T. Nohmi for his kind gift of *E. coli*, NM759 and BHB2688 and Dr. K. Masumura for his technical direction in making packaging extracts. This research is supported in part by the grant from Ministry of the Environment, Japan.

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## HX531, a retinoid X receptor antagonist, inhibited the 9-cis retinoic acid-induced binding with steroid receptor coactivator-1 as detected by surface plasmon resonance

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Received 3 September 2004; accepted 29 November 2004

### Abstract

HX531 is a retinoid X receptor (RXR) antagonist that inhibits 9-cis retinoic acid-induced neutrophilic differentiation of HL-60 cells. In order to elucidate the inhibitory mechanism of HX531, we have developed a novel ligand sensor assay for RXR in which the receptor-coactivator interaction is directly monitored using surface plasmon resonance (SPR) biosensor technology. A 20-mer peptide from steroid receptor coactivator-1 (SRC-1), containing nuclear receptor interaction motif LXXLL was immobilized on the surface of a BIAcore sensor chip. Injection of human recombinant RXR with or without 9-cis retinoic acid resulted in ligand-dependent interaction with the SRC-1 peptide. Kinetic analysis revealed dissociation constants (KD) of 9-cis RA-preincubated RXR to SRC-1 was  $5.92 \times 10^{-8}$  M. Using this technique, we found that 1  $\mu$ M HX531 reduced the  $k_a$  value of liganded-RXR with SRC-1, suggesting that HX531 reduced the affinity of RXR to SRC-1. This SPR assay system was applied to obtain quantitative kinetic data of RXR ligand binding to the SRC-1 peptide and the alteration of these data by antagonists.

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**Keywords:** Retinoid X receptor; Steroid receptor coactivator-1; 9-cis Retinoic acid; HX531; PA024; HL-60 cells; Surface plasmon resonance

### 1. Introduction

Retinoids have a chemopreventive and therapeutic potency in oncology and dermatology. All-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA) are well-known natural retinoids. Retinoidal activities are mainly achieved through the transcriptional regulations of specific genes via two kinds of nuclear receptors (NRs): (a) RAR $\alpha$ ,  $\beta$  and  $\gamma$ , and (b) RXR $\alpha$ ,  $\beta$  and  $\gamma$ . ATRA binds RARs and 9-cis RA to both RARs and RXRs.

HL-60 cells, which are an established cell line from an acutely promyelocytic leukemia patient [1], differentiate into neutrophilic cells in response to both ATRA [2] and 9-cis RA [3]. This differentiation ability of HL-60 cells has been useful for screening of chemically synthesized agonists or antagonists of NRs; HX531 was discovered as an antagonist of RXRs from the synthetic chemical library in this screening system [4]. Since HX531 inhibits the activation of both RXR homodimer and the RAR/RXR heterodimer, HX531 is expected to modulate the gene expression through these NRs. The precise mechanism of antagonistic effects by this compound, however, has not been fully elucidated.

The p160 steroid receptor coactivator (SRC) gene family contains three homologous members, which serve as transcriptional coactivators for NRs and certain other transcription factors [5]. These coactivators interact with ligand-bound NRs to recruit histone acetyltransferases (HAT) and methyl-

**Abbreviations:** 9-Cis RA, 9-cis retinoic acid; ATRA, all-trans retinoic acid; NRs, nuclear receptors; RXR, retinoid X receptor; rhRXR, recombinant human RXR; SRC-1, steroid receptor coactivator-1; SPR, surface plasmon resonance; RU, resonance unit; KD, dissociation constants;  $k_a$ , association rate constant

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transferases to specific enhancer/promotor regions, which in turn facilitates chromatin-remodeling [6,7], assembly of general transcription factors, and transcription of target genes. The relatively conserved central region of the SRC family contains three LXXLL motifs that are responsible for interaction with ligand-bound NRs [8–11]. Recently, it was reported that 9-cis RA-liganded RAR/RXR showed enhanced binding with SRC-1 [12]. Hence, we suspected that a binding assay utilizing liganded-RXR with SRC-1 would be useful for the analysis of RXR kinetic parameters.

HX531 inhibited the increase in the nitro blue tetrazolium (NBT) reduction ability of HL-60 cells induced by 9-cis RA [4]. In this study, we have examined the antagonistic effect of HX531 against 9-cis RA on cell growth and on expression of the formyl-Met-Leu-Phe receptor (fMLP-R) and CD11b on HL-60 cells. Furthermore, we have developed an analytical method for agonist or antagonist effects on RXR using surface plasmon resonance (SPR) biosensor technology. Here, we also discuss the interaction of SRC-1 and RXR incubated with 9-cis RA and HX531 using this new method.

## 2. Materials and methods

### 2.1. Materials

Biotinylated peptides (>95% of purity) were purchased from Biologica (Nagoya, Japan). Recombinant human RXR $\beta$  (rhRXR $\beta$ ) was obtained from Affinity BioReagents (Golden, Co). 9-Cis RA and anti-human CD11b monoclonal antibody were from Sigma Chemical (St. Louis, MO) and DAKO (Glostrup, Denmark), respectively.

### 2.2. Cell culture and neutrophilic differentiation in relation to neutrophilic granulocytes lineage

HL-60 cells were kindly supplied by the Japanese Collection of Research Resources Cell Bank. Cells were maintained in RPMI 1640 medium, containing 10% heat-inactivated FBS and 30 mg/L kanamycin sulfate at 37 °C under moisturized air, containing 5% CO<sub>2</sub>. HL-60 cells were differentiated into neutrophilic cells by addition of 9-cis RA. HX531 was preincubated with cells for 30 min before addition of 9-cis RA. Five days after addition of the differentiating agent, expressions of both fMLP-R and CD11b, and cell numbers were measured.

### 2.3. fMLP-R and CD11b expression

The differentiated cells were collected and incubated with FITC-conjugated fMLP or phycoerythrin-conjugated anti-CD11b antibody, and then labeled cells were subjected to flow cytometric analysis (FACSCalibur, Becton and Dickinson).

### 2.4. SPR assay

The measurements were performed using a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) at 25 °C in a running buffer comprising 0.2% bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl<sub>2</sub> and 0.1% Tween 20. Streptoavidin chips (BIAcore AB) were first treated with three 1-min pulses of 50 mM NaOH, containing 1 M NaCl at a flow rate of 10  $\mu$ l/min. Wild-type peptide from human SRC-1 (wtSRC-1, CPSSHSSLTARHKILHRLQLQEGSPS-CONH<sub>2</sub>, residues 676–700), containing the consensus NR interaction motif (LXXLL), and the consensus-mutated SRC-1 peptide (mtSRC-1, CPSSHSSLTARHKIAHRLQLQEGSPS-CONH<sub>2</sub>) were biotinylated and immobilized on individual surfaces. Recombinant human RXR $\beta$  dissolved in the running buffer was preincubated with 9-cis RA or PA024, an agonist of RXRs, in either the presence or absence of HX531 at room temperature for 1 h, and then injected over the surfaces at a flow rate of 10  $\mu$ l/min. Ligands were dissolved in ethanol and the final concentration of ethanol was adjusted to 1.0%. After the injection was completed (300 s), the formed complex was washed with the running buffer for an additional 300 s. The chip surfaces were regenerated down to the peptide level by subsequent washing for 60 s pulses with 0.1% SDS and 3.3 mM EDTA solution and 10 mM NaOH solution. Ligand-treated RXR interaction with SRC-1 was represented by resonance unit (RU). Both the KD and *k*<sub>a</sub> values were calculated from the experimental curve with BIAcore evaluation 3.1 software package. The formation of surface-bound complexes was analyzed according to the interaction type of A + B  $\leftrightarrow$  AB.

### 2.5. Statistical analysis

Each experiment was repeated three or more times, and representative data are indicated. Statistical analysis was performed using the unpaired t-test. Values of *P* < 0.05 were considered to indicate statistical significance.

