

Fig. 1. Evaluation of the reliability of the cDNA microarray-based CGH method by comparison with Southern blotting. (A) Portion of typical array patterns of in-house microarrays (red, HL60; green, PBMC). The spot located by the white arrow indicates the c-myc gene. (B) Southern blot analysis of HL60 and PBMC DNA hybridized with c-myc probes. (C) DNA copy number of c-myc in HL60 cells, N-myc in IMR-32 cells, and the luciferase gene in RCF-26 cells determined by cDNA microarray and by Southern blotting. *Abbreviation:* F-Luc, firefly luciferase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

our method, we found that each of these genes was significantly amplified in their respective cell lines and that the estimated copy number determined by our cDNA array-CGH was well correlated with that from Southern blotting analysis (Fig. 1). Our cDNA array-CGH method also found that the DNA copy number of the p53 gene in HL60 cells was about half that in PBMCs (data not shown), in agreement with previously reported results [33].

Amplified and/or deleted genes detected by cDNA array-CGH

A typical scatter plot of intensities generated by Cy5 or Cy3 immobilized at the target sequence on the micro-

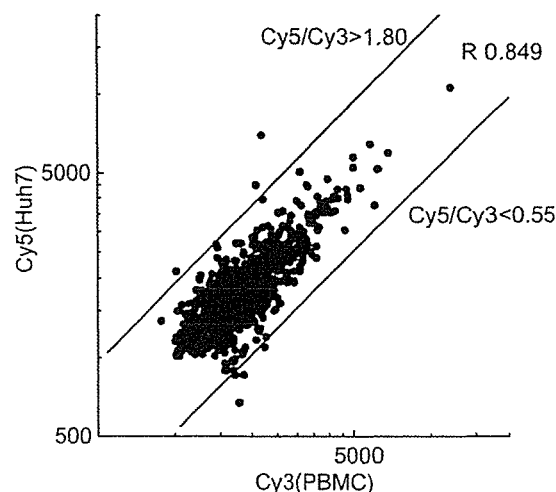


Fig. 2. \log_{10} (intensity of Cy5) and \log_{10} (intensity of Cy3) of Huh7 cells are graphed on a scatter plot, and their linear relationship was examined statistically.

array slides is shown in Fig. 2. Using a fold-change of 1.8 or higher as the filtering criteria, we selected those genes with a >95% probability of being changed differentially. The seven hepatoma cell lines tested differed with respect to the number and identity of genes amplified or deleted (Table 1). As expected, we found that the HBV copy number was increased in the Hep3B and PLC/PRF/5 cell lines [1,34]. Although few genes were commonly amplified or deleted among the seven hepatoma cell lines, there were common biological characteristics among amplified or deleted genes in the individual cell lines. In the Hep3B and HepG2 cell lines, apoptosis-related gene copy numbers [e.g., glutathione-S-transferase T1 (GST T1), fas-associated via death domain (FADD), defender against apoptotic cell death (DAD1), and mammalian inhibitors of apoptosis homolog B (MIHB)] changed, whereas in the HLE cell line, cell adhesion and receptor molecule encoding gene copy numbers (e.g., integrin, glutamate receptor, and endothelin receptor) changed, and, in the PLC/PRF/5 cell line, the cytokine-related gene copy numbers (e.g., small inducible cytokine A2, interferon- α , and interferon regulatory factor 2) changed. Differences observed in amplified and deleted genes in these cell lines may reflect differences in their oncogenetic pathways and tumor phenotypes.

Southern blotting data

The changes in DNA copy number determined by our cDNA array-CGH were reevaluated by Southern blotting analysis (Fig. 3). In agreement with array-CGH results, we observed amplification of the HBV genome in the Hep3B and PLC/PRF/5 cell lines, and amplification of cyclin-dependent kinase 3 in Huh7 cells

Table 1
Genes amplified or deleted in seven hepatoma cell lines

Cell line	Gene name	Cy5/Cy3 (array-CGH)	Cy5/Cy3 (expression profiles)	Function	GenBank	Chromosome location
Huh7						
>1.80	RNA-binding motif protein 4 (RBM4)	3.15 ± 0.70	1.60	RNA-binding	NM_002896	11q13
	Cyclin-dependent kinase 3	2.19 ± 0.23	7.75	Cell cycle	NM_001258	17q22-qter
	Alpha platelet-derived growth factor receptor precursor	1.83 ± 0.40	1.77	Cell receptor	NM_006206	4q11-q13
<0.55	Inhibin, alpha	0.49 ± 0.05	1.28	Growth factor	NM_002191	2q33-q36
	Granzyme A	0.54 ± 0.11	0.49	Immune response	NM_006144	5q11-q12
Hep3B						
>1.80	Glutathione-S-transferase T1	8.91 ± 8.74	3.32	Apoptosis	NM_000853	22q11.23
	HBV-P	6.84 ± 7.16		Virus genome		
	HBV-full	3.60 ± 1.61		Virus genome		
	<i>Homo sapiens</i> CGI-137 protein	2.40 ± 1.66	1.46	Transcription factor	NM_003187	5q11.2-q13.1
	Fas-associated via death domain (FADD)	2.23 ± 0.39	1.54	Apoptosis	NM_003824	11q13.3
	<i>H. sapiens</i> PAC clone DJ0855D21	2.02 ± 0.90	1.48	PAC clone	AC004908	Unknown
	BCL-2 homologous antagonist/killer (BAK) protein	2.02 ± 1.37	1.81	Apoptosis	NM_001188	6p21.3
	Immunoglobulin mu-binding protein 2	1.91 ± 0.37	2.2	DNA-binding	NM_002180	11q13.2-q13.4
	Discs, large (<i>Drosophila</i>) homolog 1	1.88 ± 1.32	2.03	Guanylate kinase	NM_004087	3q29
	RNA-binding motif protein 4 (RBM4)	1.83 ± 0.04	4.02	RNA-binding	NM_006144	11q13
<0.55	Burkitt lymphoma receptor 1, GTP-binding protein	0.53 ± 0.18	2.27	Cell receptor	NM_032966	11q23.3
HepG2						
>1.80	Glutathione-S-transferase T1	2.70 ± 0.57	2.16	Apoptosis	NM_000853	22q11.23
	Defender against cell death (DAD1)	2.31 ± 0.27	1.46	Apoptosis inhibitor	NM_001344	14q11-q12
	Oxidase (cytochrome c) assembly 1-like	2.26 ± 0.19	2.66	Electron transport	NM_005015	14q11.2
	Transcriptional regulator ISGF3 gamma subunit	1.99 ± 0.53	11.1	Transcription factor	NM_006084	14q11.2
	Mammalian inhibitor-of-apoptosis homolog B (MIHB)	1.92 ± 0.85	0.59	Apoptosis inhibitor	NM_001166	11q22
	Guanine nucleotide-binding protein (G protein), alpha stimulating activity polypeptide 1	1.85 ± 0.31	1.85	Transcription factor	NM_080425	20q13.2-q13.3
<0.55	Nothing					
SKHep1						
>1.80	<i>H. sapiens</i> PAC clone DJ0855D21	2.10 ± 0.27	0.4	PAC clone	AC004908	Unknown
	Decorin	1.83 ± 0.17	1.28	Cell-cell interaction	NM_133503	12q13.2
<0.55	Inhibin, alpha	0.44 ± 0.02	0.34	Growth factor	NM_002191	2q33-q36
	<i>H. sapiens</i> insulin-like growth factor II receptor (IGF2R)	0.53 ± 0.01	0.3	Cell receptor	NM_000876	6q26
HLE						
>1.80	Interleukin enhancer-binding factor 1	2.90 ± 0.21	NA	Cytokine	NM_004514	17q25
	Integrin, beta 4	2.70 ± 0.45	NA	Cell-cell interaction	NM_000213	17q11-qter
	RB130 retinoblastoma-like 2	2.33 ± 0.47	NA	Protein kinase	NM_004203	16p13.11
	Glutamate receptor, ionotropic, AMPA 1	1.93 ± 0.26	0.8	Cell receptor	NM_000827	5q31.1
	E2D2	1.85 ± 0.38	1.95	Protein ligase	NM_003339	5q31.3
<0.55	Human vitamin K-dependent protein Z	0.38 ± 0.11	0.52	Protein-binding	NM_003891	13q34
	Endothelin receptor type B	0.40 ± 0.05	NA	Cell receptor	NM_000115	13q22
PLC/PRF/S						
>1.80	HBV-P	19.3 ± 4.62	NA	Virus genome		
	HBV-full	9.04 ± 5.36	NA	Virus genome		
	<i>H. sapiens</i> PAC clone DJ0855D21	2.81 ± 0.32	1.19	PAC clone	AC004908	Unknown
	Small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-je)	1.81 ± 0.02	4.05	Cytokine	NM_002982	17q11.2-q21.1
<0.55	Interferon-alpha	0.40 ± 0.10	1.44	Cytokine	NM_000605	9p22
	Interferon regulatory factor 2	0.45 ± 0.05	1.22	Cytokine	NM_002199	4q34.1-q35.1
	<i>H. sapiens</i> transcriptional coactivator p52	0.55 ± 0.29	1.13	Transcription factor	NM_033222	9p22.2
Huh6						
>1.80	Nothing					
<0.55	Nothing					

Notes. Clones showing a copy number ratio >1.80 were considered to be amplified and those with a copy number ratio <0.55 were considered to be deleted. The array-CGH data are expressed as means ± SD. Abbreviation: NA, data not available.

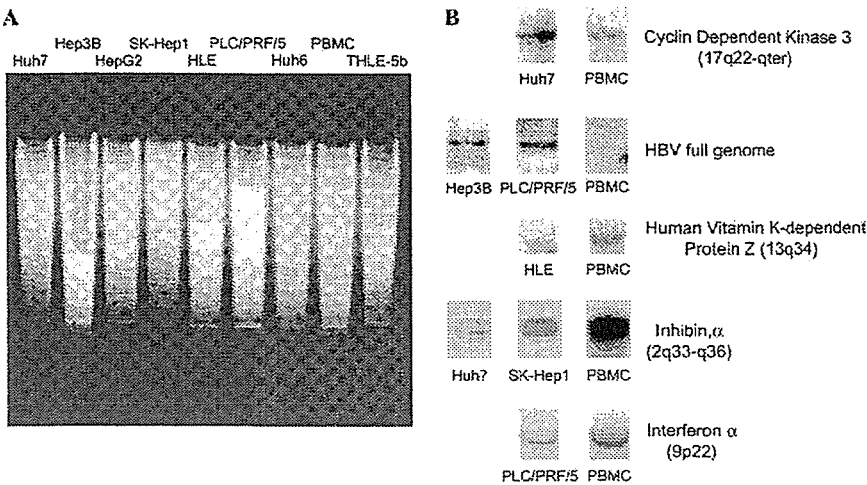


Fig. 3. Southern blot analyses of DNA from seven hepatoma cell lines. (A) Gel electrophoresis image of seven hepatoma cell lines and two normal cell lines. (B) Two amplified genes and three deleted genes in hepatoma cell lines, as shown by the cDNA array-CGH method.

by Southern blotting. Similarly, Southern blotting confirmed the deletion of inhibin α in Huh7 and SK-Hep1 cells, of human vitamin K-dependent protein Z in HLE cells, and of interferon- α in PLC/PRF/5 cells.

Comparison with expression microarray data

We proceeded to determine the relationship between genomic alterations and changes in mRNA expression in these cell lines (Table 1). We found that 40% of amplified genes were associated with mRNA overexpression and, conversely, 2–3% of overexpressed genes were accompanied by genomic alterations. When we plotted the global effect of copy number on gene expression, we found a direct relationship between these parameters

(Fig. 4). Thus, changes in DNA copy number closely correlated with gene expression in these hepatoma cell lines.

