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Differential gene alteration among hepatoma cell lines demonstrated by cDNA microarray-based comparative genomic hybridization

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Abstract

We assayed chromosomal abnormalities in hepatoma cell lines using the microarray-based comparative genomic hybridization (array-CGH) method and investigated the relationship between genomic copy number alterations and expression profiles in these hepatoma cell lines. We modified a cDNA array-CGH assay to compare genomic DNAs from seven hepatoma cell lines, as well as DNA from two non-hepatoma cell lines and from normal cells. The mRNA expression of each sample was assayed in parallel by cDNA microarray. We identified small amplified or deleted chromosomal regions, as well as alterations in DNA copy number not previously described. We predominantly found alterations of apoptosis-related genes in Hep3B and HepG2, cell adhesion and receptor molecules in HLE, and cytokine-related genes in PLC/PRF/5. About 40% of the genes showing amplification or loss showed altered levels of mRNA ($p < 0.05$). Hierarchical clustering analysis showed that the expression of these genes allows differentiation between α-fetoprotein (AFP)-producing and AFP-negative cell lines. cDNA array-CGH is a sensitive method that can be used to detect alterations in genomic copy number in tumor cells. Differences in DNA copy alterations between AFP-producing and AFP-negative cells may lead to differential gene expression and may be related to the phenotype of these cells.

Keywords: Microarray-based comparative genomic hybridization; Hepatocellular carcinoma; α-Fetoprotein

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1]. Among the factors implicated in the etiology of HCC are infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), dietary aflatoxin, alcohol consumption, and exposure to chemical carcinogens [2–4]. Alterations in genomic DNA copy number are key genetic events in the development and progression of human cancers, including HCC [5–7].

Comparative genomic hybridization (CGH) is a highly specific molecular cytogenetic approach that allows positional identification of gains and losses of DNA sequences throughout the entire genome [8–12]. CGH is based on the use of competitive fluorescence in situ hybridization (FISH) on normal chromosome spreads of differently labeled total genomic DNA from appropriate control and tumor tissue [13]. CGH, however, is unable to detect DNA copy number changes within narrow regions of chromosomes, and alterations of <1 Mb are difficult to detect [14].

Microarray-based CGH (array-CGH) was developed to detect genome-wide alterations in tumor samples by Pinkel et al. using cDNA microarray slides [14–16]. This technique has enabled rapid surveys of known copy number alterations in tumor samples, but resolution can be hampered when only small regions of the genome are amplified [17,18]. Using cDNA clones instead of BAC or PAC clones as probes, however, would make it possible to directly detect amplification and deletion

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of copy numbers of individual genes. Moreover, using cDNAs arrayed on slides would enable parallel measurements of mRNA levels, which may reveal the degree to which variation in gene copy number contributes to variation in gene expression in tumor cells [19,20]. We previously analyzed the alterations of mRNA expression in hepatoma cell lines using an in-house cDNA microarray [21,22], and we showed that α-fetoprotein (AFP)-producing and AFP-negative hepatoma cell lines had different gene expression profiles [23].

In this study, we assayed chromosomal abnormalities in hepatoma cell lines using the cDNA array-CGH method and investigated the relationship between genomic copy number alterations and mRNA expression profiles in these hepatoma cell lines.

Materials and methods

Cell lines and cells. We used three positive control cell lines: the HL-60 promyelocytic leukemia cell line, the IMR-32 neuroblastoma cell line, and the RCF-26 hepatoma cell line, which contain amplified copy numbers of the c-myc, N-myc, and luciferase genes [24–26]. Five AFP-producing hepatoma cell lines: Huh7, Hep3B, HepG2, PLC/PRF/5, and Huh6; two AFP-negative hepatoma cell lines: SK-Hep1 and HLE; two non-hepatoma cell lines: HeLa and KMBC (bile duct cancer cell derived) were used for gene expression profiling and analysis of genomic copy number alterations. As a reference sample in these microarray experiments, we used peripheral blood mononuclear cells (PBMCs) from healthy volunteers and a SV40-T antigen-immortalized normal human hepatocyte cell line (THLE-5b) [27].