## 3. Results

### 3.1. Effects of HX531 on proliferation and CD11b and fMLP-R expression of 9-cis RA-treated HL-60 cells.

To confirm the antagonistic effects of HX531, we examined the effects of 1.0  $\mu$ M HX531 on the growth of 0.1  $\mu$ M 9-cis RA-treated HL-60 cells 5 days after cultivation. As shown in Fig. 1A, HX531 could not completely restore but partially antagonized the inhibitory effect of 9-cis RA on proliferation of HL-60.

The expression of fMLP-R is one of suitable maker, since we have previously reported that it positively correlated the differentiation of HL-60 cells, i.e. migration and superoxide producing abilities [13–15]. Next, the expression of both fMLP-R and CD11b was analyzed by flowcytometry 5 days

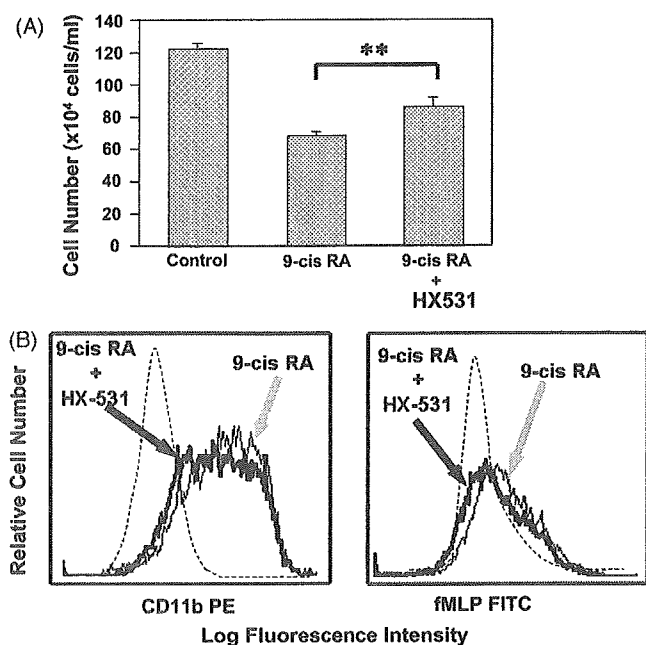


Fig. 1. Effects of HX531 on 9-cis RA-induced-proliferation and differentiation. (A) HL-60 cells were treated HX531 and 9-cis RA. Five days after treatment, cellular number was determined. Columns and bars represent the mean  $\pm$  S.D. of triplicate wells (\*\*,  $P < 0.01$ ). (B) fMLP-R and CD11b expression were analyzed by flow cytometry. Non-treated cells are indicated by dotted lines. The gray arrow indicates cells cultured with 9-cis RA (gray lines) and the black arrow the cells cultured with 9-cis RA in the presence of HX531 (black lines).

after addition of 9-cis RA in the presence or absence of HX531 (Fig. 1B). HX531 reduced not only CD11b expression but also fMLP-R expression compared with 9-cis RA-treated HL-60 cells. These results confirmed the antagonistic effects of HX531 on 9-cis RA-induced neutrophilic differentiation and growth arrest.

### 3.2. Establishment of SPR assay and inhibitory effects of HX531

To elucidate the antagonistic effects of HX531 against 9-cis RA, we undertook establishment of an SPR assay system using the ligand-dependent interaction of NRs and SRCs. First, we tried to detect the signals of ligand-dependent RXR binding with SRC-1 using SPR. When rhRXR $\beta$  was injected over the sensor chip surface immobilized with a 20-mer peptide, containing the NR interaction consensus motif LXXLL of SRC-1 (wtSRC-1), a small response was observed in the absence of 1  $\mu$ M 9-cis RA (Fig. 2A, upper panel; 0.225  $\mu$ M (–)). The signal observed from the injection of 0.225  $\mu$ M rhRXR $\beta$  was markedly enhanced by preincubation with 9-cis RA (+) compared with control (–), and the signal increased with rhRXR $\beta$  in a dose-dependent fashion. On the other hand, when mutated SRC-1 peptide (mtSRC-1), containing alanine substitutions in the core motif (AXXAL) was immobilized on the sensor chip surface as a control, only a

Table 1

Treatment ( $\mu$ M)	HX531 ( $\pm 1 \mu$ M)	$k_a$ ( $\times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ )
Control	–	0.50
	+	0.18
PA024	–	4.89
	10.0	–
	1.0	–
	1.0	+
9-cis RA	–	2.16
	10.0	–
	1.0	–
	1.0	+

The  $k_a$  values of rhRXR $\beta$  treated with PA024 and 9-cis RA in the presence or absence of HX531.

very weak signal was observed (Fig. 2A, lower panel) compared to that of wtSRC-1 peptide. Thus, these data indicated that the 9-cis RA-elicited signal observed in the injection of RXR with the SRC-1 peptide was specific to the LXXLL motif.

To obtain the sensorgrams showing the LXXLL motif-specific interaction, we subtracted the sensorgrams using mtSRC-1 peptide from those produced by the wtSRC-1 peptide in the following experiments. The result by sensorgrams (Fig. 2B) clearly shows the specific binding of liganded-RXR to the SRC-1 peptide, and the kinetic analysis established an association constant ( $K_A$ ) for 9-cis RA-preincubated RXR of  $1.69 \times 10^7 \text{ M}^{-1}$ , which was three-fold greater than its dissociation constants ( $K_D$ ,  $5.92 \times 10^{-8} \text{ M}$ ).

Next, rhRXR was incubated with PA024 (1  $\mu$ M or 10  $\mu$ M) or 9-cis RA (1  $\mu$ M or 10  $\mu$ M), and then the incubation mixture was subjected to SPR analysis subtracting the sensorgrams using mtSRC-1 peptide from those produced by the wtSRC-1. In this analysis, the concentration of the rhRXR protein was fixed at 0.9  $\mu$ M (Fig. 3A), and the association rate constant ( $k_a$ ) from liganded-RXR binding with SRC-1 was calculated. The addition of 1  $\mu$ M and 10  $\mu$ M of PA024 markedly increased the  $k_a$  from  $0.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  in the control to  $3.41 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  and  $4.89 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. 9-cis RA (1  $\mu$ M and 10  $\mu$ M) also increased the  $k_a$  value to  $1.45 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  and  $2.16 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ , respectively, although the  $K_D$  value of RXR did not change. These data indicate that RXR agonists caused an increase in the binding of RXR to SRC-1 in this system. The binding of RXR to the SRC-1 peptide may increase due to ligand presence in a dose-dependent manner.

When rhRXR $\beta$  was preincubated with (HX) or without (control) 1  $\mu$ M HX531 alone, no signal difference was observed (Fig. 3B), suggesting that HX531 dose not affect the ligand-independent binding of rhRXR $\beta$  to SRC-1 peptide. When rhRXR $\beta$  was incubated with either 1  $\mu$ M PA024 or 1  $\mu$ M 9-cis RA in the presence of HX531, the signals were reduced compared with those in the absence of HX531 (Fig. 3B). All  $k_a$  values are shown in Table 1. These results indicated that it is possible to analyze the kinetics param-