Chromosome mapping

The chromosome locations of amplified and deleted genes were determined (Table 1, Fig. 5). In Hep3B cells, we observed a cluster of amplified and deleted genes at chromosome 11q13; whereas, in HepG2 cells, there was a cluster at 14q11. Similarly, we observed clusters at 5q31 in HLE cells and at 9p22 in PLC/PRF/5 cells. These results indicate that relatively long-range genomic DNA rearrangements take place within these cell lines. In addition to these clusters, DNA copy number

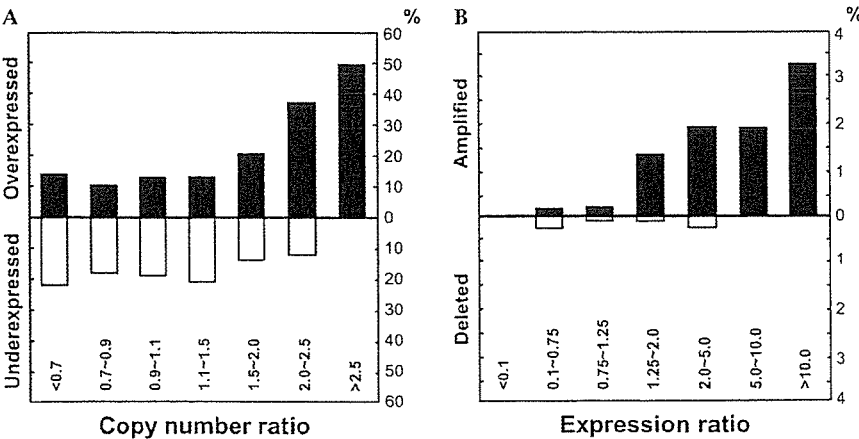


Fig. 4. Impact of gene copy number on global gene expression levels. (A) Percentage of over- and underexpressed genes (vertical axis) according to copy number ratios (horizontal axis). Threshold values used for over- and underexpression were >1.80 and <0.55 , respectively. (B) Percentage of amplified and deleted genes according to expression ratios, using the same threshold values for amplification and deletion.

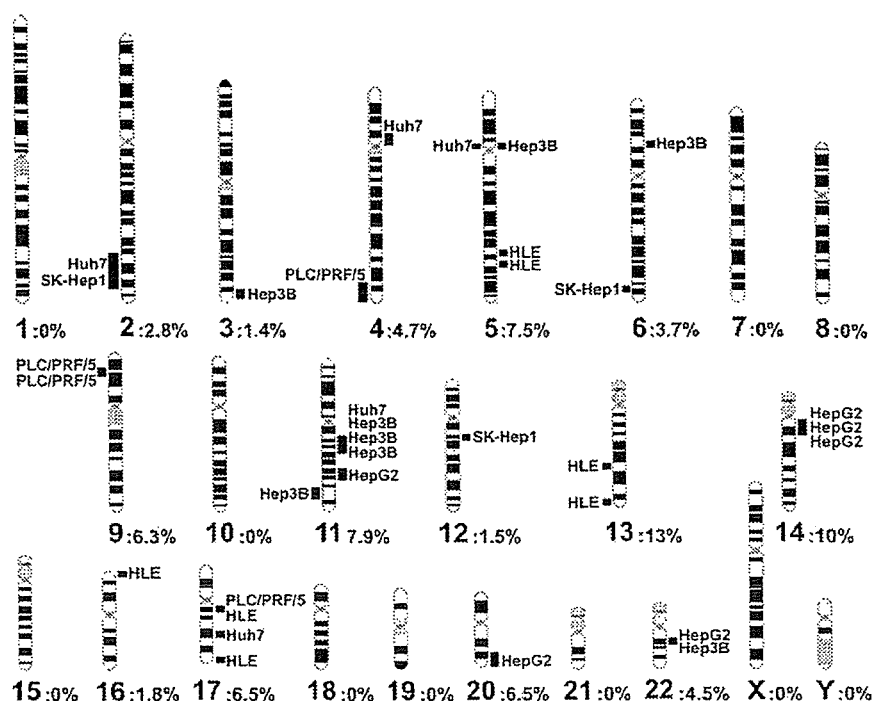


Fig. 5. Chromosome locations of genes amplified or deleted, as shown by cDNA array-CGH. Squares on the right side of each chromosome indicate genomic amplifications, squares on the left side indicate deletions. The percentages of genes amplified or deleted at each chromosomal location are indicated.

alterations were scattered at other chromosomal locations, reflecting genomic alteration in small regions, including multiple copies of single genes, in tumor cells. Overall, we found that there were many alterations in genes located on chromosomes 5 (7.5% amplified or deleted genes/analyzed genes), 11 (7.9%), 13 (13%), 14 (10%), and 17 (6.5%). In contrast, we did not identify any alterations on chromosomes 1 and 8 (Fig. 5), locations at which chromosomal alterations have been frequently observed in previous CGH analyses.

Genomic copy number profiling of hepatoma and non-hepatoma cells relative to clinical parameters

To determine the clinical relevance of our data, we evaluated the genomic copy number in each of these hepatoma and non-hepatoma cell lines with respect to their clinical parameters. Each hepatoma cell line in this study could be differentiated by various parameters, including HBV integration, AFP-production, and p53 mutation. Hierarchical clustering analysis of all copy number alterations did not differentiate these cell lines according to any clinical parameter, reflecting the high conservation of DNA copy number compared with mRNA expression. Using the class comparison method in BRB-Array tools ($p < 0.05$), however, we identified 57 genes that were differentially amplified between AFP-producing (40 genes) and AFP-negative (17 genes) cell

lines (Fig. 6A, Table 2). Importantly, expression of mRNA encoded by these genes differentiated AFP-producing from AFP-negative cell lines, except for one (Huh7 cells, Fig. 6B), suggesting a physiological role of genomic copy number alterations in gene expression and the phenotype of these cell lines. Clinical parameters other than AFP-production, however, did not differentiate these cell lines.

Surprisingly, the genes differentially amplified in AFP-producing and AFP-negative cells were clustered at specific chromosomal locations (Table 2). Genes amplified in AFP-producing cell lines included interleukin-1 receptor types 1 (IL-1R1), 2 (IL-1R2), and transforming growth factor β receptor-1 (TGF β R1), located at cytokine receptor cluster 2q11-12; the apoptosis regulatory genes granzyme H, Bcl-w, and DAD1, located at 14q11-12; and the immune response genes cytokine receptor (EBI3), intercellular adhesion molecule 3 (ICAM3), CD79A, and interferon regulatory factor 3, located at 19q13. In AFP-negative cell lines, amplified genes were clustered at chromosomes 5q11-13 and 17q11. When these differentially expressed genes were classified by function, we found that many cell-cell interaction genes, including cytokine and chemokine receptors (IL-1R1, IL-1R2, TGF β R1, chemokine (C-C motif) receptor 5, IGF2R, EBI3, and erythropoietin receptor), and cell adhesion molecules (collagen IX, ICAM3, Jagged1, and integrin), as well as cell cycle

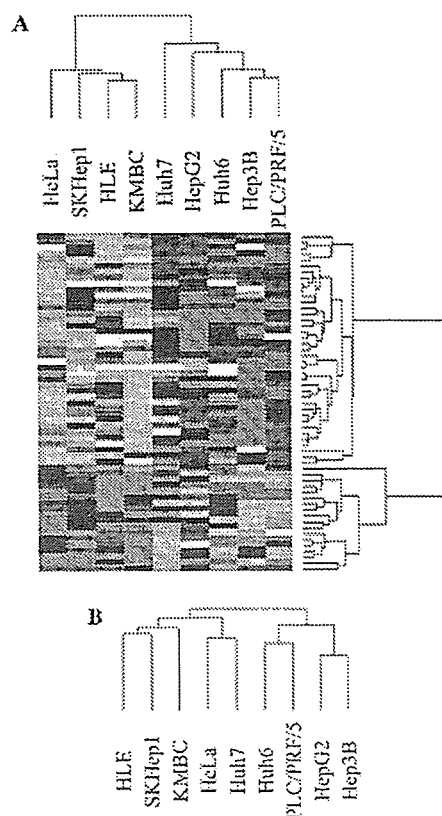


Fig. 6. Genes used to differentiate α -fetoprotein (AFP)-positive from AFP-negative cell lines. Using BRB-Array Tools Ver. 3.1.0 (<http://linus.nci.nih.gov/BRB-ArrayTools.html>), a modified *t* test was used to identify genes that could differentiate the AFP-positive hepatoma cell lines, Huh7, Hep3B, HepG2, PLC/PRF/5, and Huh6, from the AFP-negative hepatoma (SKHep1 and HLE) and non-hepatoma (HeLa and KMBC) cell lines. (A) Dendrogram of 57 genes determined by the class comparison method, showing the relatedness of genome gain or loss among these cell lines. (B) Expression of mRNA encoded by these 57 genes differentiated AFP-producing and AFP-negative cell lines, except for Huh7 cells.

(p19, KAP1, and B-myb) and apoptosis-related genes (granzyme H, Bcl-w, and DAD1), were up-regulated in AFP-producing cells.

Discussion

Microarray-based CGH was developed to detect genome-wide alterations in tumor samples [14]. We utilized cDNA as probes instead of BAC or PAC clones, making it easier to determine genes amplified and/or deleted in these cell lines and enabling us to evaluate the effect of changes in DNA copy number on mRNA expression [19]. In addition, the cDNA array-CGH method enabled us to detect changes in small regions of DNA, especially intron-less genes, including interferon- α and BCL-2 homologous antagonist/killer protein. Using cDNA

probes eliminated binding to intron sequences of the genome, thus decreasing the background signal. We verified the reliability of our cDNA array-CGH method using cell lines known to have high copy numbers of c-myc (HL60), N-myc (IMR-32), and luciferase (RCF-26) genes, all of which were significantly amplified by our cDNA array-CGH method and shown to correlate with Southern blot results. Our cDNA array consisted of suitable gene sets for analyzing the hepatoma cell lines. Using this series of in-house cDNA microarrays, gene expression profiling clearly distinguished between AFP-positive and AFP-negative cell lines [23], differential gene expression in chronic hepatitis B and C tissue lesions was demonstrated [21], gene expression profiling of hepatocellular carcinoma was performed [22], and genes for systemic vascular complications were found to be differentially expressed in the livers of type 2 diabetic patients [35]. Thus, the cDNA microarray used in this study was equipped with gene sets suitable and advantageous for the evaluation of human liver-derived materials. Furthermore, the sensitivity and specificity of our cDNA microarray had been properly evaluated. As we previously reported, the sensitivity of our array system is sufficient to detect 10^3 copies/ml (in the case of plasmid DNA) to 10^5 copies/ml (in the case of serum HBV virus) of the HBV genome, which corresponds to 4–400 ng/ml of human genomic DNA [36]. The high sensitivity of our array system enabled us to detect gene amplification or deletion properly, as confirmed by Southern blotting in this study. To reduce non-specific binding of intronic sequences to the cDNA probe on the slide, genomic DNA preparation was modified from previously described methods. Specifically, nuclei were isolated from cells, mitochondrial DNA was removed, and the nuclei were sonicated before *DpnII* restriction enzyme digestion. These changes increased the specificity and sensitivity of our cDNA array-CGH system.

Although few genes were amplified or deleted in common among the seven hepatoma cell lines analyzed, we found that there were common biological characteristics among the genes amplified or deleted in the individual cell lines. As the sharp contrast in function of the genes amplified and deleted in each cell line may reflect their different oncogenetic pathways and tumor phenotypes, a greater number of genes must be analyzed in these cell lines before more confident conclusions can be made.