cDNA clones and sequence verification. The cDNA microarrays used in this study were made in collaboration with Hitachi Software Engineering (Yokohama, Japan), using an SPBIO2000 robotic arraying machine. The cDNA clones used for making microarrays were selected from UniGene cluster (http://www.ncbi.nlm.nih.gov/UniGene/), and most of them were obtained from the IMAGE Consortium libraries (http://image.llnl.gov/) through Research Genetics (Huntsville, AL). Each microarray contained a total of 1080 cDNA clones, consisting of 930 unique sequence-verified clones and four housekeeping genes. The 930 clones included 141 apoptosis-related genes, 99 cell cycle-related genes, 154 cell–cell interaction-related genes, 198 cytokine and growth factor genes, 123 oncogenes, 81 transcription factor genes, 26 DNA repair-related genes, 93 stress response-related genes, and 87 hematology-related genes. Polymerase chain reaction (PCR) products from the clones were prepared and printed onto glass slides as previously described [28,29].

Copy number analyses by cDNA microarrays. CGH experiments on cDNA microarrays were performed using a modification of a previously described procedure [30]. Nuclei were isolated from cells and dissolved in "hypotonic buffer," consisting of 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dichlorodiphenyltrichloroethane-1,1,1–trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Following digestion in proteinase K in the presence of SDS, genomic DNA was isolated by phenol–chloroform extraction. From each sample, 20 μg of genomic DNA was sonicated for 10 min and digested for 32 h with *Dpn*II (New England Biolabs, Beverly, MA), followed by phenol–chloroform extraction. Each digested hepatoma cell line or positive control DNA was labeled with Cy5-dUTP (Amersham-Pharmacia Biotech, Uppsala, Sweden), and each normal DNA was labeled with Cy3-dUTP (Amersham-Pharmacia) using a Bioprime Labeling Kit (Invitrogen, Carlsbad, CA). Briefly, digested sample DNA was mixed with 15 μg random octamers in a total volume of 41 μl reaction buffer, heated at 100 °C for 5 min, and chilled on ice. Five microliters of 10× dNTPs (i.e., 1.2 mM each of dATP, dGTP, and dCTP, and 0.6 mM dTTP), 3 μl Cy5-dUTP or Cy3-dUTP, and 1 μl Klenow fragment were added to the DNA and then incubated at 37 °C for 2 h. Reactions were stopped by adding 5 μl of 0.5 M EDTA, pH 8.0, and labeled probes were purified on a Microcon 30 column (Millipore, Bedford, MA). Thirty micrograms of human Cot-1 DNA (Invitrogen), 100 μg yeast tRNA (Sigma–Aldrich, St. Louis, MO), and 20 μg poly(A) (Sigma–Aldrich) were added as blocking reagents and then the samples were concentrated to 12 μl. 2.55 μl of 20× SSC and 0.45 μl of 10% SDS were added, and each 15 μl sample was heated at 100 °C for 90 s and then used as a hybridization probe for the DNA-spotted slides. The slides were covered with glass coverslips (22 mm × 22 mm), fixed in a Hybridization Cassette (TeleChem, Sunnyvale, CA), and hybridization was performed at 70 °C for 12 h. The slides were washed in 2× SSC, 0.03% SDS at 70 °C for 5 min, 1× SSC at 70 °C for 5 min, and 0.2× SSC at room temperature for 5 min.

Expression analyses by cDNA microarrays. Total RNA was isolated using a ToTally RNA Kit (Ambion, Austin, TX), and mRNA was isolated from total RNA samples using a MicroPoly(A)Pure Kit (Ambion), according to the manufacturer's instructions. RNA prepared from THLE-5b cells was used as a reference for all cDNA microarray analyses. Fluorescently labeled cDNA probes were made from 2 μg aliquots of mRNA by reverse transcription using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Labeling and hybridization were performed as previously described [23].

Imaging and analyses. Fluorescence intensities generated by the Cy5 and Cy3 probes immobilized on the microarray slides were measured by a laser confocal microscope equipped with a scanning system (ScanArray 5000, GSI Lumonics, Billerica, MA) with appropriate excitation and emission filters. The fluorescence images for Cy5 and Cy3 were scanned separately and stored for image analysis using ImaGene Ver. 3.0 Software (BioDiscovery, Marina del Rey, CA). The signal intensity of each spot was corrected by subtracting background signals in the immediate vicinity, and each Cy5/Cy3 ratio was calculated using global normalization. Clones with a copy number ratio >1.80 were considered to be amplified and those with a ratio <0.55 were considered to be deleted. Over- and underexpression of mRNAs used the same ratios.