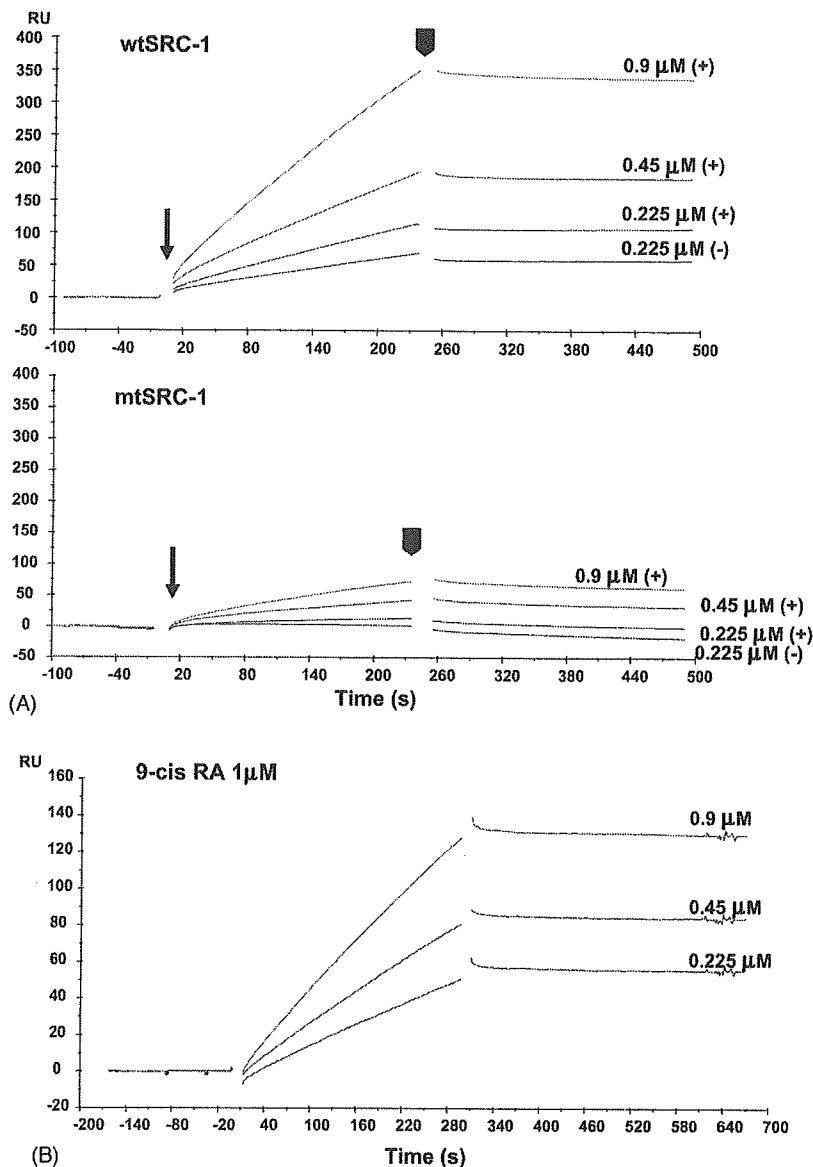


Fig. 2. (A) 9-cis RA caused specific interaction between RXR and SRC-1 peptides, containing a consensus LXXLL motif. Each concentration of rhRXR preincubated with (+) or without (-) 1 μM 9-cis RA was injected over the surfaces immobilized with wtSRC-1 peptide (upper panel) or mtSRC-1 peptide (lower panel). The arrow and the arrowhead indicate the beginning and end of the injections, respectively. (B) Kinetic analysis of 9-cis RA-liganded-rhRXRβ binding to the SRC-1 peptide. Subtraction sensorgrams of mtSRC-1 from those of the wtSRC-1 peptide represent the consensus-specific interaction of rhRXR with the SRC-1 peptide in the presence of 1 μM 9-cis RA. KD value was calculated by BIAcore evaluation 3.1.

ters of agonists or antagonists in this system. As shown in Table 1, the  $k_a$  value of rhRXRβ treated with either PA024 or 9-cis RA was reduced by the addition of HX531, indicating that HX531 lowers the affinity of liganded-rhRXRβ to SRC-1.

Finally, rhRXRβ was preincubated in the presence or absence of HX531 with 0.1 μM 9-cis RA, a sufficient but minimal dose to promote differentiation of HL-60 cells (Fig. 1), and the signals were compared. In this SPR assay, the 9-cis RA-dependent signal was completely inhibited by the preincubation with 1.0 μM HX531 (Fig. 3C). These data indicated the possibility that the antagonistic mechanism of

HX531 is the inhibition of the binding of liganded-RXR with SRC-1.

#### 4. Discussion

Many synthetic agonists or antagonists of NRs have been developed to control specific gene expression [7]. These agonists or antagonists are expected to be used as medical treatment for patients through the control of etiologic gene expression related to specific diseases. The search for the candidate agonists and antagonists for NRs have been performed us-



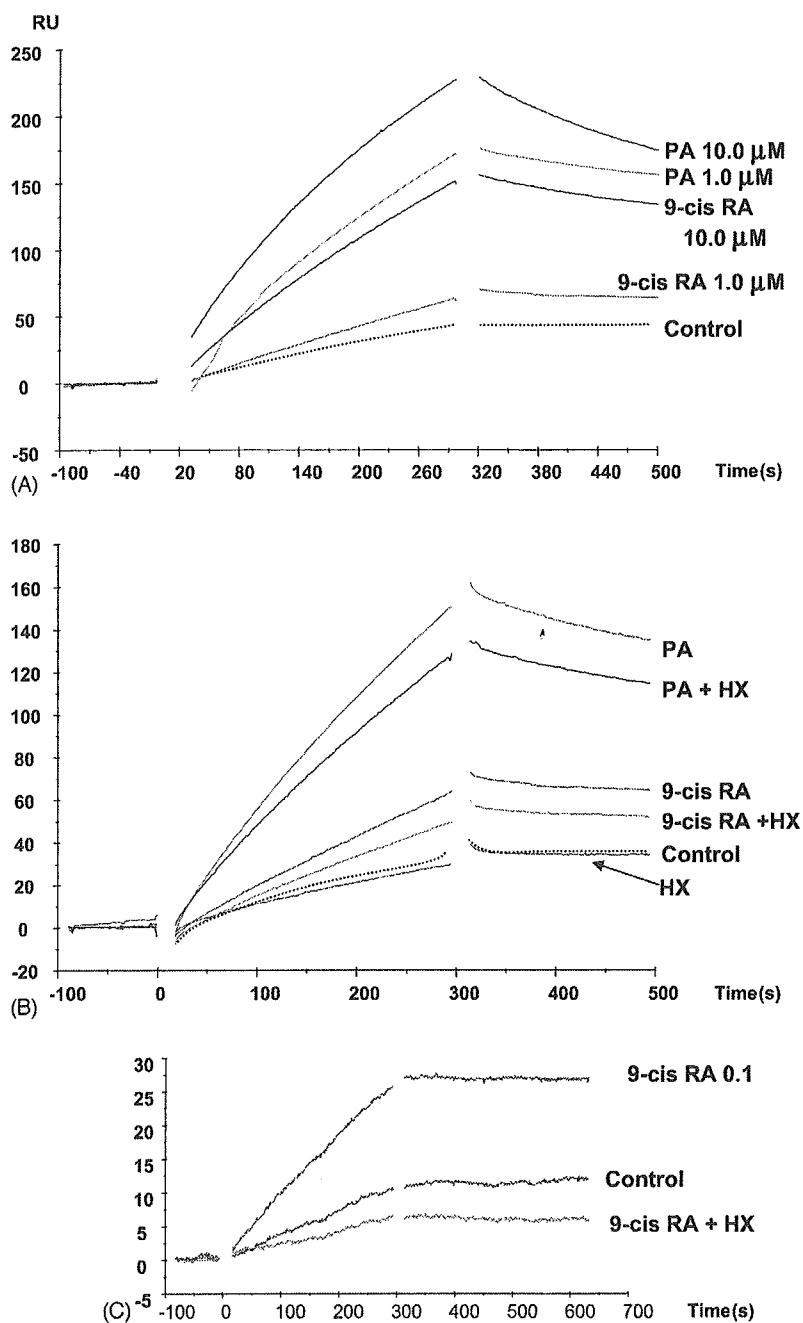


Fig. 3. Comparison of 9-cis RA and PA024 on the binding of rhRXR with SRC-1 and the inhibition of HX531 on these binding interactions. (A) 1.0 μM and 10 μM of 9-cis RA or PA024 (PA) were added to rhRXR, and the solution was subjected to SPR assay system. (B) 1.0 μM HX531 (HX) and agonists were added to rhRXR simultaneously. (C) 0.1 μM 9-cis RA was preincubated with rhRXRβ in the presence or absence of HX531.

ing specific cell culture assays or reporter assays. Using nitro blue tetrazolium (NBT) reduction assay of neutrophilic differentiation in HL-60 cells, either PA024 [16], an agonist for RXR, or HX531 [4], an antagonist for RXR, could be detected. The inhibition of cell growth and the stimulatory expression of fMLP-R and CD11b by 0.1 μM 9-cis RA were antagonized by 1.0 μM HX531 (Fig. 1A and B); these results corresponded with those of previous reports [4,16]. From this assay, however, it was impossible to evaluate or assess the molecular mechanism producing these agonistic or antago-

nistic effects. In the present study, in order to elucidate the kinetics of the HX531 antagonist, we developed an SPR assay system using the interaction of NR with SRC. We have investigated here the effect of agonists and antagonists on the apparent affinity of RXRβ for an SRC peptide. As shown in Table 1, the apparent  $k_a$  of RXRβ to the SRC-1 peptide in the presence of PA024 was higher than that of 9-cis RA, confirming that PA024 is stronger than the natural agonist. Furthermore, the addition of 1 μM HX531 markedly reduced the apparent  $k_a$  of RXRβ to the SRC-1 peptide in the pres-

ence of either 1  $\mu$ M PA024 or 1  $\mu$ M 9-cis RA. In this context, using SPR assay systems with SRC-1 peptides, HX531 was confirmed to possess the ability to reduce the affinity of RXR $\beta$  to a SRC.