In hepatoma cells, CGH analysis showed that the most frequent DNA copy number gains had been localized to 1p34.3-35, 1p33-34.1, 1q21-23, 1q31-32, 6p11-12, 7p21, 7q11.2, 8q24.1-24.2, 11q11-13, 12q11-13, 12q23, 17q11.2-21, 17q23-24, and 20p11.1-q13.2, whereas recurrent losses had been mapped to 3p12-14, 3q25, 4p12-14, 4q13-34, 5q21, 6q25-26, 8p11.2-23, 9p12-24, 11q23-24, 13q12-33, 14q12-13, 15q25-26, 18q11.2-22.2, and 21q21-22 [37]. Our data did not show amplified or deleted genes on chromosomes 1q or 8q where amplification had been

Table 2

Identification of 57 genes differentially amplified in AFP-producing and AFP-negative cell lines

Genes	Chromosome location	Fold (genome: AFP-producing cells/ AFP-negative cells)	Fold (expression: AFP-producing cells/ AFP-negative cells)	Function	GenBank
AFP-producing cell lines dominant					
Protein tyrosine phosphatase, non-receptor type 7	1q32.1	1.314	1.087	Signal transduction	NM_080588
MAX dimerization protein	2p13-p12	1.871	1.025	Transcriptional repressors	NM_002357
Ribosomal protein	2q11.1-q11.2	1.349	1.393	Housekeeping Genes	NM_014763
Interleukin 1 receptor 1	2q12	1.626	0.949	Cytokine	NM_003856
Interleukin 1 receptor 2	2q12-q22	1.359	0.807	Cytokine	NM_004633
TGFbeta receptor 1	2q12.1	1.315	0.797	Cell receptor	NM_004257
ERCC3	2q21	1.427	1.240	DNA repair	NM_000122
Homeobox protein HOX-D3	2q31-q37	1.627	1.087	Transcriptional factors	NM_006898
Chemokine (C-C motif) receptor 5	3p21	1.313	1.025	Cell receptor	NM_000579
Special AT-rich sequence-binding protein 1	3p23	1.301	0.726	Transcriptional factors	NM_002971
Sno oncogene snoN protein ski-related	3q26	1.326	0.948	Oncogenes	NM_005414
Epidermal growth factor	4q25	1.373	0.958	Growth factors	NM_001963
Collagen, type IX, alpha 1	6q12-q14	1.44	1.040	Cell-Cell interaction	NM_078485
Cytosolic acetoacetyl-coenzyme A thiolase	6q25-q27	1.347	2.234	Stress and toxicology response	NM_005891
IGF2R	6q26	1.4	1.270	Cell receptor	NM_000876
Paraoxonase 3	7q21.3	1.265	1.608	Metabolism	NM_000940
Deoxynucleotidyltransferase, terminal	9q34.3	1.427	1.433	Cell receptor	NM_002957
Granzyme H	14q11.2	2.503	0.942	Immune response	NM_033423
Ref-1	14q11.2-q12	1.468	1.375	DNA repair	NM_080648
Bcl-w	14q11.2-q12	1.424	1.057	Apoptosis	NM_004050
DAD1	14q11-q12	1.638	0.858	Apoptosis inhibitor	NM_001344
Chromosome 16 BAC clone CIT987SK-A-233A8	16	1.309	1.027	BAC clone	Unknown
p19	19p13	1.425	0.734	Cell cycle	NM_079421
Human protein phosphatase (KAP1)	19p13.2	1.45	1.077	Cell cycle	NM_005192
EB virus-induced gene 3 (EBI3)	19p13.3	1.319	0.927	Cytokine	NM_005755
Nuclear factor I/X (CCAAT-binding transcription factor)	19p13.3	1.304	1.037	Transcriptional factors	NM_002501
Erythropoietin receptor	19p13.3-p13.2	1.475	1.005	Cell receptor	NM_000121
Intercellular adhesion molecule 3 (ICAM 3)	19p13.3-p13.2	1.441	1.528	Cell-Cell interaction	NM_002162
CD79A antigen	19q13.2	1.329	0.969	Immune response	NM_021601
Interferon regulatory factor 3	19q13.3-q13.4	1.366	1.079	Cytokine	NM_001571
Jagged 1	20p12.1-p11.23	1.384	2.144	Cell-Cell interaction	NM_000214
B-myb	20q13.1	1.473	0.708	Cell cycle	NM_002466
Glutamate receptor, ionotropic, kainate 1	21q22.11	1.326	0.794	Cell receptor	NM_000830
Integrin, beta 2	21q22.3	1.368	1.088	Cell-Cell interaction	NM_000211
Glutathione-S-transferase T1	22q11.23	2.187	1.160	Metabolism	NM_000853
Crystallin, beta B1	22q12.1	4.905	0.906	Structural components	NM_001887
Leukemia inhibitory factor	22q12.2	1.431	0.800	Growth factors	NM_002309
ESTs	22q13.1	1.306	1.168	EST	NM_002110
HBV-P	—	8.496	2.188	Virus genome	—
HBV-full	—	3.294	2.394	Virus genome	—
AFP-negative cell lines dominant					
CD58 antigen	1p13	0.737	1.086	Immune response	NM_001779
Glucose transporter-like protein-III (GLUT3)	1p22-p21	0.762	3.791	Transcriptional factors	NM_006931
Endothelin 2	1p34	0.762	0.747	Vasoconstrictor	NM_001956
Corticotropin releasing hormone-binding protein	5q11.2-q13.3	0.697	1.117	Hormone regulator	NM_001882

(continued on next page)

Table 2 (continued)

Genes	Chromosome location	Fold (genome: AFP-producing cells/ AFP-negative cells)	Fold (expression: AFP-producing cells/ AFP-negative cells)	Function	GenBank
Granzyme A	5q11-q12	0.684	0.960	Immune response	NM_006144
XRCC4	5q13-q14	0.718	0.911	DNA repair	NM_022406
Human protocadherin 42	5q32-q33	0.697	0.308	Cell–Cell interaction	NM_032420
Human KIAA0056	11q25	0.787	0.839	Unidentified human genes	Unknown
P120 antigen	12p13	0.703	1.097	Cell Cycle	NM_006170
Myelodysplasia/myeloid leukemia factor 2 (MLF2)	12p13	0.764	1.178	Leukemia factor	NM_005439
Alpha-2-macroglobulin	12p13.3-p12.3	0.649	3.886	Cell–Cell interaction	NM_000014
HIF-1	14q21-q24	0.735	1.103	p53 Pathway	Unknown
Monocyte chemotactic protein 2	17q11.2	0.721	1.031	Chemokine	NM_005623
Ecotropic viral integration site 2B	17q11.2	0.71	0.707	Oncogene	NM_006495
CDC18	17q21.3	0.751	0.751	Cell Cycle	NM_001254
Topoisomerase (DNA) II alpha	17q21-q22	0.74	1.048	Transcriptional factors	NM_001067
Transmembrane 4 superfamily member 2	Xq11	0.69	1.109	Cell–Cell interaction	NM_004615

frequently observed in other CGH analyses [37,38]. This may be due to the relatively low number of analyzed genes located on chromosome 8, although many genes located on chromosome 1 have been analyzed (data were not shown). The other possibility is that our criteria used for identifying genes may have been too strict and may have failed to find genes located in these chromosome lesions. By setting over 1.5-fold as significant, several chromosome 1q or 8q genes were listed, but, in addition, many chromosome 11q or 17q genes were listed as well. The last possibility might be that the use of intron-less cDNA probe sequences reduced the sensitivity of gene alteration detection, alterations which involved both intron and exon sequences. We failed to detect c-myc amplification in Huh7 cells by Southern blotting using cDNA probes that had successfully detected c-myc amplification in HL60. It is possible that only portions of genes, such as c-myc intronic sequences, are amplified in Huh7 cells.

When we compared DNA copy number and mRNA expression of individual genes, we found that about 40% of amplifications and/or deletions were associated with changes in mRNA expression level. Other reports have hypothesized that global genome-wide analysis of expression profiles may reflect chromosomal aberrations in hepatoma [7]. These findings indicate that alterations in DNA copy number have a marked effect on gene expression in hepatoma cell lines. Among the differentially expressed genes in AFP-producing cells, as determined by hierarchical clustering [23], we found that 16 out of 325 genes were frequently changed at the genome-wide level (data was not shown). Thus, the data described in this report support our previous data.

We previously reported that the five AFP-producing hepatoma cell lines could be differentiated from two AFP-negative cell lines by their global gene expression

profiles using cDNA microarrays [23]. In this study, we found that these AFP-producing and AFP-negative hepatoma cell lines could not be clearly differentiated by hierarchical clustering using all the genes analyzed. We were able to identify many genes that could differentiate AFP-producing from AFP-negative hepatoma cell lines. We found that many cell–cell interaction genes, including cytokine and chemokine receptors, cell adhesion molecules, and cell cycle and apoptosis-related genes, were up-regulated in AFP-producing cells. Inflammation-related cytokine receptor family genes were up-regulated in AFP-producing cells, suggesting that these receptors may serve to mediate growth and inflammatory signaling, although functional studies must be performed to confirm this hypothesis. Thus, these data strongly suggest that alterations in DNA copy number are common to AFP-producing and AFP-negative cells, and that alterations in these genes, accompanied by altered mRNA expression, might determine the specific phenotype of each cell line. Differential expression of many of these genes did not exceed the 1.8-fold threshold, suggesting that differences lower than 1.8-fold may have physiological significance. Considering that non-synchronized cell populations contain cells at various points in the cell cycle, gene amplification or deletion may not be correlated with mRNA over- or underexpression, especially in those cells undergoing DNA synthesis or those in the mitotic phase of the cell cycle.

In this study, we have demonstrated that cDNA array-CGH analysis is a sensitive method for identifying altered genes in hepatoma cell lines. Although we found that alterations in DNA copy number can be correlated with gene expression, other types of alterations, including single nucleotide polymorphisms (SNPs) and epigenetic methylation, should also influence gene

expression levels. The biological significance of these chromosomal alterations on tumorigenesis in HCC requires further study, including the analysis of a greater number of genes and the use of more primary tumor samples.

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HEPATOBIILIARY DISEASE

Ephrin-A1 expression contributes to the malignant characteristics of α -fetoprotein producing hepatocellular carcinoma

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Background and aims: α -Fetoprotein (AFP), a tumour marker for hepatocellular carcinoma (HCC), is associated with poor prognosis. Using cDNA microarray analysis, we previously found that ephrin-A1, an angiogenic factor, is the most differentially overexpressed gene in AFP producing hepatoma cell lines. In the present study, we investigated the significance of ephrin-A1 expression in HCC.

Methods: We examined ephrin-A1 expression and its effect on cell proliferation and gene expression in five AFP producing hepatoma cell lines, three AFP negative hepatoma cell lines, and 20 human HCC specimens.

Results: Ephrin-A1 expression levels were lowest in normal liver tissue, elevated in cirrhotic tissue, and further elevated in HCC specimens. Ephrin-A1 expression was strongly correlated with AFP expression ($r=0.866$). We showed that ephrin-A1 induced expression of AFP. This finding implicates ephrin-A1 in the mechanism of AFP induction in HCC. Ephrin-A1 promoted the proliferation of ephrin-A1 underexpressing HLE cells, and an ephrin-A1 antisense oligonucleotide inhibited the proliferation of ephrin-A1 overexpressing Huh7 cells. Thus ephrin-A1 affects hepatoma cell growth. cDNA microarray analysis showed that ephrin-A1 induced expression of genes related to the cell cycle (p21), angiogenesis (angiopoietin 1 and thrombospondin 1), and cell-cell interactions (Rho, integrin, and matrix metalloproteinases) in cultured hepatoma cells. These ephrin-A1 induced genes are also activated in HCC tissues that overexpress AFP.

Conclusion: These findings suggest that the poor prognosis of patients with AFP producing HCC is partially caused by ephrin-A1 expression, which induces expression of genes related to tumour cell growth, angiogenesis, invasion, and metastasis.

