Expression Profiling Suggested a Regulatory Role of Liver-enriched Transcription Factors in Human Hepatocellular Carcinoma

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ABSTRACT

By using a cDNA array representing 14,000 cDNA clusters, we studied the expression profiles in paired clinical hepatocellular carcinoma (HCC) samples and the distal nontumorous liver tissues from the same patients. Despite the significant heterogeneity among the clinical samples, 72 genes (including 30 novel genes) were down-regulated and 84 genes (including 48 novel genes) were up-regulated in >50% of the cancer samples that were identified. The alterations in gene expression levels were confirmed by Northern blot and reverse-transcription PCR in all of 4 randomly selected genes. It was conspicuous that 21 of 38 hepatocarcinoma (HCC) down-regulated genes studied previously were reportedly regulated by a group of liver-enriched transcription factors (LETFs), and 12 of 36 HCC up-regulated genes studied previously were involved in protein translation. Reexamination of the cDNA array data further revealed that most of the genes known to be regulated by LETFs were down-regulated in at least a portion of the HCC samples. Among the LETFs, the expression level of CCAAT/enhancer-binding protein (C/EBP) α was down-regulated in cancer, whereas hepatocyte nuclear factor 1 (HNF-1), HNF-3β, HNF-4α, and HNF-4γ were up-regulated. The expression profiling thus suggested multiple regulatory pathways involved in HCC, especially that related to LETFs.

INTRODUCTION

HCC is a common and highly malignant tumor that is prevalent in Saharan African and Southeast Asia (1). Worldwide HCC affects approximately 250,000–1,000,000 individuals annually and causes at least 200,000 deaths/year (2). Hepatitis B virus infection and aflatoxin B1 exposure were considered major etiological factors associated with at least 200,000 deaths/year (2). Hepatitis B virus infection and aflatoxin B1 exposure were considered major etiological factors associated with at least 200,000 deaths/year (2).

The molecular mechanisms involved in hepatocarcinogenetic processes are far from clear. It was postulated that cell cycle regulators controlling the G1 phase progression might be involved in carcinogenesis, including inactivated p16INK4 and reduced p21(WAF1/CIP1) and p27Kip1 (3). Various HCC subtypes might be caused by different genetic events. For example, c-met mutation was associated with childhood HCC (4), whereas mutant porphobilinogen deaminase gene was detected in HCC associated with acute intermittent porphyria (5). Complicated genetic alterations including DNA mutations and varied gene expressions were observed in clinical HCC. For example, loss of heterozygosity in chromosomes 1p, 5q, 8p, 9p, 10q, 11p, 13q, and 17p were detected frequently (6, 7) and might increase as liver lesions became more severe (6). Chromosome gains in HCC often occur in 1q, 5p, 6p, 8q, 17q, and 20q (7, 8). However, none of these genetic changes was observed in all HCC patients, suggesting a significant heterogeneity in hepatocarcinogenesis.

Genes that are differentially expressed in HCC and normal hepatocytes might be involved in carcinogenesis, progression, or malignancy of HCC. However, conventional methods to search for those genes such as differential display RT-PCR and representational difference analysis were usually time consuming and limited by the view scope. CDNA array provided a powerful alternative with an unprecedented view scope in monitoring gene expression levels (9) and led to discoveries of regulatory pathways involved in complicated biological processes (10, 11). Application of cDNA array in cancer research resulted in the identification of a number of genes with the potentials of drug target, molecular diagnosis, and molecular classification of cancer (12–14). We have developed a cDNA array representing 14,000 cDNA clusters to profile the gene expression patterns in nine sets of clinical HCC samples. In this report we show that on average 1165 genes were differentially expressed in paired cancer and distal nontumorous liver tissues. However, only 156 genes were consistently up- or down-regulated in >50% of the HCC samples tested. A set of genes involved in protein translation were up-regulated. Most interestingly, the majority of the genes known to be regulated by LETFs were down-regulated. Among the LETFs, C/EBPα was down-regulated, and most of other LETFs were up-regulated. The involvement of LETFs, especially C/EBPα, in hepatocarcinogenesis was discussed.

MATERIALS AND METHODS

cDNA Array Construction. CDNA clones were from liver and hepatocarcinoma cell lines and hypothalamus-pituitary-adenal libraries (15) or purchased from Research Genetics (Huntsville, AL, USA). A cDNA array was assembled with 14,000 CDNA clones representing the same number of independent CDNA clusters, of which 7,565 clusters were homologous to that in the UniGene Database. All CDNA fragments were amplified and verified by gel electrophoresis. The average length of the CDNA fragments was ~1 kb. PCR products were precipitated in isopropanol, redissolved in 10 μl of denaturing buffer (1.5 M NaCl, 0.5 M NaOH), and spotted on two 8 × 12-cm Hybond-N nylon membranes (Amersham Pharmacia, Buckinghamshire, United Kingdom) using an arrayer (BioRobotics, Cambridge, United Kingdom). Each spot carried ~100 nl in volume and was 0.4 mm in diameter, and each CDNA fragment was placed in two different spots (double-offset). Lambda phage and pUC18 vector DNA were spotted as negative controls.