Farnesoid X receptor (FXR) is a NR that forms a heterodimer with RXR. FXR is activated by natural ligands, such as bile acids, and we previously established SPR assay system for FXR [17]. The binding of liganded-RXR $\beta$  with the SRC-1 peptide (Fig. 2) is thought to be also dependent to the LXXLL motif as described previously [6]. In the present SPR assay, 1  $\mu$ M HX531 completely inhibited 0.1  $\mu$ M 9-cis RA-induced signal, the concentration of which was a sufficient but minimal dose for promoting differentiation in HL-60 cells (Fig. 1A and B) [18]. Therefore, the present SPR assay using this concentration of 9-cis RA was easily adapted to the screening of RXR $\beta$  antagonists. The present SPR assay can also be utilized in screening for agonists that possess the ability to provide sufficient binding affinity of RXR $\beta$  to the SRC-1 peptide.

The p160 SRC complex contains histone acetyltransferases (HAT), including the CREB-binding protein (CBP), p300 and the p300/(CBP)-associated factor (p/CAF) and methyltransferases, including CRAM1 and PRMT1. These chromatin-remodeling enzymes are recruited to promoters through interaction between NRs and SRC coactivators in a ligand-dependent manner [7,19]. Although the C-terminal domains of SRC-1 and SRC-3 contain HAT activities [8,20], these activities are much weaker than those in CBP, p300 and p/CAF and inactivation of HAT in SRC-1 does not significantly affect its coactivation function [20,21]. These reports indicate that it may be more important for transcription of their target genes that SRC-1 recruits other coactivators that have high HAT or methyltransferase activity rather than that SRC-1 exhibits its HAT activity. Poujol et al. [12] reported that 9-cis RA caused increase in binding of RAR/RXR with SRC-1. Moreover, in our SPR assay system, the function of agonists and antagonists could be distinguished, and HX531 inhibited the 9-cis RA- or PA024-induced binding of RXR with SRC-1 (Fig. 3).

Recently, new agonists and antagonists of NRs have been synthesized, and simple assay systems are needed immediately. Our SPR assay system is a simple and useful technique for analyzing the binding of NRs and coactivators.

## Acknowledgments

This work was supported by a grant (MF-16) from the Organization for Pharmaceutical Safety and Research and a grant from the Japan Health Sciences Foundation.

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## REGULAR ARTICLE

# Proteomic analysis of sera from hepatocellular carcinoma patients after radiofrequency ablation treatment

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Comparative proteomic analysis was used to search for characteristic alterations in the sera of hepatocellular carcinoma (HCC) patients who had undergone curative radiofrequency ablation treatment. Serum samples collected from eight patients before and after treatment were subjected to 2-DE. Eighty-eight protein spots differentially expressed with the treatment were selected by clustering analysis, and the proteins were identified by MS based on MALDI-TOF/TOF analysis and public database searches. The statistical analysis suggested that four proteins decreased after treatment (pro-apolipoprotein,  $\alpha$ 2-HS glycoprotein, apolipoprotein A-IV precursor, and PRO1708/PRO2044, which is the carboxy terminal fragment of albumin) and that seven proteins were increased after treatment, including leucine-rich  $\alpha$ 2-glycoprotein and  $\alpha$ 1-antitrypsin. These data facilitate the identification of differentially expressed proteins that are involved in HCC carcinogenesis and provide candidate biomarkers for the development of diagnostic and therapeutic tools.

Received: September 6, 2004

Revised: February 9, 2005

Accepted: February 11, 2005

**Keywords:**

2-DE / Hepatocellular carcinoma / Serum proteome

## 1 Introduction

The current methods for the diagnosis of hepatocellular carcinoma (HCC) rely on serological markers, such as alpha-fetoprotein (AFP) and certain liver enzymes, together with

physical assessments and imaging technologies (e.g., ultrasound and computed tomography) [1]. Increases in the serum levels of des-gamma-carboxyprothrombin (DCP) and the fucosylated isoform of AFP have also been associated with HCC. Nevertheless, particularly in hepatitis B virus (HBV) infection, a diagnosis of HCC is sometimes made when the disease is too far advanced for effective treatment, and thus the 5-year survival rates are very low [2]. Clearly, better methods for screening and early diagnosis are important for improved prognosis.

Radiofrequency ablation treatment (RFA) has recently been introduced in the therapeutic modality of HCC [3]. Assuming that the tumor is diagnosed as HCC at an early stage, i.e., at a tumor diameter of less than 3 cm and with restricted growth in several nodules, it can be curatively treated with RFA. In RFA treatment, heat is generated locally

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**Abbreviations:** AFP, alpha-fetoprotein; AHS, alpha-2-HS glycoprotein; DCP, des gamma carboxyprothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RFA, radiofrequency ablation therapy; TAE, transcatheter arterial embolization

by a high-frequency alternating current that flows from the electrodes. A probe is inserted into the lesion in the liver and local heat is generated to melt the tissue adjacent to the probe, causing tissue coagulative necrosis. In the case of RFA, as compared with percutaneous ethanol injection therapy [3, 4], the area of necrosis can be controlled precisely by adjusting the duration of the radiofrequency emission, which increases concentrically from the electrode. Because RFA can shorten the period of hospitalization, it is rapidly gaining popularity as a treatment modality for HCC in Japan and elsewhere [5].

In an effort to identify protein markers that have pathological significance for various cancers, proteomic analysis with 2-DE and MS has been used. Presently, proteomic analysis is widely used for the molecular analysis of various tumors and for the identification of new targets for cancer therapy [6, 7]. Proteomic analysis of disease is based on the profiling of the differential expression levels of various proteins between healthy and diseased states. Thus, identified disease markers may be involved in the etiology of the disease. Recently, two groups applied proteomic analysis to the sera of HBV-infected patients in a search for serological biomarkers of the disease [8, 9]. They found that apolipoprotein AI was associated with the HBV-infected status or HCC of HBV-infected patients. In order to identify candidate biomarkers, they used a 2-DE gel separation technique and MALDI-TOF MS. These findings are attributable to the development of MS/MS. Moreover, one of the latest advances in the accurate, sensitive resolution of mass is TOF/TOF technology [10, 11]. Therefore, in the present study, we used the new high-resolution MALDI-TOF/TOF mass spectrometer (Applied Biosystems), in combination with gel-based proteomic analysis, to profile the proteins in the sera of HCC patients. Eight pairs of pre- and post-treatment serum samples were analyzed for alterations in protein expression.

## 2 Materials and methods

### 2.1 Materials

Eight human serum samples were collected with the informed consent of patients with HCC, who had been admitted to the University of Tokyo Hospital. The samples were analyzed in accordance with procedures approved by the ethics committee of the hospital. The collection interval after treatment was 1 wk.