Alpha-fetoprotein (AFP), a tumour marker for hepatocellular carcinoma (HCC), is expressed in fetal liver but is not expressed in normal adult liver. Approximately 70% of HCCs are positive for AFP, and levels of AFP increase with tumour progression.¹ HCCs with high levels of AFP have a poor prognosis and exhibit multicentric growth more frequently than AFP negative HCCs.^{2–4} In a previous study, we identified a gene cluster that was characteristically expressed in AFP producing hepatoma cell lines but not in non-AFP producing hepatoma cell lines.⁵ In this cluster, ephrin-A1 was the most differentially overexpressed gene in AFP producing hepatoma cell lines.

Ephrin-A1, a ligand for the Eph receptor tyrosine kinase, is involved in vascular development, tissue border formation, cell migration, axon guidance, synaptic plasticity, and adult neovascularisation.⁶ Several lines of evidence suggest that ephrin-A1 plays roles in multiple aspects of tumorigenesis, including abnormal cell growth, angiogenesis, invasion, and metastasis.^{7–12} Overexpression of ephrin-A1 in melanoma cells correlates with an increase in tumour cell growth, indicating that ephrin-A1 acts as a cell survival factor or a promoter of abnormal cell growth in tumour cells.^{9–11} Ephrin-A1 and its receptor are consistently expressed in endothelial cells of tumour associated vessels in a variety of human tumours, including lung, stomach, and colorectal cancers,⁷ and blocking the EphA receptor inhibits tumour angiogenesis and tumour progression *in vivo*.⁸ Inappropriate expression and regulation of ephrin ligands and Eph receptors affects cell-matrix interaction by modulating integrin activity.¹² Although these findings suggest that ephrin-A1 expression

participates in tumorigenesis, the biological significance and expression pattern of ephrin-A1 in HCC are unknown.

In the present study, we investigated ephrin-A1 expression in HCC specimens and hepatoma cell lines, and we examined the effect of ephrin-A1 on cell proliferation and gene expression. We also discuss the significance of ephrin-A1 expression in AFP producing HCC.

MATERIALS AND METHODS

Cell lines

Huh7, Hep3B, HepG2, Huh6, PLC/PRF/5, SK-Hep1, and HLE cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The simian virus 40 large-T antigen-immortalised normal human hepatocyte cell line (THLE-5b) was maintained in PMFR-4.^{13, 14}

Tissue specimens

Cancerous and non-cancerous tissues were obtained from patients who underwent partial hepatectomy for HCC. Normal controls were histologically normal tissues and were obtained from patients who underwent partial hepatectomy for metastatic liver tumours. These patients had no obvious underlying disease, tested negative for all hepatitis virus markers, and had normal levels of serum transaminase.

Abbreviations: AFP, α -fetoprotein; HCC, hepatocellular carcinoma; RIA, radioimmunoassay; IGF-II, insulin-like growth factor II; TGF- β , transforming growth factor β ; BMP, bone morphogenetic protein; TSP-1, thrombospondin 1; MMP-2, matrix metalloproteinase 2; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

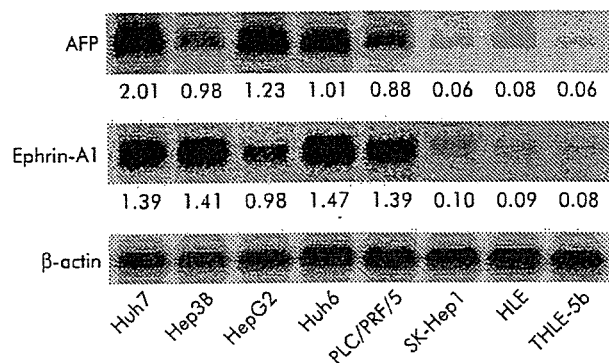


Figure 1 Expression of α -fetoprotein (AFP) and ephrin-A1 mRNA in eight human hepatoma cell lines determined by northern blotting (20 μ g RNA/lane). Expression levels of AFP and ephrin-A1 mRNA were quantified. The ratio of AFP or ephrin-A1 to β -actin is shown below each northern blot.

Cancerous and non-cancerous tissues were separately enucleated from resected tissues and were then immediately frozen in liquid nitrogen. Histological characterisation of HCC and normal liver tissue was performed as described previously.^{15, 16} Informed consent was obtained from all patients and ethics approval for the study was obtained from the ethics committee for human genome/gene analysis research at Kanazawa University Graduate School of Medical Science.

Northern blotting

Total RNA was isolated from cultured cells and frozen tissues using a ToTally-RNA kit (Ambion, Austin, Texas, USA). Northern blotting was performed using a NorthernMax kit (Ambion). cDNA probes containing unique sequences from human AFP, ephrin-A1, EphA1, EphA2, p21^{WAF1/CIP1}, thrombospondin 1 (TSP-1), matrix metalloproteinase 2 (MMP-2), and β -actin were labelled with [α -³²P] dCTP by random priming. mRNA expression levels were quantified with a Fujix-MacBas1000 Bio-imaging analyser (Fujix, Tokyo, Japan).

Immunohistochemistry

Tissue specimens were fixed in 10% formalin, embedded in paraffin blocks, and cut into 5 μ m section. Sections were

incubated overnight at 4°C with anti-ephrin-A1 (Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-EphA1 (R&D Systems, Minneapolis, Minnesota, USA), or anti-AFP antibodies (DakoCytomation, Glostrup, Denmark), and were subjected to the ABC immunostaining procedure using a Vectorstain ABC kit (Vector, Burlingame, California, USA), followed by DAB (Sigma-Aldrich Co., St. Louis, Missouri, USA). Negative controls were normal liver tissues in which northern blotting and western blotting confirmed the absence of ephrin-A1, AFP, and EphA1 expression.

Antisense-oligodeoxynucleotide and cell proliferation assay

We designed an antisense phosphorothioate oligodeoxynucleotide (5'-CCA GAG GAA CTC CAT AGC GC-3') complementary to the ephrin-A1 gene nucleotide sequence that spans from five nucleotides upstream of the predicted translational initiation site to 15 nucleotides downstream from this site. We also used a sense oligodeoxynucleotide (5'-GCG CTA TGG AGT TCC TCT GG-3') as a negative control. For positive controls, we used an antisense oligodeoxynucleotide for the potent HCC growth factor, insulin-like growth factor II (IGF-II; 5'-TCT GCC TCG CAG TTG G-3'), and its control sense oligodeoxynucleotide (5'-TGT CTC CCA GGC GGT T-3').¹⁷ For the proliferation assay, 3×10^5 cells were cultured in 96 well plates for 24 hours. Various concentrations of oligodeoxynucleotides were then added to the medium using the FuGENE6 transfection reagent (Boehringer Mannheim, Mannheim, Germany), and cultures were incubated for 72 hours. Cell growth was measured using the MTS assay kit (Promega, Madison, Wisconsin, USA).

Clonal growth assay

For studies using soluble ephrin-A1-Fc, 250 cells were plated in triplicate directly onto 12 well cell culture dishes in the presence of 0.3 μ g/ml ephrin-A1-Fc. The medium was changed every two days. After 8–10 days of culture, cell growth was measured using the MTS assay kit (Promega).

Ligand stimulation and cDNA microarray analysis

Subconfluent HLE cells were cultured with or without 1 μ g/ml of ephrin-A1-Fc for 10 hours in serum free Dulbecco's

Table 1 Characteristics of the 20 hepatocellular carcinomas

Case	Virus	Size of tumour (cm)	Histological grading of HCC*	Non-cancerous tissue staging and grading
1	C	4.7	Well differentiated	F4A3
2	B + C	5.5	Well differentiated	F4A1
3	C	3.0	Poorly differentiated	F3A1
4	C	2.7	Poorly differentiated	F4A1
5	C	13.5	Poorly differentiated	F3A2
6	C	3.5	Poorly differentiated	F4A2
7	C	5.5	Moderately differentiated	F3A1
8	C	4.0	Moderately differentiated	F4A3
9	B	10.0	Poorly differentiated	F3A2
10	C	5.5	Moderately differentiated	F3A2
11	C	3.5	Poorly differentiated	F4A2
12	C	2.5	Poorly differentiated	F4A2
13	C	6.0	Well differentiated	F2A1
14	C	2.3	Moderately differentiated	F3A1
15	B	3.5	Poorly differentiated	F4A2
16	C	2.0	Moderately differentiated	F4A2
17	C	1.4	Moderately differentiated	F3A1
18	C	2.5	Moderately differentiated	F3A1
19	C	2.5	Well differentiated	F4A2
20	C	4.0	Well differentiated	F3A1

*Histological grading of HCCs was decided according to the Classification of Liver Cancer Study Group of Japan. Histological grading and staging of non-cancerous tissue were decided according to the method of Desmet and colleagues.

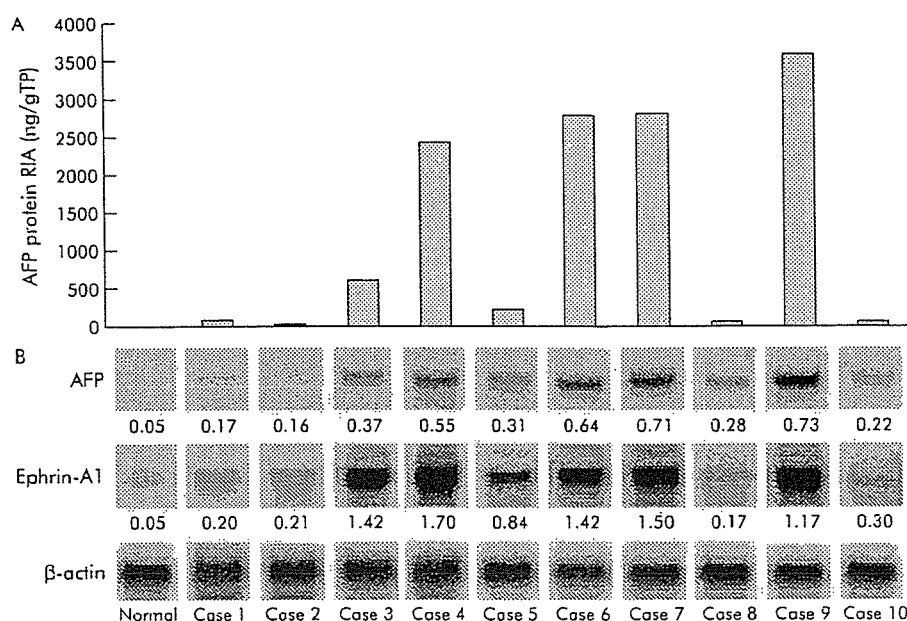


Figure 2 Expression of α -fetoprotein (AFP) and ephrin-A1 in one normal liver tissue specimen and 20 cancerous tissue specimens from patients with hepatocellular carcinoma. (A) Amount of AFP protein per gram of total protein (gTP) was calculated after AFP protein expression was quantified by radioimmunoassay. (B) AFP mRNA and ephrin-A1 mRNA expression analysed by northern blotting (20 μ g RNA/lane). Bands for AFP, ephrin-A1, and β -actin mRNA were quantified by densitometry. The number under each band indicates AFP and ephrin-A1 fragment signal normalised against the signal for β -actin.