HIC. Eight housekeeping genes encoding ribosomal protein S9 (RPS9), β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase, hypoxanthen phosphoribosyltransferase 1, Mf, 23,000 highly basic protein (RPL13A), ubiquitin C, phospholipase A2, and ubiquitin thiolesterase (UCHL1) were evenly distributed, each in 12 places, on each 8 × 12-cm array as an intramembrane control. Hybridization data was considered invalid if among the 12 spots representing the same gene, the intensity of the darkest spot exceeded 1.5-fold of that of the weakest spot.

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3 The abbreviations used are: HCC, hepatocellular carcinoma; RT-PCR, reverse transcription-PCR; LETF, liver-enriched transcription factor; C/EBP, CCAAT/enhancer binding protein; HIC, hybridization intramembrane control; HNF, hepatocyte nuclear factor.
Clinical Samples. HCC samples were collected via collaboration with local hospitals. Each sample consisted of two parts from the same patient, including the cancer tissue and liver tissue that was at least 3 cm distal from the lesion, and was nontumorous by microscopic examination. All tissues were verified by histological examination (Table 1).

RNA Extraction and Probe Preparation and RT-PCR. Total RNA was extracted using standard Trizol RNA isolation protocol (Life Technologies, Inc., Grand Island, NY). The poly(A)+ mRNA was isolated from the total RNA using a poly(dT) resin (Qiagen, Hilden, Germany). Approximately 1–2 μg of mRNA were labeled in a reverse transcription reaction in the presence of 200 μCi [α-33P]-deoxyadenosine 5′-triphosphate (DuPont NEN, Boston, MA) using Moloney murine leukemia virus reverse transcriptase as per the manufacturer’s instruction (Promega Corp., Madison, WI). For RT-PCR, 1% of the reverse transcription reaction was amplified using Taq DNA polymerase for 30 s at 94°C, 1 min at 55°C, and 1.5 min at 72°C for 35 cycles.

Hybridization and Image Processing. Prehybridization was carried out in 20 ml of prehybridization solution (6× SSC, 0.5% SDS, 5× Denhardt’s, and 100 μg/ml denatured salmon sperm DNA) at 68°C for 3 h. Overnight hybridization with the 33P-labeled cDNA in 6 ml of hybridization solution (6× SSC, 0.5% SDS, and 100 μg/ml salmon sperm DNA) was followed by stringent washing (0.1× SSC, 0.5% SDS, at 65°C for 1 h). Membranes were exposed to Phosphor Screen overnight and scanned using an FLA-3000A Plate/Fluorescent Image Analyzer (Fuji Photo Film, Tokyo, Japan). Radioactive intensity of

![Fig. 1. Scatterplot of the two independent cDNA array analyses of the same liver sample. Each point stands for a gene or cDNA cluster with the X coordinate value as the gene expression level in one test and the Y coordinate value the other test. An R^2 of 0.97 indicates high reproducibility of the cDNA array assay.](image1)

![Fig. 2. Histological pictures of two representative HCC samples and the corresponding distant liver tissues. Scatterplots under the histological pictures illustrated the differential gene expression patterns between the paired cancer and normal liver tissues from the same individual, and those on the left of the histological pictures indicate the differences between patients.](image2)
each spot was linearly digitalized to 65,536 gray-grade in a pixel size of 50 μm in an Image Reader and recorded using the Array Gauge software (Fuji Photo Film, Tokyo, Japan). After subtraction of background (3σ) chosen from an area where no cDNA was spotted, genes with intensities >10 were considered as positive signals to ensure that they were distinguished from background with statistical significance 99.9%. Normalization among arrays was based on the sum of background-subtracted signals from all genes on the membrane.

**Northern Blot.** Total RNA, 35 μg/sample, was run on a 1% agarose gel in 1.8 M formaldehyde and transferred onto a Hybond-N nylon membrane. rRNA was used as the control to normalize the total RNA quantity. The DNA fragments representing unique sequence of ADH2, MT2A, ASS, C/EBPα, HNF-1, HNF-3β, HNF-4α, and HNF-4γ were labeled with [α-32P]dATP (Amersham Pharmacia, Piscataway, NJ). Hybridization was carried at 42°C overnight in a 5× SSC, 0.1% SDS, and 50% deionized formamide. Membranes were washed with 0.1× SSC, 0.1% SDS for 2–4 h in 37°C, exposed to Phosphor Screen, and subsequently scanned using FLA-3000A Plate/Fluorescent Image Analyzer.