CHAPS, DTT, SDS, iodoacetamide, TEMED, ammonium bicarbonate, Tris, glycine, methanol, urea, and TFA of HPLC grade were purchased from Wako Pure Chemicals (Osaka, Japan). The IEF system (IPGphor), Immobiline DryStrips (18 cm, pH 4–7), and Protein A-Sepharose Fast Flow were purchased from Amersham Pharmacia (Little Chalfont, Buckinghamshire, UK). Acrylamide was obtained from Bio-Rad (Hercules, CA, USA). The MS experiments were carried out in a AB4700 MALDI-TOF/TOF mass spec-

trometer (Applied Biosystems, Framingham, MA, USA). A 99% grade CHCA was purchased from Sigma-Aldrich (St. Louis, MO, USA), sequence-grade trypsin was obtained from Promega (Madison, WI, USA), and the standard peptide mixture used for calibration was from Applied Biosystems.

### 2.2 Sample preparation

Each serum sample of 200  $\mu$ L was absorbed with 200  $\mu$ L volume of Protein A-Sepharose Fast Flow, which had been previously equilibrated with sodium phosphate buffer (pH 7.1). After the flow-through fractions were collected, the resin was washed with an equal volume of sodium phosphate buffer (pH 7.1), and each wash solution was collected, together with the previously described flow-through fraction. The protein concentration was estimated using Bradford reagent (Bio-Rad), and the samples were stored at  $-80^{\circ}\text{C}$  until use.

### 2.3 2-DE and gel staining

IEF was carried out with 18-cm, pH 4–7 IPG strips according to the manufacturer's instructions, with minor modifications. The 2-DE gels were run on two separate occasions for each sample. The IPG strips were located in the IPG ceramic holders and rehydrated overnight with 300  $\mu$ g of the sample, which was solubilized in 340  $\mu$ L of a solution that contained 8 M Urea, 2% CHAPS, 18.3 mM DTT, 0.5% v/v IPG buffer (pH 4–7), and a trace of bromophenol blue. The proteins were separated by the IEF system using a programmed voltage gradient at  $20^{\circ}\text{C}$ . After overnight rehydration, the voltage was set at 500 V for 1 h, increased to 4000 V with a 3-h linear gradient, and then increased further to 8000 V for 5.5 h. The samples were maintained at 8000 V until a total run of 47.5 kWh was accomplished (about 9.5 h in total). After IEF, the strips were equilibrated for 15 min in 10 mL of equilibration buffer (6 M Urea, 2% SDS, 0.05 M Tris-HCl (pH 8.8), 30% glycerol, 1% DTT) and then transferred for a further 15 min into equilibration buffer that contained 2.5 iodoacetamide instead of DTT. The equilibrated IPG strips were transferred onto 12.5% uniform second-dimensional polyacrylamide gels, and electrophoresis was carried out in vertical uniform slab SDS-PAGE at 30 mA per gel for about 4.5 h. Each gel measured 20  $\times$  18 cm. Protein spots in the gels were visualized by silver staining using conventional protocols [12], with minor modifications. Briefly, the gels were fixed overnight in 40% ethanol and 10% acetic acid in water. After the gels were washed twice with 30% ethanol in water, they were incubated for exactly 1 min in 0.05% w/v  $\text{Na}_2\text{S}_2\text{O}_3$ . The gels were then washed twice with water for 20 s each and incubated for 20 min in 0.2% w/v  $\text{AgNO}_3$ . The gels were washed three times for 20 s each in water and developed with 3% w/v  $\text{Na}_2\text{CO}_3$  in 0.05% v/v formaldehyde in water until the desired contrast was reached (usually after 2.5 min). The reaction was stopped by the addition of 0.5% acetic acid and incubation for 20 min.

## 2.4 Gel image and data analyses

After silver staining, the 2-DE gels were scanned with an ES-2200 image scanner (Epson, Tokyo, Japan). The intensity of each spot was calculated in ppm using the PDQuest software (Bio-Rad). The volume of each spot was normalized for the total density of the gel. To exclude spots that showed variable intensity in duplicated experiments, we calculated the SD of each spot; spots that showed deviations of more than  $2 \times$  SD and spots with missing values in over 25% of the samples were eliminated (Fig. 2) [13]. The averages of the intensity values from duplicate experiments for the remaining 812 spots were used for further analysis. Agglomerative hierarchical clustering of protein spots was performed using the Cluster and TreeView programs (<http://rana.lbl.gov/EisenSoftware.htm>) [14]. The changes in spot intensities after RFA treatment were evaluated using the *t*-statistic. The level of statistical significance was determined by the random permutation test with false discovery rate correction. Corrected *p* values less than 0.05 were considered significant.

## 2.5 In-gel digestion

Protein spots were excised from gels as  $1 \times 1$ -mm pieces and were digested with trypsin using a previously published procedure [15, 16], which was modified as described in this section. Gel slices were destained in a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, and then washed with water. After the addition of ACN and complete drying in a SpeedVac<sup>®</sup>, the gels were reswollen in 2  $\mu$ L of trypsin solution (20  $\mu$ g/mL in 25 mM ammonium bicarbonate) and 0.1% *n*-octyl glucoside. After the solvents had penetrated into the gels, 10  $\mu$ L of 25 mM ammonium bicarbonate that contained 0.1% *n*-octyl glucoside was added, and the mixture was incubated at 37°C overnight. After in-gel digestion with trypsin, the tryptic peptides were extracted, first with 50  $\mu$ L and then with 25  $\mu$ L of extraction buffer (50% ACN/0.1% TFA). The supernatants were collected and dried in the SpeedVac<sup>®</sup>, and the samples were dissolved in 25  $\mu$ L of 0.1% TFA.

## 2.6 Data examination and protein identification

Using ZipTip<sup>®</sup>  $\mu$ C18, the tryptic peptides were desalted and concentrated, and then eluted with 1  $\mu$ L of matrix solution (5 mg/mL CHCA in 50% ACN/0.1% TFA) onto a MALDI-TOF/TOF 2  $\times$  96-well target plate. MS/MS analysis was performed on a model 4700 Proteomics Analyzer (Applied Biosystems), and air was used as the collision gas. The spectra were obtained by the accumulation of 2000–3500 consecutive laser shots.

Proteins that were expressed differentially in the pre- and post-treatment samples were analyzed by MALDI-TOF, which permitted the identification of peptide masses for subsequent MS/MS analysis. Spectrum peak harvesting and baseline correction were carried out automatically. The query

was made for all species, with the minimum number of matched masses being set at four. The maximum tolerance level for the peptide masses was 50 ppm, and the modifications accepted were carbamidomethyl cysteines and artifactual oxidation of methionines. The NCBI non-redundant databases were used for the searches, and the MS/MS studies were carried out with the same parameters as those described previously for the PMF research, using the MASCOT PMF search tools (Matrix Science, London, UK; [http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html)). The precursor peak error was set at 2 Da, and fragment tolerance was defined as 0.6 Da. Internal calibration of the MS/MS data was not performed.