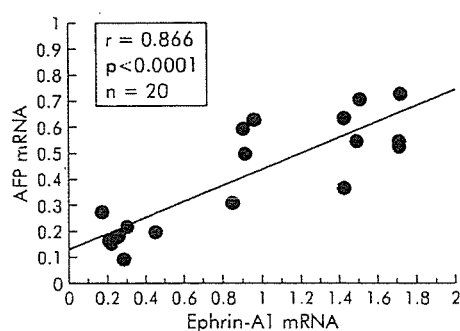
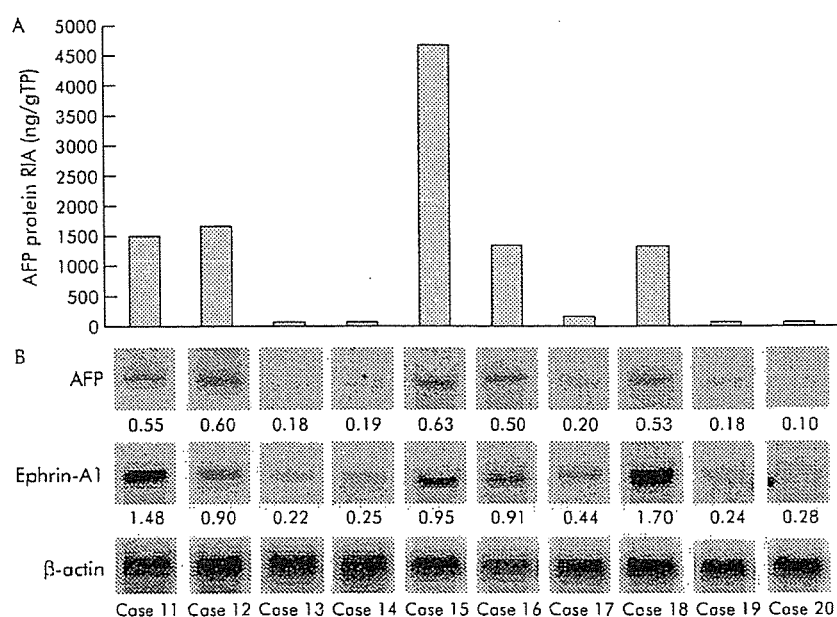


Figure 3 α -Fetoprotein (AFP) mRNA and ephrin-A1 mRNA values for individual patients were displayed on a scatterplot, and their linear correlation was evaluated.

modified Eagle's medium. Total RNA was then isolated. The cDNA microarrays were produced in collaboration with Hitachi Software Engineering Co. Ltd (Yokohama, Japan) using a SPBIO2000 robotic arrayer, and contained a total of 1080 cDNA clones, as described previously.^{5, 16, 18} Fluorescence intensities, generated by Cy5 or Cy3 immobilised to the target sequences on the microarray slides, were measured with a ScanArray5000 laser confocal microscope equipped scanning system (General Scanning, Watertown, Massachusetts, USA) with appropriate excitation and emission filters, as described previously.⁵ The signal from each immobilised cDNA target on the microarray slide was localised, and the expression ratio between the experimental and reference samples (Cy5/Cy3 ratio) was determined using ImageGene version 3.0 software (Biodiscovery, Los Angeles, California, USA). Microarray analysis was performed in triplicate, and the values of Cy5/Cy3 ratio represent averages.

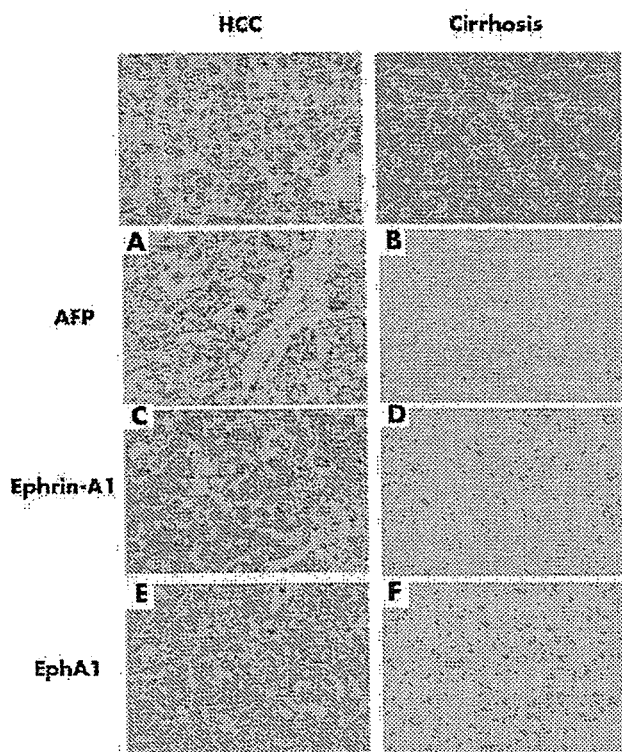


Figure 4 Immunohistochemistry of α -fetoprotein (AFP), ephrin-A1, and EphA1 in cancerous (A, C, E) and non-cancerous (B, D, F) tissues of hepatocellular carcinoma (HCC) (case 4). Immunoperoxidase labelling of these specimens using anti-AFP antibody (A, B), anti-ephrin-A1 antibody (C, D), or anti-EphA1 antibody (E, F). Top figures show haematoxylin-eosin staining of cancerous and non-cancerous tissues of HCC (case 4).

Immunoprecipitation and immunoblotting

Cell extracts were prepared in RIPA buffer (50 mM HEPES, pH 7.2, 150 mM sodium chloride, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10% glycerol, 1% TritonX-100, 0.1% sodium dodecyl sulphate, and 1% sodium deoxycholate) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin). For immunoblotting, whole cell lysates were boiled in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, separated by 4–20% gradient SDS-PAGE, and then electrophoretically transferred to PVDF membranes. Immunoblotting was carried out according to the manufacturer's instructions. Anti-ephrin-A1, anti-EphA2, anti-AFP, anti-p21, anti-TSP-1, and anti-MMP-2 antibodies were purchased from Santa Cruz Biotechnology, while anti-EphA1 antibody was purchased from R&D Systems. For immunoprecipitation, cell lysates were first incubated for two hours at 4°C with anti-EphA1 antibodies (R&D Systems) followed by incubation for 1.5 hours at 4°C with Gamma-Bind Sepharose beads. Immunoprecipitates were boiled in SDS-PAGE loading buffer, separated by 4–20% gradient SDS-PAGE, and then electrophoretically transferred to PVDF membranes. An anti-phosphotyrosine antibody, PY-20, conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, Kentucky, USA) was used for immunoblotting at a dilution of 1:2000. An anti-EphA1 antibody (R&D Systems) was used at a dilution of 1:1000, followed by an antirabbit antibody conjugated to horseradish peroxidase. Immunoreactive bands were revealed with the Amersham ECL immunoblot detection system.

Radioimmunoassay for AFP

AFP protein concentrations in clinical specimens were measured by radioimmunoassay (RIA) (SRL, Tokyo, Japan).¹⁶

Statistical analysis

In order to investigate correlations between expression levels of ephrin-A1 mRNA and AFP mRNA in clinical specimens, linear relationships were measured using scatterplots. Statistical differences between the two groups were determined by the Student's *t* test. A *p* value of <0.05 was considered statistically significant.

RESULTS

Ephrin-A1 expression correlates with AFP expression

Using cDNA microarray analysis, we previously found that ephrin-A1 was the most differentially overexpressed gene in AFP producing hepatoma cell lines.⁹ In the current study, we investigated expression of ephrin-A1 mRNA in eight hepatoma cell lines using northern blotting. As shown in fig 1, AFP producing hepatoma cell lines (Huh7, Hep3B, HepG2, Huh6, and PLC/PRF/5) displayed elevated ephrin-A1 expression compared with non-AFP producing hepatoma cell lines (SK-Hep1, HLE, and THLE-5b).

We then investigated expression of ephrin-A1 mRNA in cancerous tissue obtained from 20 patients with HCC by northern blotting (table 1, and fig 2A, B). Interestingly, in 11 (cases 3, 4, 5, 6, 7, 9, 11, 12, 15, 16, and 18) HCC specimens with markedly increased ephrin-A1 mRNA expression, the cancerous tissue showed high levels of AFP expression. Ephrin-A1 mRNA expression in HCC tissue strongly correlated with AFP mRNA expression ($r = 0.866$), suggesting a close association between expression of both genes (fig 3). Immunostaining (fig 4, case 4) showed that ephrin-A1, AFP, and EphA1 receptors were strongly expressed in the cytoplasm and on the cell surfaces of tumour cells in cancerous tissue. In particular, in AFP overexpressing HCC (cases 3, 4, 5, 6, 7, 9, 11, 12, 15, 16, and 18), ephrin-A1 was strongly expressed in tumour cells in cancerous tissue, but in AFP underexpressing HCC (cases 1, 2, 8, 10, 13, 14, 17, 19, and 20), expression of ephrin-A1 was markedly lower. Furthermore, in non-cancerous (cirrhotic) tissue, only slight expression of ephrin-A1, AFP, and EphA1 receptor was seen in hepatocytes. In normal liver tissues, expression of ephrin-A1, AFP, and EphA1 was not seen (data not shown).

Effect of ephrin-A1 on the proliferation of hepatoma cells

In order to determine whether overexpression of ephrin-A1 plays a significant role in the malignant growth of hepatoma cells, we examined the effect of an ephrin-A1 antisense oligonucleotide on proliferation of Huh7 and HLE cells (fig 5A). The specificity of the antisense oligonucleotide was confirmed by northern blotting. Suppression of ephrin-A1 by antisense oligonucleotide led to dose dependent growth inhibition of ephrin-A1 overexpressing Huh7 cells. The degree of growth inhibition caused by ephrin-A1 suppression was equal to or greater than the reduction of growth caused by suppression of IGF-II, a growth factor that strongly induces Huh7 cell proliferation.¹⁷ Compared with the sense oligonucleotide, the antisense oligonucleotide did not increase apoptosis in Huh7 cells, as determined by DNA content analysis and acridine orange/ethidium bromide double staining (data not shown). These results indicate that reduced cell proliferation, rather than increased apoptosis, was responsible for the reduction in Huh7 cell number by the ephrin-A1 antisense oligonucleotide. The antisense oligonucleotide against ephrin-A1 did not have an antiproliferative effect on HLE cells, which produce little ephrin-A1.

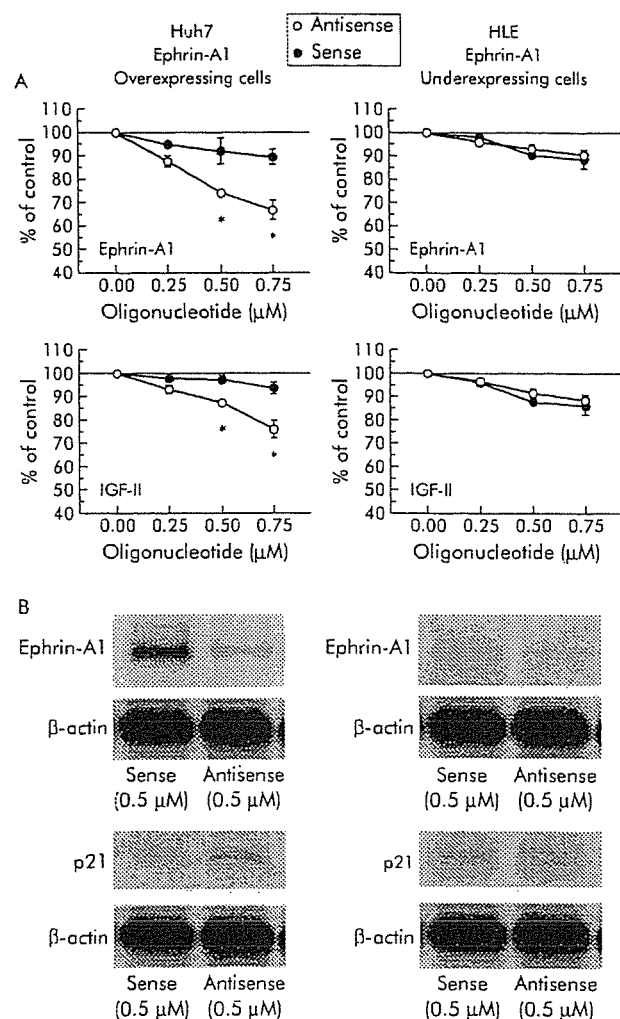


Figure 5 Dose dependent inhibition of cell proliferation by an ephrin-A1 antisense oligodeoxynucleotide. (A) Cell proliferation assay. Cells were incubated for 72 hours with various concentrations of antisense or sense oligodeoxynucleotides. Cell proliferation was measured by MTS assay. "Control" indicates absorbance at 490 nm with no oligodeoxynucleotide added. Mean (SEM) of three independent experiments is plotted. The upper figures show the effect of ephrin-A1 antisense or sense oligodeoxynucleotide, while the lower figures show the effects of insulin-like growth factor II (IGF-II) antisense or sense oligodeoxynucleotides. Bands under the graphs confirm that the antisense oligodeoxynucleotide suppresses ephrin-A1 mRNA expression by northern blotting. Total RNA was extracted from cells 20 hours after transfection (20 μg/lane). * $p < 0.05$. (B) Changes in expression of p21 mRNA after treatment with ephrin-A1 antisense or sense oligodeoxynucleotide by northern blotting. Total RNA was extracted from cells 24 hours after transfection (20 μg/lane).