Southern hybridization. Genomic DNAs from each cell line and PBMCs were digested overnight with the appropriate restriction enzyme, electrophoresed in 0.8% agarose gels, transferred onto Hybond-N^+ nylon membranes (Amersham-Pharmacia), and hybridized with a ^{32}P-labeled probe previously shown to be amplified or deleted in our array-CGH experiments, according to the standard method [31,32].

Statistical analysis. BRB-Array Tools Ver. 3.1.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) were used to compare gene amplification in AFP-producing and AFP-negative hepatoma cell lines. Genes showing significant differences were determined by univariate significance test, with a threshold of $p < 0.05$, using a randomized variance model. Hierarchical clustering was performed using centered genes and correlations were determined using average linkage analysis.

Results

Establishment of a sensitive CGH analysis of cDNA arrays

To evaluate the reliability of our CGH method using cDNA microarray slides, we first assayed our positive control cell lines, IMR-32, HL60, and RCF-26, which are known to have amplified copies of c-myc (HL60), N-myc (IMR-32), or luciferase (RCF-26) [24–26]. Using

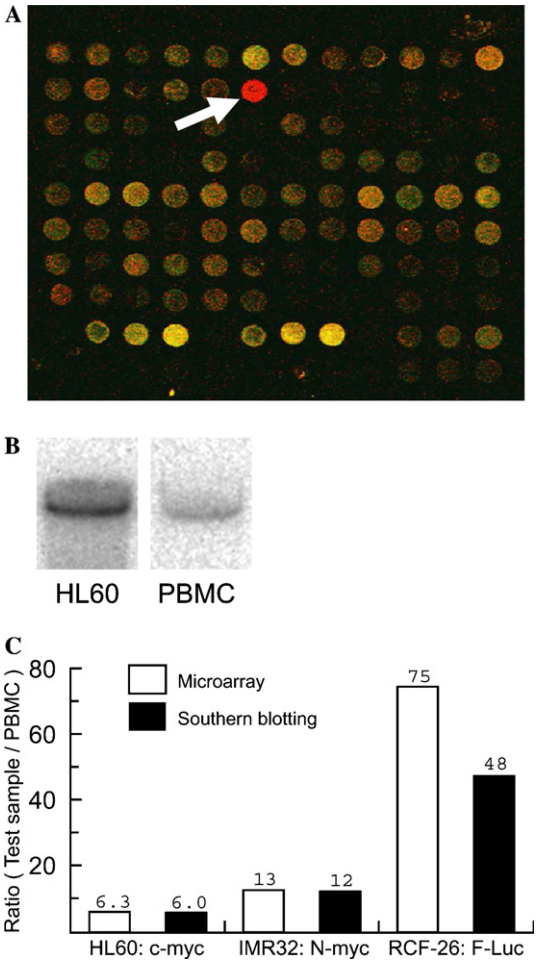

Fig. 1. Evaluation of the reliability of the cDNA microarray-based CGH method by comparison with Southern blotting. (A) Portion of typical array patterns of in-house microarrays (red, HL60; green, PBMC). The spot located by the white arrow indicates the c-myc gene. (B) Southern blot analysis of HL60 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 microarray 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.

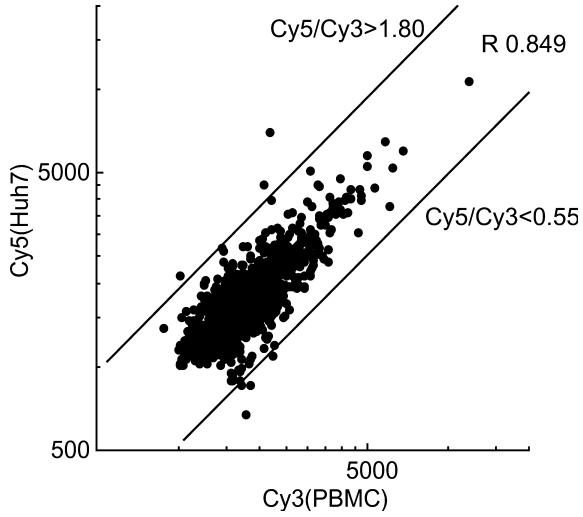

Fig. 2. Log_{10} (intensity of Cy5) and log_{10} (intensity of Cy3) of Huh7 cells are graphed on a scatter plot, and their linear relationship was examined statistically.