## 2.7 Western blotting

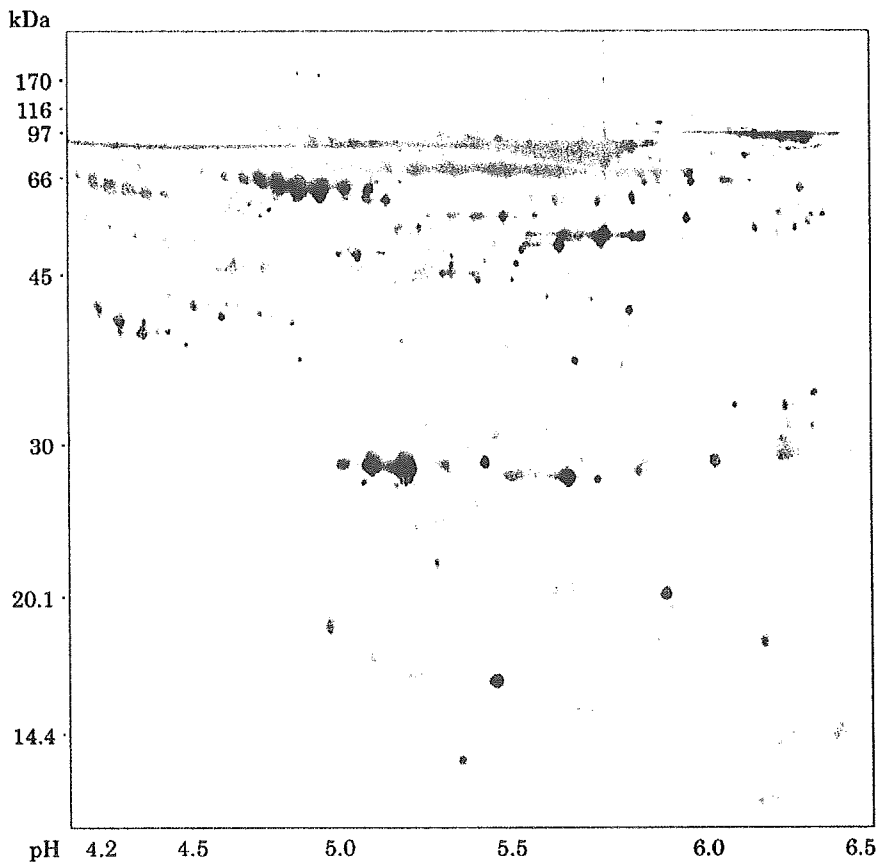
Western blotting was performed as described previously [17]. Three paired samples were separated on 10–20% gradient SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 100% Block Ace (Dainippon Pharmaceutical, Osaka, Japan) at 4°C overnight and incubated with sheep anti-AHSG antibody (ab8939) (Abcam, Cambridgeshire, UK) in TBST that contained 10% Block Ace at 4°C overnight. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies, followed by chemiluminescence detection according to the manufacturer's instructions (Amersham Biosciences).

## 3 Results

### 3.1 Sample preparation and gel image analysis

We characterized the proteomic serum patterns of eight HCC patients following treatment with RFA or RFA plus transcatheter arterial embolization (TAE). A total of 16 serum samples (before and after treatment for eight patients) were analyzed by 2-DE; an image of a 2-DE gel is shown in Fig. 1. The clinical characteristics of the patients are shown in Table 1. Eight of the patients were infected with HBV or HCV and suffered from HCC (maximum diameter 3.2 cm). These patients were treated with curative RFA or curative RFA plus TAE. The changes in the levels of the HCC markers (AFP/L3/DCP), owing to the therapeutic procedure, are indicated in Table 1 (before vs. after treatment).

The serum samples were first prepared using Protein A-Sepharose Fast Flow beads to deplete the immunoglobulins, which are proteins that are abundant in the serum. Another abundant serum protein, albumin, was not depleted from the samples in this study, as albumin is also a carrier protein that conveys a large variety of compounds, including hormones, lipoproteins, and amino acids [18, 19]. Therefore, the removal of albumin from serum might result in the specific removal of low-abundance cytokines, peptide hormones, and lipoproteins of interest [20].



**Figure 1.** Serum protein patterns on a 2-DE gel (12.5%) after the removal of immunoglobulin. The 2-DE running conditions are described in the text. Protein (300 µg) was loaded onto the IEF gel strip (pH 4–7). Following 2-DE separation, the protein spots were visualized by silver staining and were numbered using the PDQuest software. A representative gel image is shown.

**Table 1.** Profiles of patients. Eight cases infected with hepatitis virus with HCC were enrolled in this analysis. Second column is TNM staging and max size of hepatocellular carcinoma(cm). TNM is tumor staging system, which uses three criteria to judge the stage of the cancer – primary tumor(T), regional lymph nodes(N), and distant metastasis(M) – stage I through IV. AFP, L3 and DCP are HCC tumor markers, which are often clinically used. L3 fraction is a fucosylated AFP subtype and more specific for HCC than AFP. If TAE was done, it was stated in the last column

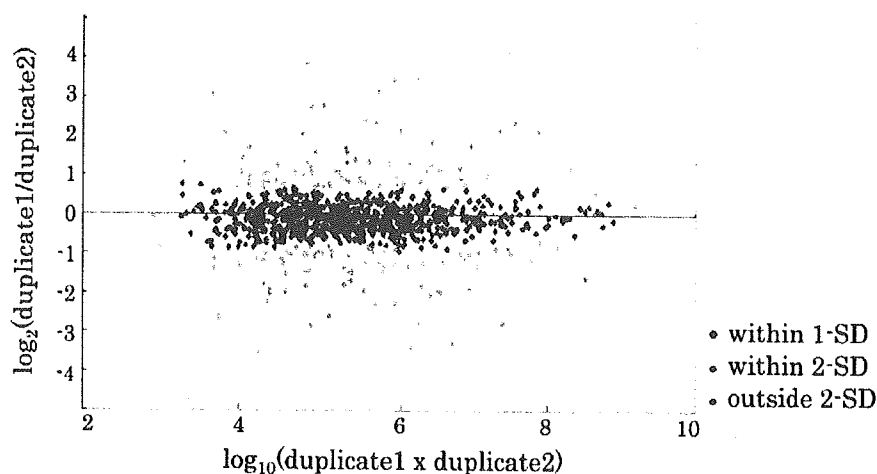
	cases	TNM/radius	changes by the treatment			
			AFP (IU/L)	L3 (%)	DCP (IU/L)	
1.	70F C-LC	II/φ 1,4 cm	7 ⇒ 7	N.D.	18 ⇒ 16	
2.	81M C-CH	I/φ 1,2 cm	32 ⇒ 29	2.0 ⇒ 0.5	13 ⇒ 15	
3.	64M C-CH	II/φ 2,1 cm	11 ⇒ 12	0.5 ⇒ 0.5	13 ⇒ 14	
4.	56F B-LC	II/φ 3,0 cm	162 ⇒ 55	43.3 ⇒ 33.2	10 ⇒ 10	TAE (+)
5.	74F C-LC	III/φ 2,9 cm	8855 ⇒ 912	5.2 ⇒ 7.0	134 ⇒ 16	TAE (+)
6.	55F B-CH	II/φ 3,2 cm	7 ⇒ 5	N.D.	59 ⇒ 18	TAE (+)
7.	58M C-LC	I/φ 1,5 cm	195 ⇒ 132	12.0 ⇒ 5.3	12 ⇒ 13	
8.	64F C-CH	II/φ 3,0 cm	1375 ⇒ 108	1.7 ⇒ 2.4	24 ⇒ 13	TAE (+)

TAE: transarterial embolization, N.D.: not detected

In order to ensure reproducibility, the 2-DE pattern of each patient serum sample was analyzed in two separate experiments. The image analysis showed that these 2-DE maps were similar, as shown in Fig. 1. Using the PDQuest

image analysis software, 1133 spots were resolved on the 2-DE gels.

After excluding spots with intensities that varied by over 2 SD in duplicate gels (Fig. 2), hierarchical clustering



**Figure 2.** Reproducibility of 2-DE-based protein spot quantification. The spot intensity-dependent SD was calculated based on the ratio-intensity plot, using the data from duplicate experiments. Protein spots with intensities that varied by  $>2 \times \text{SD}$  between experiments were eliminated from the subsequent analysis.

was performed with the remaining 812 spots. As shown in Fig. 3A, the unsupervised clustering analysis demonstrated that each pair of samples before and after RFA treatment lined up side by side, except in case 2, which means that the treatment did not produce any drastic change in the protein abundance in common across the eight patients. We may have missed subtle changes that could be associated with the tumor. Therefore, we divided the serum samples into two groups, *i.e.*, before and after treatment, and performed supervised clustering analysis to discover proteins expressed differentially with the treatment. Of 812 protein spots, 88 protein spots were selected as changing significantly in this analysis (Fig. 3B).

### 3.2 Protein identification of spots

The 88 protein spots shown in Fig. 3B were subjected to in-gel trypsin digestion and MS, and 45 protein spots (51%) were identified. Many of these identified spots represented PTM variants; they were collapsed into 11 distinct proteins after homology and similarity searches to eliminate redundant protein annotations. Although 15 protein spots are shown in Table 2 and Fig. 3B (asterisks), they still contain PTM variants. The remaining differential protein spots were not identified at this point, because the spot intensities were too faint to obtain sufficient amounts of tryptic peptide for protein identification. Several spots were identified by MALDI-TOF/TOF analysis.