We next used recombinant ephrin-A1 that was dimerised by fusion to human immunoglobulin G (ephrin-A1-Fc) to investigate the effect of ephrin-A1 on proliferation of hepatoma cells (fig 6A).¹⁹ We detected activated (tyrosine phosphorylated) EphA1 receptor in ephrin-A1 overexpressing Huh7 cells prior to addition of ephrin-A1-Fc, suggesting autocrine activation of the EphA1 receptor by endogenous ephrin-A1. In contrast, activated EphA1 receptor was not observed in HLE cells prior to addition of ephrin-A1-Fc, consistent with low expression of ephrin-A1 in these cells. Addition of ephrin-A1-Fc significantly increased the level of activated EphA1 receptor in both Huh7 and HLE cells. However, ephrin-A1-Fc promoted the proliferation of only ephrin-A1 underexpressing HLE cell (16% increase in cell growth, $p = 0.014$).

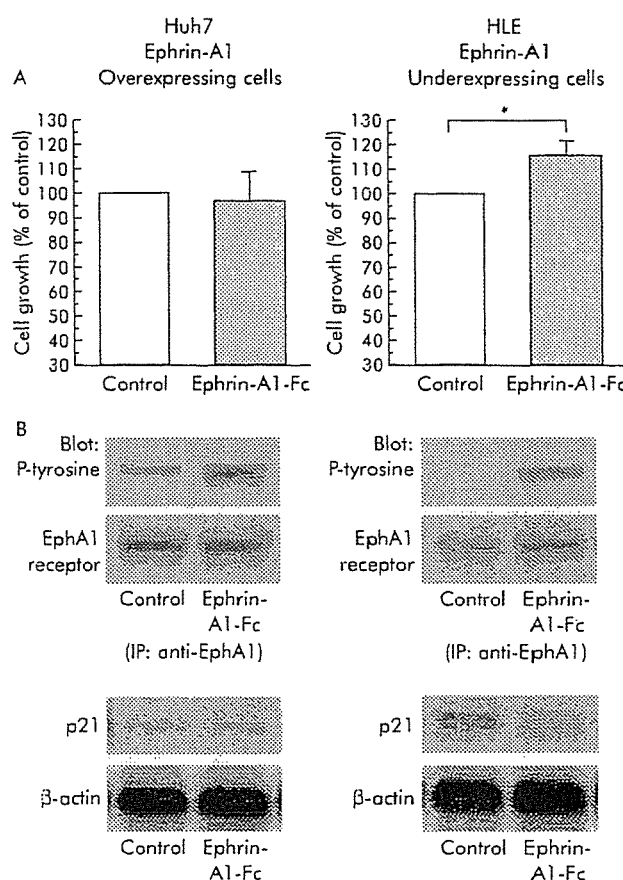


Figure 6 Effect of ephrin-A1 on proliferation of hepatoma cells. (A) Clonal growth assay. Cells were plated in 12 well cell culture dishes in the presence of 0.3 μg/ml ephrin-A1-Fc. Representative results from three independent experiments are shown. Bands under the graphs show that ephrin-A1-Fc stimulates tyrosine phosphorylation of the EphA1 receptor. Huh7 cells and HLE cells were treated for 60 minutes with 0.3 μg/ml ephrin-A1-Fc prior to protein extraction. Extracts were immunoprecipitated (IP) with antibodies against EphA1. Immunoprecipitates were subjected to western blotting, probed with antibodies against phosphorylated tyrosine (P-tyrosine), and reprobed with antibodies against EphA1. Data in the bar chart are mean (SEM) ($n = 9$). * $p < 0.05$. (B) Changes in expression of p21 mRNA 10 hours after stimulation with ephrin-A1-Fc by northern blotting (20 μg RNA/lane).

In order to clarify these results, we investigated expression levels of p21, a universal inhibitor of cyclin dependent kinases (figs 5B, 6B).²⁰ In HCC, p21 is an important inhibitor of cell cycle progression.²¹⁻²³ There was little basal expression of p21 in Huh7 cells in which the EphA1 signalling pathway was already activated due to autocrine stimulation (fig 6A, B). Consistent with autocrine activation of EphA1 and a role for p21, suppression of ephrin-A1 by the antisense oligonucleotide caused increased expression of p21 and inhibited Huh7 cell growth (fig 5B). Ephrin-A1-Fc stimulated tyrosine phosphorylation of the EphA1 receptor in Huh7 cells but ephrin-A1-Fc did not alter the already suppressed expression of p21, and it did not affect cell growth (fig 6B). In contrast, in HLE cells, EphA1 signalling was not activated in the basal state, and p21 was expressed (fig 6A, B). Furthermore, ephrin-A1-Fc stimulated EphA1 signalling, suppressed p21 expression, and promoted the proliferation of HLE cells (fig 6B). These findings strongly suggest that ephrin-A1 enhances cell proliferation, at least in part, through a p21 dependent signalling pathway.

Table 2 Changes in HLE gene expression by ephrin-A1-Fc stimulation

Gene category	Gene name	Cy5/Cy3 ratio
Cell cycle	<i>H.sapiens</i> RBQ-1	3.12
	RB107 retinoblastoma-like 1 (p107)	2.38
	RB130 retinoblastoma-like 2	2.13
	Cyclin D2	0.46
	p21	0.40
Angiogenesis	Endothelin 2	2.30
	Endothelin 3	2.22
	Laminin, $\alpha 4$	2.22
	Bone morphogenetic protein 4 (BMP4)	2.16
	Angiopoietin 1 (Ang1)	2.12
	Inhibin, βA (activin A, activin AB alpha polypeptide)	2.07
	Fibroblast growth factor receptor 3	1.85
	Laminin, $\gamma 1$ (formerly LAMB2)	0.68
	Bone morphogenetic protein 1 (BMP1)	0.68
	Thrombospondin 1 (TSP-1)	0.47
	CD22 antigen	2.41
Cell-cell interaction	RhoG protein	1.95
	CD36 antigen (collagen type I receptor, thrombospondin receptor)	1.94
	Matrix metalloproteinase 12 (MMP-12)	1.89
	Ras homologue gene family, member H	1.88
	Manic fringe (Drosophila) homologue	1.88
	Integrin, $\alpha 2$ (CD49B, alpha 2 subunit of VLA-2 receptor)	1.83
	Integrin, αM	1.81
	Matrix metalloproteinase 2 (MMP-2)	1.56
	CD44 antigen (homing function and Indian blood group system)	0.68
	Alpha-fetoprotein (AFP)	1.91
	Met proto-oncogene (hepatocyte growth factor receptor)	0.52
Oncogene	Vav 2 oncogene	0.59
	Tachykinin 2	2.31
Growth factors and cytokines	Interleukin 5	0.59
	Fibroblast growth factor 7 (keratinocyte growth factor)	0.50
Cell receptor	Interleukin 5 receptor, α	3.12
	Erythropoietin receptor	2.12
	Glutamate receptor, ionotropic, kainate 1	1.90
Signal transduction	EphA1 receptor	1.82
	Human 76 kDa tyrosine phosphoprotein SLP-76 extracellular signal regulated kinase 2 (ERK2)	0.53
Stress and toxicology response	Cytochrome P450, subfamily IIC	1.95
Transcription factors	Hepatic leukaemia factor	2.30
	Nuclear factor 1/X (CCAAT binding transcription factor)	2.11
	Human Ikaros/LyF-1 homologue (hIk-1)	2.04
Tumour suppressors	BRCA1 associated RING domain 1	1.95
Cytoskeleton	Keratin, type II cytoskeletal 6D	0.42

Changes in gene expression caused by ephrin-A1

We used cDNA microarrays to identify the genes that are induced by ephrin-A1-Fc stimulation during hepatoma cell proliferation (table 2). Ephrin-A1-Fc (fig 7A) increased expression of AFP, indicating that enhanced ephrin-A1 expression induced AFP expression in hepatoma cells. Furthermore, we performed northern blotting to confirm that when mRNA expression of ephrin-A1 in Huh7 cells was suppressed by 77% using the antisense oligonucleotide, mRNA expression of AFP decreased by 24% (fig 7B). This agrees with our finding that ephrin-A1 expression strongly correlates with AFP expression in cultured hepatoma cells and HCC tissue (figs 1–4). There was also an increase in expression of the EphA1 receptor, one of the seven EphA receptors that bind to ephrin-A1 (fig 7A).^{24–27} Although the EphA2 receptor is reported to mediate the majority of effects of ephrin-A1 in various cancers,^{7,9,28–32} little or no change in EphA2 receptor expression was observed (data not shown).

In the category of cell cycle related genes, we observed decreased expression of p21 caused by ephrin-A1. This is consistent with the results of cell proliferation assays (fig 6B). Our microarray data confirmed that ephrin-A1 suppresses p21 expression. In addition, ephrin-A1 increased expression of other cell cycle related genes, such as RB family proteins (table 2).

Among angiogenesis related genes, we found a decrease in the tumour angiogenesis inhibitor TSP-1 and an increase in the tumour angiogenesis promoter angiopoietin 1 (table 2, fig 7A). We also found increased expression of the TGF- β superfamily, as well as angiogenesis related vasoactive substances, such as bone morphogenetic protein (BMP), inhibin, endothelin, fibroblast growth factor receptor, and laminin.

Among cell-cell interaction related genes, we observed increased expression of Rho family members and integrins, as well as MMP-2 and MMP-12 (table 2, fig 7A).

Expression of ephrin-A1 induced genes in HCC tissue

We studied the association between expression of ephrin-A1 mRNA and AFP, EphA1 receptor, EphA2 receptor, p21, TSP-1, and MMP-2 mRNA in 11 HCC tissues that overexpress AFP by northern blotting (cases 3, 4, 5, 6, 7, 9, 11, 12, 15, 16, and 18) (table 3). In all 11 of these cases, cancerous tissue showed increased expression of ephrin-A1 mRNA compared with non-cancerous tissue.