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		
	Homo sapiens 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
	H. sapiens 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 (*Drosophila*) 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	*H. sapiens* 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
	H. sapiens 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/5						
>1.80	HBV-P	19.3 ± 4.62	NA	Virus genome		
	HBV-full	9.04 ± 5.36	NA	Virus genome		
	H. sapiens 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
	H. sapiens 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.

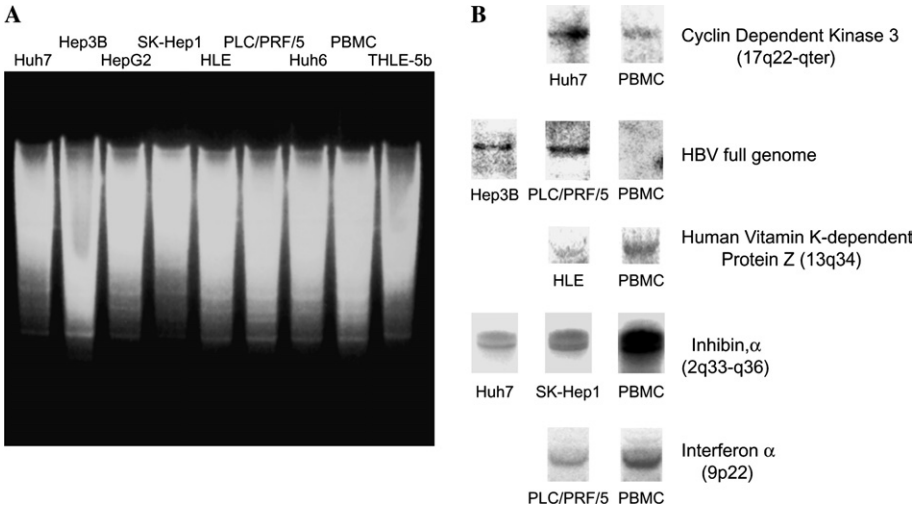

Fig. 3. Southern blot analyses of DNA from seven hepatoma cell lines. (A) Gel electrophoresis image of seven hepatoma cell lines and two normal cell lines. (B) Two amplified genes and three deleted genes in hepatoma cell lines, as shown by the cDNA array-CGH method.

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

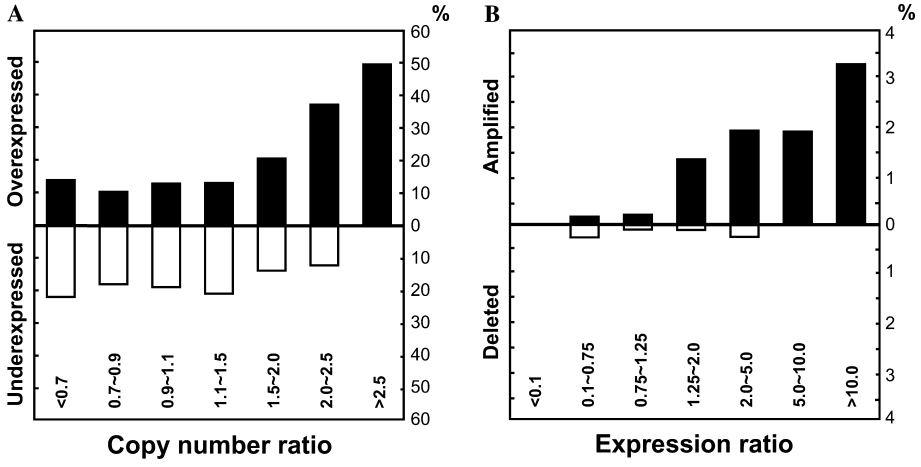

Fig. 4. Impact of gene copy number on global gene expression levels. (A) Percentage of over- and underexpressed genes (vertical axis) according to copy number ratios (horizontal axis). Threshold values used for over- and underexpression were >1.80 and <0.55, respectively. (B) Percentage of amplified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