The levels of four proteins were decreased after RFA: PRO1708/PRO2044 (the C-terminal fragment of albumin), pro-apolipoprotein,  $\alpha 2$ -HS glycoprotein (AHSG), and apolipoprotein A-IV precursor (Table 2 and Fig. 4A). The levels of seven proteins were increased after treatment: leucine-rich  $\alpha 2$ -glycoprotein,  $\alpha 1$ -antitrypsin, macroglobulin  $\alpha 2$ , haptoglobin (precursor), serum paraoxonase, complement C3 precursor, and C4A (Fig. 4B).

### 3.3 Western blotting with the anti-AHSG antibody

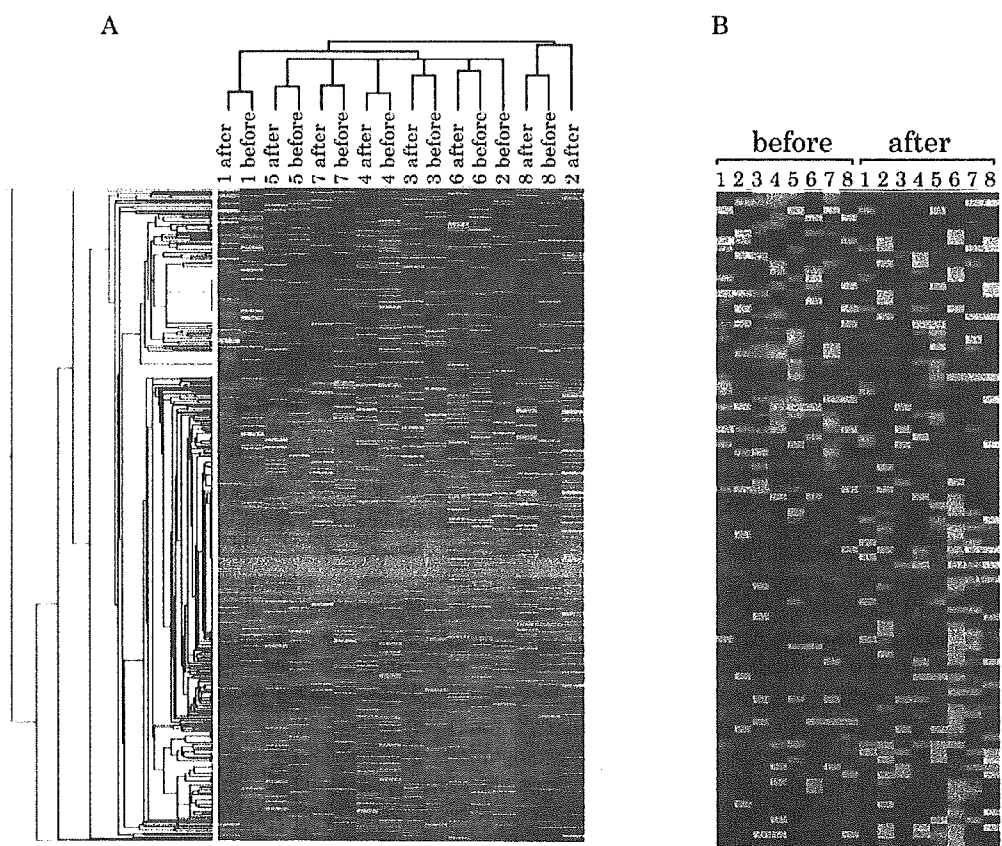
The expression of AHSG was confirmed by Western blot analysis with the anti-AHSG antibody. Paired sera from the patients were dissolved in SDS-PAGE gel buffer and blotted onto PVDF membranes. As shown in Fig. 5, the AHSG protein level was decreased after RFA treatment in three of six patients.

## 4 Discussion

Chronic infection with HBV or HCV is a significant risk factor for the development of HCC [18, 19]. The monitoring of disease progression and the prediction of outcome currently depend on a combination of physical and serological assessments. Unfortunately, these methods often lack the sensitivity required to detect HCC at an early stage, when therapeutic options are the most effective, especially when all three of the HCC markers AFP, L3 (fucosylated fraction of AFP), and DCP, are within normal ranges. There is a very real need for the discovery of markers that can detect the disease at an earlier stage in a higher proportion of patients and that are suitable for screening populations known to be at high risk for the development of HCC.

In the search for new biomarkers, several groups have analyzed differences in the levels of RNA expressed in normal and tumor-derived liver tissues [21–24] or in cultured cells [22, 25–27]. Similarly, some groups have studied differences in the protein profiles, or proteomes, of normal and tumor-derived liver tissues, cell lines, and serum [8, 9]. These studies have provided new insights into tumor carcinogenesis. For the discovery of disease progression markers that can be used for HCC patient screening, it is desirable to uncover changes in specific gene products that can be found in samples, such as serum, that are easily collected from patients.

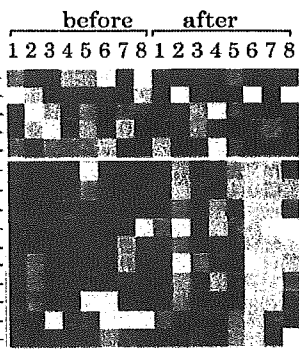




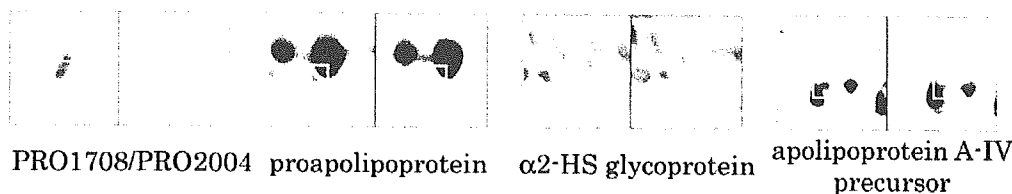
**Figure 3.** Hierarchical clustering analysis of serum protein spots. (A) Unsupervised clustering of eight cases and 812 spots. (B) Eighty-eight protein spots that were expressed differentially before and after treatment for HCC were selected in the supervised clustering analysis. Of these, 45 protein spots were identified using MS. The 15 spots indicated by asterisks (also listed in Table 2) still contain variants modified post-translationally, and the final number of proteins that changed with treatment is 11. Gray: missing value.

**Table 2.** Identified proteins by mass spectrometry. 2-DE gel images were analyzed by PDQuest software and detected spots were numbered (Spot no.). The values of score,  $M_r$  and  $pI$  are indicated in MASCOT MS/MS Ion search. The ratio between before and after treatment is indicated as Ratio (before/after). The scaling factor of the spot intensity used in PDQuest software is PPM (parts per million) and Mean and SE (standard error) of each spot was calculated. Corrected  $p$  values  $<0.05$  were considered to be significant. Right panel is the heat map extracted from the Fig. 3 of each protein. The line order is as same as Table 2.