We examined the relationship between ephrin-A1 and the EphA2 receptor because it has been reported that an autocrine loop between ephrin-A1 and the EphA2 receptor is responsible for the biological activity of ephrin-A1 in various cancers.^{7,9,28–32} Surprisingly, EphA2 receptor mRNA

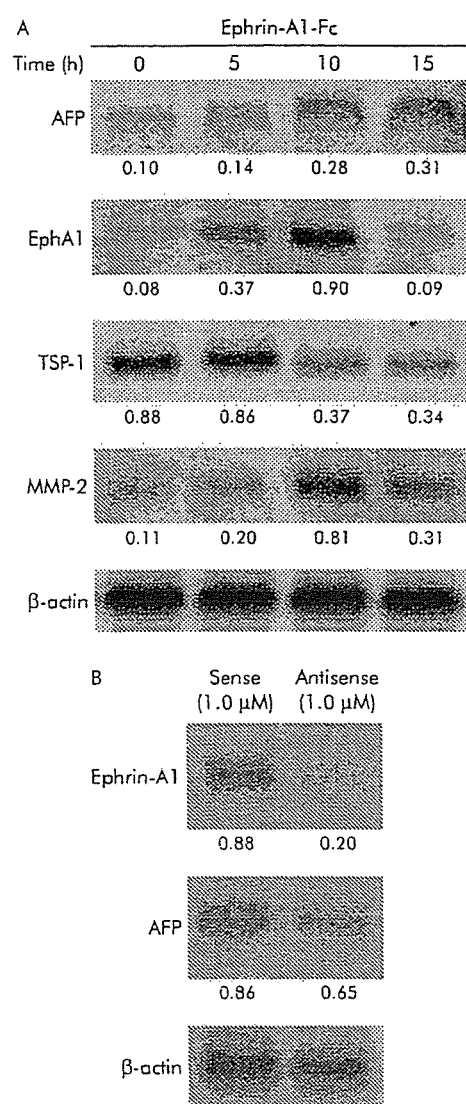


Figure 7 (A) Time course (0, 5, 10, and 15 hours) of changes in expression of α -fetoprotein (AFP), EphA1, thrombospondin 1 (TSP-1), and matrix metalloproteinase 2 (MMP-2) mRNA levels in HLE cells after stimulation with ephrin-A1-Fc by northern blotting (20 μ g RNA/lane). The relative amounts of AFP, EphA1, TSP-1, and MMP-2 mRNA fragments were measured by densitometry and normalised against β -actin levels. (B) Suppression of ephrin-A1 mRNA by antisense oligonucleotide inhibited expression of AFP in Huh7 cells. Total RNA for ephrin-A1 was extracted from cells 20 hours after transfection (20 μ g/lane), and total RNA for AFP was extracted from cells 30 hours after transfection (20 μ g/lane). Expression levels of ephrin-A1 and AFP mRNA were quantified. The ratio of ephrin-A1 or AFP to β -actin is shown below each northern blot.

was strongly expressed in non-cancerous tissue but its expression was suppressed in cancerous tissue. This expression pattern is the opposite of the ephrin-A1 mRNA expression pattern in HCC tissues. Expression of EphA1 receptor mRNA was more enhanced in cancerous tissue than in non-cancerous tissue and was closely correlated with ephrin-A1 mRNA expression levels. Identical expression patterns for ephrin-A1 and the EphA1 receptor protein were also found by immunohistochemistry (fig 4). Because ephrin-A1 can induce tyrosine phosphorylation of EphA1 receptors in Huh7 and HLE cells (fig 6A), these findings suggest that ephrin-A1-EphA1 signalling is activated in HCC.

Microarray analysis of ephrin-A1 stimulated HLE cells revealed reduced expression of p21 and TSP-1 mRNA and

increased expression of MMP-2 mRNA (table 2, figs 6B and 7A). These proteins play important roles in malignant proliferation, angiogenesis, invasion, and metastasis of HCC.^{21, 33-35} In agreement with these results, p21 mRNA expression levels in cancerous tissues were significantly reduced in comparison with those in non-cancerous tissues in eight (cases 4, 5, 6, 7, 11, 12, 16, and 18) of the 11 HCC specimens that overexpressed AFP (table 3). These eight cases exhibited enhanced ephrin-A1 mRNA expression and reduced p21 mRNA expression in cancerous tissue compared with non-cancerous tissue. In both cancerous and non-cancerous tissues from the remaining three cases (cases 3, 9, and 15), p21 mRNA expression was inhibited while ephrin-A1 was strongly expressed.

TSP-1 mRNA expression was lower in cancerous tissue than in non-cancerous tissue in eight (cases 3, 4, 6, 7, 9, 11, 12, and 15) of the 11 cases of HCC that overexpressed AFP. Thus TSP-1 mRNA expression correlated negatively with ephrin-A1 expression. MMP-2 mRNA expression was higher in cancerous tissue than in non-cancerous tissue in 10 (cases 3, 4, 5, 6, 7, 9, 12, 15, 16, and 18) of the 11 cases of HCC that overexpressed AFP. This revealed a positive correlation between MMP-2 mRNA expression and ephrin-A1 mRNA expression.

Furthermore, we performed northern blotting and western blotting in order to confirm that mRNA expression of AFP, ephrin-A1, EphA1, EphA2, p21, MMP2, and TSP-1 in cancerous and non-cancerous tissues was correlated with expression of their proteins (fig 8).

The above findings indicate that ephrin-A1 induced genes identified by cDNA microarray analysis are activated not only in cultured hepatoma cells but also in clinical cases of HCC that overexpress AFP.

DISCUSSION

Expression of ephrin-A1 was very low in normal liver tissue, slightly increased in liver tissues from patients with cirrhosis, and was further increased in HCC. Expression of ephrin-A1 was greater in poorly differentiated HCC than in well differentiated HCC (table 1, figs 2-4). Thus expression of ephrin-A1 may be related to the malignant phenotype of HCC. It is possible that local ischaemia in the cirrhotic state may induce angiogenic factors, including ephrin-A1. Immunohistochemistry showed high levels of ephrin-A1 protein in tumour tissue. Although tumour tissue includes not only hepatocytes but also non-parenchymal cells (for example, stellate cells, endothelial cells, Kupffer's cells, and infiltrated lymphocytes), immunohistochemical staining confirmed that increased expression of ephrin-A1 was in tumour cells rather than in the surrounding normal hepatocytes (fig 4). This indicates that increased expression of ephrin-A1 in HCC tissue mainly originated from hepatocytes within the tumour.

Expression levels of ephrin-A1 were strongly correlated with those of AFP in hepatoma cell lines and human HCC tissues. The cDNA microarray analysis showed that ephrin-A1 induced AFP expression in hepatoma cells, implicating ephrin-A1 in the mechanism of AFP induction. This may explain why most patients with liver cirrhosis have elevated AFP levels without any detectable HCC. Therefore, ephrin-A1 may play an important role in the transformation of normal hepatocytes to tumour cells.

Enhanced expression of ephrin-A1 may lead to tumorigenesis by promoting abnormal cell growth, angiogenesis, invasion, or metastasis.⁷⁻¹² However, the biological significance of ephrin-A1 in hepatocellular carcinogenesis has not yet been clarified. We found that reduction of ephrin-A1 expression by an antisense oligonucleotide inhibited the proliferation of ephrin-A1 overexpressing Huh7 cells and

Table 3 Expression of ephrin-A1 induced genes in 11 α -fetoprotein (AFP) overexpressing hepatocellular carcinomas

Case	AFP RIA* (ng/gTP)	AFP †	Ephrin-A1†	EphA1†	EphA2 †	p21†	TSP-1†	MMP-2†
3	612	++	+	++	-	+/-	-	++
4	2454	++	++	++	-	-	-	++
5	185	++	++	++	-	-	+/-	++
6	2776	++	+	++	-	-	-	++
7	2816	++	++	++	+/-	-	-	++
9	3613	++	++	++	-	+/-	-	++
11	1526	++	++	++	-	-	-	-
12	1684	++	++	++	-	-	-	+
15	4671	++	++	++	-	+/-	-	++
16	1339	++	++	++	-	-	+/-	++
18	1311	++	++	++	-	-	+/-	+

*AFP RIA(ng/gTP) indicates the quantity of α -fetoprotein per g total protein after quantification of AFP expression by the radioimmunoassay method.

†Relative amounts of AFP, ephrin-A1, EphA1, EphA2, p21, TSP-1, and MMP-2 mRNA fragments by northern blotting were measured by densitometry and normalised to the level of β -actin. The ratios of the cancerous mRNA expression level to non-cancerous mRNA expression level (T/N 18 ratios) are indicated (T/N ratio <0.50, -; 0.50 <T/N ratio <0.67, -; 0.67 <T/N ratio <1.50, +/-; 1.50 <T/N ratio <2.00, +; 2.00 <T/N ratio, ++).

increased expression of p21, an inhibitor of cyclin dependent kinases.²⁹⁻³¹ In contrast, inhibition of ephrin-A1 expression did not inhibit basal proliferation of HLE cells because they express little ephrin-A1. In HLE cells, ephrin-A1-Fc promoted cellular proliferation, activated the EphA1 receptor, and suppressed p21 expression. However, ephrin-A1-Fc did not promote proliferation of ephrin-A1 overexpressing Huh7 cells in which the EphA1 receptor is already activated and p21 expression is suppressed by autocrine ephrin-A1. These results indicate that ephrin-A1 promotes the proliferation of cultured hepatoma cell lines and that p21 might be involved in the regulation of the cell cycle induced by ephrin-A1.

Using cDNA microarray analysis we found that ephrin-A1 induced genes related to the cell cycle, angiogenesis, and cell-cell interactions. In agreement with the results of clonal growth assay, we observed decreased p21 expression in response to ephrin-A1. Reduced expression of p21 has been reported to participate in hepatocarcinogenesis and is associated with larger tumour size and poor prognosis in HCC patients.³⁰⁻³³ In HCC tissues that overexpress AFP, we found that p21 expression is suppressed in cancerous tissue compared with non-cancerous tissue and that p21 expression correlates negatively with ephrin-A1 expression. Although we also found that ephrin-A1-Fc increased expression of RB family cell cycle arresting genes, many studies have reported mutations and inactivation of RB family proteins and p53 in HCC tissue.^{21, 22, 36, 37} Therefore, increased expression of these genes does not necessarily imply that they cause arrest of the cell cycle in HCC.

We found that ephrin-A1 induces expression of the angiogenesis factor angiopoietin 1³⁸ as well as angiogenesis related vasoactive substances, including BMP, inhibin, endothelin, fibroblast growth factor receptor, and laminin. We also found that ephrin-A1 inhibits the expression of the tumour angiogenesis inhibitor TSP-1. Reduced TSP-1 expression promotes angiogenesis in melanoma, lung cancer, breast cancer, and cholangiocarcinoma.³⁹⁻⁴¹ TSP-1 expression, which correlated negatively with ephrin-A1 expression, was suppressed in HCC tissue compared with non-cancerous tissue. These results suggest that ephrin-A1 downregulates TSP-1 and acts upstream of angiogenesis related factors in promoting HCC vascular growth.

Our microarray studies also showed that ephrin-A1 increases expression of cell-cell interaction related genes, such as Rho family proteins, integrins, MMP-2, and MMP-12. Ephrin-A1 regulates cellular migration and cell-cell and cell-matrix interactions via Rho and integrin, thus influencing tumour cell motility, invasion, and metastasis in various human cancers.^{9-12, 42-43} Changes in expression of these proteins play a key role in the invasion and metastasis of HCC. MMPs play a crucial role as proteinases during cancer invasion and metastasis, and overexpression of MMP-2 correlates closely with portal invasion and intrahepatic metastasis of HCC.^{33, 35} We found that MMP-2 expression is higher in cancerous tissue than in non-cancerous tissue in cases of HCC that overexpress ephrin-A1. In HCC cases that underexpress ephrin-A1, MMP-2 expression is lower in cancerous tissue than in non-cancerous tissue. These findings suggest that ephrin-A1 increases expression of MMP-2,

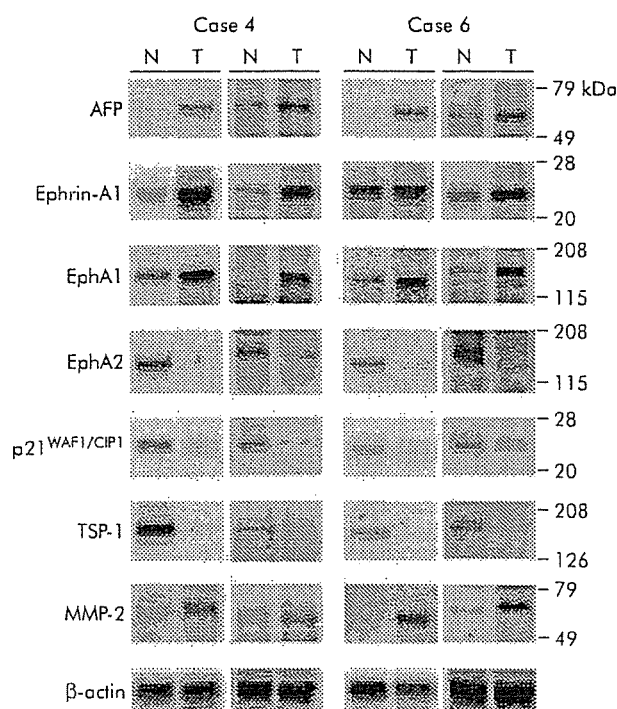


Figure 8 Expression of α -fetoprotein (AFP), ephrin-A1, EphA1, EphA2, p21, thrombospondin 1 (TSP-1), and matrix metalloproteinase 2 (MMP-2) mRNA and protein in AFP overexpressing HCC patients (cases 4 and 6) by northern blotting and western blotting. In each figure, the left side shows the results of northern blotting (total RNA: 20 μ g/lane) while the right side shows the results of western blotting. T, tumour; N, adjacent non-cancerous tissue.

thereby promoting cancer invasion and metastasis in cases of HCC that overexpress AFP. The ephrin-A1 induced changes in gene expression of hepatoma cells in vitro recapitulate the changes in gene expression of cancerous and non-cancerous tissues from patients with HCC in vivo. This is particularly the case in HCC tissues that overexpress AFP and have high levels of ephrin-A1 expression; a large percentage (72.7%) of these HCC cases are highly malignant and poorly differentiated.