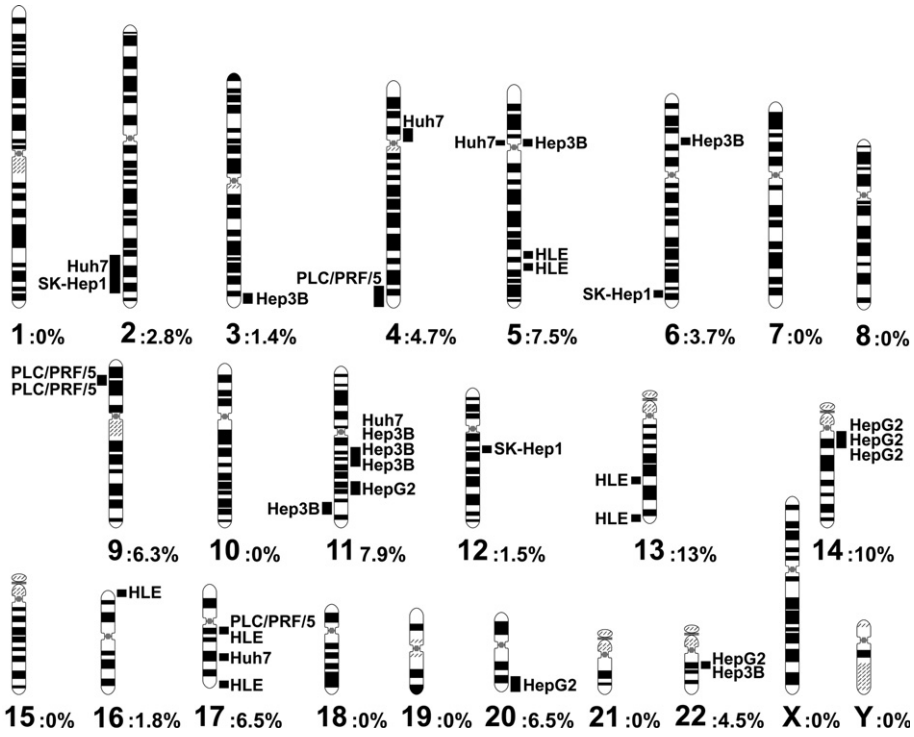

Fig. 5. Chromosome locations of genes amplified or deleted, as shown by cDNA array-CGH. Squares on the right side of each chromosome indicate genomic amplifications, squares on the left side indicate deletions. The percentages of genes amplified or deleted at each chromosomal location are indicated.

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 (TGFβR1), 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, TGFβR1, chemokine (C–C motif) receptor 5, IGF2R, EB13, and erythropoietin receptor), and cell adhesion molecules (collagen IX, ICAM3, Jagged1, and integrin), as well as cell cycle

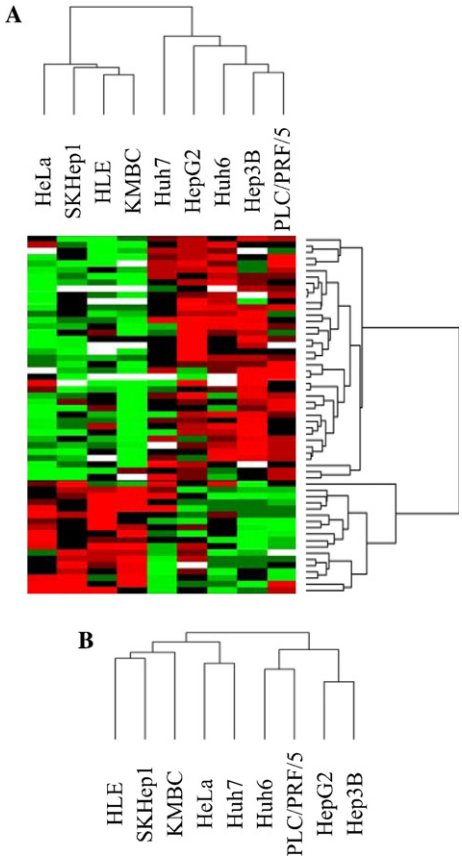

Fig. 6. Genes used to differentiate α-fetoprotein (AFP)-positive from AFP-negative cell lines. Using BRB-Array Tools Ver. 3.1.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html), a modified *t* test was used to identify genes that could differentiate the AFP-positive hepatoma cell lines, Huh7, Hep3B, HepG2, 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 *Dpn*II 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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