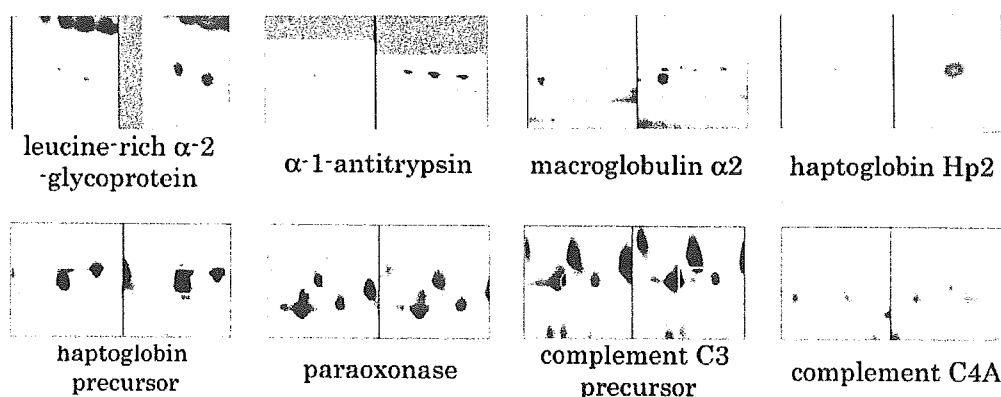
Spot no.	Protein identified	Accession no.	Score	Ratio (before/after)	$M_r$ (kDa)	$pI$	Mean	SE
9010	PRO1708 /PRO2044	gi 7959791 /gi 66650826	62	3.85	29.2	7.0	2467	433
3115	proapolipoprotein	gi 178775	124	2.08	28.9	5.5	1112	251
638	alpha2-HS glycoprotein	gi 2521981	180	1.44	35.6	5.2	6882	629
1605	alpha2-HS glycoprotein	gi 2521981	30	1.39	35.6	5.2	190	34
3401	apolipoprotein A-IV precursor	gi 178779	109	2.33	43.4	5.2	990	205
501	leucine-rich $\alpha$ -2-glycoprotein	gi 72059	63	0.44	34.3	5.7	1365	209
504	leucine-rich $\alpha$ -2-glycoprotein	gi 16418467	37	0.54	38.2	6.5	1520	169
2808	alpha-1-antitrypsin	gi 177831	68	0.52	46.7	5.4	1145	196
8801	macroglobulin $\alpha$ 2	gi 224053	115	0.61	160.7	6.0	323	32
6003	haptoglobin Hp2	gi 223976	154	0.55	41.7	6.2	5235	1036
2414	haptoglobin precursor	gi 306882	106	0.52	45.2	6.1	2966	2349
1507	serum paraoxonase	gi 130675	112	0.41	39.7	5.1	2470	576
1514	serum paraoxonase	gi 130675	112	0.43	39.7	5.1	6798	1798
1412	complement C3 precursor	gi 4557385	129	0.41	187.1	6.0	459	231
4207	complement C4A	gi 443671	158	0.54	193.5	6.8	525	97



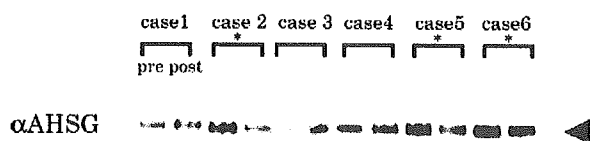
A.



B.



**Figure 4.** Identification of spots that exhibited changed levels in response to HCC treatment. (A) Identified spots with decreased levels after treatment. PRO1708/PRO2204 is the C-terminal fragment of albumin. (B) Identified spots with increased levels after treatment. Each spot is enclosed in a square, and representative gels images are shown.



**Figure 5.** Western blot using the anti-AHSG antibody. Sera were run on a 10–20% gel. The Western blot shows that, in cases 2, 5, and 6, the levels of AHSG were higher before treatment than after treatment.

Steel *et al.* [9] properly dealt with the variability between individuals by combining an equal mass of total serum protein from individuals to form a composite sample for each of the four groups and by comparing the composite 2-DE gels of the four groups. As shown in Table 1, we lacked sufficient samples to detect significant differences, and the patients had different etiological backgrounds. We tried to minimize the variance between individuals by comparing paired gels derived from the same patient's serum. The constituents of serum after treatment must be the same as those before treatment, except for tumor-associated proteins or the consequences of treatment, in order to compare serum samples from the same patient. The timing of serum collection after

treatment is important for comparative analysis, and we collected the post-treatment sera 1 wk after treatment, at the time of discharge from hospital. We considered this period of time reasonable in the clinical setting.

As described in Section 2, we collected serum samples before and after HCC treatment. At the time of discharge, the transaminases (ALT/AST) were unchanged or slightly upregulated from before treatment (the ratios of after/before were between 0.9 and 2; data not shown). It was not practical to wait for 1 month for all the serological parameters to stabilize and the tumor makers to decrease markedly in some cases, as shown in Table 1. Thus we considered 1 wk a reasonable interval and carefully continued our analyses, introducing a statistical analysis to eliminate artifactual errors.

In this study, we identified four proteins that had decreased levels following HCC treatment (Table 2). None of these proteins has been reported to be associated with liver cancer. Apolipoprotein A-IV (precursor) is synthesized primarily in the intestine [28, 29] and is secreted in the plasma; because its expression is suppressed in the sera of HBV-infected individuals, it is assumed to be an indicator of HBV infection [9]. AHSG, which is also known as human fetuin, is a liver secretory glycoprotein found at high levels in the serum and mineralized bone. This protein has the char-

acteristics of a negative acute-phase protein, in that the serum concentrations decrease significantly after major surgical procedures, trauma, burns, and severe inflammation. Western blotting with the anti-AHSG antibody confirmed that the serum level of AHSG was decreased by HCC treatment (Fig. 5). The protein with the most significant change in abundance was PRO1708/PRO2044 (Table 2). This protein is the C-terminal part of albumin. However, it was spotted in a very different location from albumin (the  $M_r$  and  $pI$  values of PRO1708/PRO2044 are 29 kDa and 7.6, respectively, whereas those of albumin are 69 kDa and 6.0 in 2-DE gels). We postulated that the partial fragment had a special physiological meaning; therefore, we used PRO1708/PRO2044 instead of a fragment of albumin in the text and table.

PRO1708/PRO2044 and AHSG were upregulated in the sera of HCC patients, as compared with levels in HCC-eradicated sera. Although the levels of AHSG may fluctuate as a result of liver damage or inflammation, it is not clear why the levels of the other proteins decrease after treatment. These results suggest roles for these proteins in HCC, but further confirmatory studies are necessary.

The levels of seven different proteins were elevated after HCC treatment. The increases in the levels of these proteins may be attributable to acute stress reactions or leakage from damaged liver tissues. Serum haptoglobin levels are widely used to study various liver diseases and are reported to be linked to liver damage in HBV liver infection [9]. Liver injury caused by RFA treatment may have increased the intensities of the spots. As a powerful inhibitor of apoptosis and caspase activation,  $\alpha$ 1-antitrypsin inhibits many of the proteases that are released from dying cells, and thus protects normal tissues during periods of stress, such as inflammation [9]. The observed increases in the  $\alpha$ 1-antitrypsin levels of the post-treatment sera may represent self-protective responses of the liver. Although Steel *et al.* [8] found that a fragment of complement C3 was downregulated in HCC, we identified complement C3 as being upregulated after RFA treatment. This could indicate recovery of the liver, although more study is necessary.

As shown in Table 1, the types of tumor markers produced by HCC differ among patients, and neither the sensitivity nor the specificity of the three markers is satisfactory. As the sample number was small and the patients had different etiological backgrounds, the clinical significance of the proteins identified here must be explored by analyzing more cases. We propose an analytical procedure using a standard statistical algorithm [14] with carefully normalized and transformed data [13] in combination with protein identification using TOF/TOF technology.

As previously described, AFP, L3, and DCP are frequently used clinical HCC tumor markers. L3, which is a fucosylated fraction of AFP, is considered more specific than AFP for HCC. We tried to evaluate the degrees of differences in individuals around the time of treatment using these three HCC markers, but it proved too difficult. Albumin is a carrier

protein for many small proteins and peptides, is one of the most abundant proteins, and has an  $M_r$  of 69 kDa and a  $pI$  value of 6. Different forms of modified/processed albumin appear and form multiple spots in the 2-DE gel, which leave traces that make it difficult to detect spots with a  $pI$  value lower than 6. AFP, the L3 fraction, and DCP are positioned in this area, and it is technically difficult to identify these proteins on gels.

In the final analysis, we identified 45 of the 812 protein spots on the 2-DE gels using MS/MS. As listed in Table 2, many of these identified spots represented PTM variants; they collapsed into 15 distinct proteins after homology and similarity searches eliminated redundant protein annotations (indicated as asterisks in Fig. 3B, and Table 2).

The technique of comparative proteomics is an effective platform for the study of cancer. The 2-DE images presented in this study will facilitate the identification of potential tumor markers and increase our understanding of the mechanisms of HCC.

*This work was supported by a research grant from the Ministry of Culture, Science, and Sports. We thank Mitsuko Tsubouchi for technical assistance.*

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