In conclusion, ephrin-A1 induces gene expression that is also activated in AFP producing hepatoma but not in non-AFP producing hepatoma. Ephrin-A1 induces AFP and changes the expression level of genes associated with tumour cell proliferation, angiogenesis, invasion, and metastasis. It is important to examine whether the findings could be applied to HCC related to non-viral liver disease, because all HCC cases in this study were related to viral liver disease. Elucidation of gene expression patterns in AFP producing HCC is important for the development of new molecular targeting therapies. These results indicate that ephrin-A1 is a promising therapeutic target in cases of HCC that overexpress AFP.

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La Protein Is a Potent Regulator of Replication of Hepatitis C Virus in Patients With Chronic Hepatitis C Through Internal Ribosomal Entry Site-Directed Translation

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Background & Aims: Translation of hepatitis C virus is an essential step of viral replication and is mediated by an internal ribosome entry site. We previously reported that the hepatitis C virus internal ribosome entry site is most active during the synthetic (S) or mitotic (M) phases and lowest during quiescent (G_0) phase. Here, we investigated host factors responsible for the regulation of the hepatitis C virus internal ribosome entry site. **Methods:** We synchronized the cell-cycle progression and evaluated gene-expression dynamics of host factors and kinetics of hepatitis C virus internal ribosome entry site activity in cells at various points during the cell cycle by using a complementary DNA microarray. We also validated the significance of identified host factors on hepatitis C virus replication in vivo. **Results:** Hepatitis C virus internal ribosome entry site activity correlated with a gene cluster induced in the S and G_2 /M phases. It is interesting to note that most initiation factors known to bind or interact with the hepatitis C virus internal ribosome entry site [poly(rC)-binding protein 2, polypyrimidine tract binding protein, eukaryotic initiation factor 3, eukaryotic initiation factor 2 γ , eukaryotic initiation factor 2 β , La protein, and heterogeneous nuclear ribonucleoprotein L] were induced during the S and G_2 /M phases. Expression of La protein, polypyrimidine tract binding protein, and eukaryotic initiation factor 3 (p116, p170) were predominantly repressed in G_0 phase and induced in S and G_2 /M phases. Suppression or overexpression of La protein and polypyrimidine tract binding protein in RCF-26 significantly changed hepatitis C virus internal ribosome entry site activity. In the livers of patients with chronic hepatitis C, expression of La protein was significantly increased and correlated with the amount of hepatitis C virus RNA. **Conclusions:** Hepatitis C virus uses host factors induced during cell division but not during quiescence for replication. Of these, La protein is a potent regulator and enhances hepatitis C virus replication in regenerating hepatocytes in patients with chronic hepatitis C.

HCV develops chronic hepatitis, cirrhosis, and, in some instances, hepatocellular carcinoma.^{1,2} Although a combination of ribavirin and interferon has become a popular means of treating infected patients, the results are often unsatisfactory, especially in patients with a high viral load.^{3–6} Identification of host factors that regulate HCV replication in infected patients could be helpful in the development of a novel antiviral treatment strategy.

Translation of the polyproteins of the HCV RNA genome is an essential step in viral replication and is supposed to be a fruitful target of new antiviral treatment strategies, such as antisense oligonucleotide (oligo) or small interference RNA. Translation of HCV is initiated by a highly structured RNA segment, the internal ribosome entry site (IRES), that occupies most of the 5'-nontranslated (5'-NTR) RNA.^{7–15} The translation machinery of HCV is simple and, because it is a prokaryote, requires only the ribosomal 40S subunit, the eukaryotic initiation factor (eIF)2/guanosine triphosphate/Met-transfer RNA complex, and eIF3 to initiate translation.⁷ In contrast, cap-dependent translation is more complex and requires additional canonical initiation factors, such as eIF4E, eIF4G, eIF4A, and eIF4B.⁷ Many other noncanonical translation initiation factors, such as La protein,^{16,17} polypyrimidine tract binding protein (PTB),¹⁸ heterogeneous nuclear ribonucleoprotein L (RNPL),¹⁹ poly(rC)-binding protein (PCBP)-2,²⁰ and ribosomal protein S9,⁷ interact with HCV IRES and might regulate HCV translation. Thus, the machineries of cap-dependent and HCV IRES-directed translation

Abbreviations used in this paper: CMV, cytomegalovirus; eIF, eukaryotic initiation factor; FBS, fetal bovine serum; FL, firefly luciferase; IRES, internal ribosome entry site; nt, nucleotide; 5'-NTR, 5' nontranslated region; oligo, oligonucleotide; PABPC, poly(A)-binding protein, cytoplasmic; PCBP, poly(rC)-binding protein; PTB, polypyrimidine tract binding protein; RL, *Renilla* luciferase; RNPL, heterogeneous nuclear ribonucleoprotein L; RTD, real-time detection; RT-PCR, reverse-transcription polymerase chain reaction; SOM, self-organizing map.

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Hepatitis C virus (HCV), a positive-strand enveloped RNA virus, belongs to the genus *Hepacivirus* of the family Flaviviridae.¹ The human liver infected with

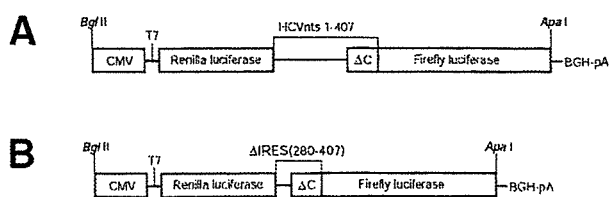


Figure 1. Organization of the transcriptional unit of plasmid pRL-HL and pRL-(ΔIRES)-HL. (A) Plasmid pRL-HL²² contains a dicistronic CMV transcriptional cassette in which upstream *Renilla* and downstream firefly luciferase genes are separated by the complete 5'-NTR and 66-nt core sequence of HCV (nts 1–407; strain 1b) placed in the intercistronic space. (B) Plasmid pRL-(ΔIRES)-HL was the control plasmid of pRL-HL in which the functional HCV IRES element (nts 1–279) was deleted. CMV, cytomegalovirus promoter; T7, Bacteriophage T7 RNA polymerase promoter; BGH-pA, bovine growth hormone polyadenylation signal.

can be differentiated in terms of requirements for canonical and noncanonical initiation factors.²¹

We previously found that HCV IRES activity varies during the cell cycle and is greatest during the synthetic (S) or mitotic (M) phases and lowest during the quiescent (G₀) phase.²² These findings suggest that HCV translation is regulated by cellular proteins that vary in abundance during the cell cycle and that viral replication is enhanced by factors that stimulate the regeneration of hepatocytes in patients with chronic hepatitis C. This finding implies that inflammation and the resulting increased turnover in hepatocytes may increase the number of actively dividing hepatocytes, resulting in increased IRES activity and enhanced HCV replication.

This study profiles the expression of cellular proteins during cell-cycle progression and identifies factors responsible for cell cycle-dependent HCV IRES-directed translation. We evaluated whether those factors are in fact related to HCV replication in the livers of patients with chronic hepatitis C.

Materials and Methods

Plasmids

Plasmid pRL-HL contains a dicistronic cytomegalovirus (CMV) transcriptional cassette in which an upstream *Renilla* luciferase (RL) gene and a downstream firefly luciferase (FL) gene are separated by the complete 5'-NTR and 66-nucleotide (nt) core sequence of HCV (nts 1–407 of a genotype 1b strain) placed within the intercistronic space²² (Figure 1A). Plasmid pRL-(ΔIRES)-HL was the control plasmid of pRL-HL in which the functional HCV IRES element (nts 1–279) had been deleted (Figure 1B). This plasmid was constructed by subcloning the 1.82-kilobase *StuI/ApaI* fragment of pRL-HL (containing the deleted HCV IRES element [nts 280–341 of the HCV-1b 5'-NTR sequence and 66 nts of the core sequence] fused directly to FL) into the multiple cloning site of

pBluescript IISK (Stratagene, La Jolla, CA). A 1.82-kilobase *NotI/ApaI* fragment was subsequently excised from this plasmid and cloned into the *NotI/ApaI* site of pRL-HL.

The La expression vector pCMV-La was constructed as previously described.²³ The PTB expression vector pRC/CMV-PTB, the RNPL expression vector pcDNA3-myc-hnRNPL, and the PCBP-1 and PCBP-2 expression vectors pcDNA3-myc-haCP1 and pcDNA3-myc-haCP2 were provided by Dr. Stanley M. Lemon,²⁴ Dr. Gideon Dreyfuss,²⁵ and Dr. Stephen A. Liebhaber,²⁶ respectively. Dr. John W. B. Hershey provided the eIF2γ full-length clone in pSP72.²⁷ We constructed the eIF2γ expression vector pCMV-eIF2-γ by excising the *EcoRI* and *EcoRI* fragment of full-length coding sequences and cloning into *EcoRI* of pCR 3.1 (Invitrogen, San Diego, CA) under the control of the CMV promoter. Dr. Keith Johnson provided the eIF3 p170 full-length clone in PUC19.²⁸ We constructed the eIF3 p170 expression vector pCMV-eIF3 p170 by excising the *KpnI* and *SnaBI* fragment of full-length coding sequences and cloning into *KpnI* and *EcoRV* of pCR 3.1 under the control of the CMV promoter. The complementary DNA (cDNA) of ribosomal protein S9 was cloned by reverse-transcription polymerase chain reaction (RT-PCR) of total RNA isolated from Huh-7 cells by using sense (5'-ACGGTGAAGCGGACGCAACATGCCAGTGG-3') and antisense (5'-GGGACAGGTGGACTTAATCCTCCTCCTCGTCG-3') primers. The resultant cDNA was cloned into the TOPO TA cloning vector (Invitrogen), and nt sequences were confirmed. The expression vector pCMV-S9 was constructed by excising and cloning *HindIII* and *XbaI* fragments from TOPO TA into the same sites of pCR 3.1.

Cell Lines

The RCF-26 was a stably transformed cell line from Huh-7 cells (human hepatocellular carcinoma cells) that constitutively express dicistronic RNA transcripts containing sequences encoding 2 reporter proteins, RL and FL, separated by a functional HCV IRES²² (Figure 1A). The ΔRCF-9 was a stably transformed cell line from Huh-7 cells that constitutively expressed dicistronic RNA transcripts in which the functional HCV IRES element (nts 1–279) had been deleted (Figure 1B).

Overexpression of Canonical and Noncanonical Initiation Factors in RCF-26

The RCF-26 cells were cultured in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 400 μg/mL of Geneticin (active compound) (Gibco BRL, Gaithersburg, MD). Cells cultured in a 5% CO₂ incubator at 37°C were transfected with 0.5–1.0 μg of plasmid DNA by using FuGENE 6 (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer's instructions. After 24–48 hours of transfection, the cells were harvested, and reporter genes were assayed.