**RESULTS**

**Establishment of the cDNA Array System.** Human cDNA clones randomly picked from cDNA libraries were terminal sequenced and compared with the Unigene database. The cDNA array was made of amplified cDNA fragments representing 14,000 genes or cDNA clusters. The reproducibility of the cDNA array analysis was evaluated in multiple replicated tests using cDNA probes independently made from the same mRNA sample to cDNA arrays of different batches. The results from the replicated experiments were almost perfectly concordant with R² (square of Pearson correlation coefficient, measuring similarity in gene expression pattern) of 0.97–0.98 in scatterplot (Fig. 1). Only 0.2% of the 14,000 genes had an expression level in the second measurement >2-fold the first measurement. Thus, the cDNA array system was highly reproducible.

**Expression Profiling Revealed Differentially Expressed Genes in HCC.** The cDNA array was applied to analyze the expression patterns of nine sets of clinical HCC samples, each including paired cancer and distal liver tissues from the same patient. All tests were considered valid because the maximal variation of HIC signal intensities was 1.45-fold. In different sample pairs, approximately 30–67% of the 14,000 genes represented on the cDNA array were informative, i.e., background subtracted intensity value >10 and the ratio of the intensities between the double-offset spots <1.5. Of the informative...
panel of clinical HCC samples for all four genes tested. (data not shown). The down-regulation in HCC was confirmed in a
ADH2, MT2A, the altered expression levels, 3 genes (genes. Eighty-four genes/cDNA clusters, including 36 known genes,
different cancer samples are highly heterogeneous. The appearance of the HCC samples (Fig. 2) were consistent with the
diversity in gene expression patterns. The heterogeneity among the
differentially expressed genes overlap in two randomly selected HCC
samples. The R² values between two sections of the same normal liver
sample was 0.97 (Fig. 1). It is evident that the expression profiles of
cancer tissues could be very different between individuals. Such heterogeneity in HCC samples was expected and might have reflected the heterogeneity in hepatocarcinogenic mechanisms probably related to different risk factors to
which HCC patients are exposed (22, 23). The different histological appearances of the HCC samples (Fig. 2) were consistent with the
heterogeneous gene expression patterns. The heterogeneity among the
normal liver samples (from 0.80 to 0.91; Fig. 2) might partially
reflected the responses of tumor-bearing livers to different HCC
lesions. Because of the heterogeneity in clinical samples, study of
additional patients is warranted to verify individual variations and to
reveal genetic changes involved in smaller fraction of patients.

Letfs in the Regulation of the HCC-related Genes. To uncover
the molecular mechanism of hepatocarcinogenesis, the regulatory
elements that reportedly affect the expression of the differentially
expressed genes were classified. It was thus revealed that 21 of the 38
known genes down-regulated (Fig. 3) were regulated by a group of
Letfs including C/EBP, HNF-1, HNF-3, HNF-4, and HNF-6 (reviewed in Refs. 19–21). Double examination of the cDNA array data
further indicated that among the genes represented on the cDNA
array, most of the genes reportedly regulated by Letfs were down-
regulated in cancer tissues in at least a portion of the samples (Fig. 5).

Because most of the Letfs were not included in the cDNA array
used, the expression levels of five Letfs, C/EBPα, HNF-1α, HNF-3β, HNF-4α, and HNF-4γ, were examined by Northern blot in
a panel of HCC samples. The expression level of C/EBPα was reduced in five of the six clinical HCC samples, whereas that of
HNF-1, HNF-3β, and HNF-4α, and HNF-4γ were mostly increased (Fig. 4).

Discussion

Reproducibility of cDNA Array Analysis. A cDNA array rep-
resenting 14,000 human genes or cDNA clusters was established and
applied in expression profiling of clinical HCC samples. Compared
with the cDNA array without the HIC (data not shown), it was evident
that the unique HIC in the cDNA array system significantly contrib-
uted to the evenness of hybridization among different parts of the
array membrane and therefore improved the reliability of the array
analysis. Replicated examinations of the same sample indicated that
only 0.2–0.3% of the genes spotted, or 0.5% of informative genes,
might be false-positive signals, whereas 7.4–39.2% of the informative
genes were differentially expressed in HCC. Because we chose the
genes that were differentially expressed in >50% of the clinical HCC
samples, the possibility of false-positives was remote.

Heterogeneity in Both HCC Cancer Samples and Cancer-bear-
ing Livers. The R² values of expression profiles among the nine HCC
samples (from 0.22 to 0.81; for an example, see Fig. 2) indicated that
the global gene expression patterns of hepatoma tissues could be very
different between individuals. Such heterogeneity in HCC samples
was expected and might have reflected the heterogeneity in hepatocar-
cinogenic mechanisms probably related to different risk factors to
which HCC patients are exposed (22, 23). The different histological appearances of the HCC samples (Fig. 2) were consistent with the
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lesions. Because of the heterogeneity in clinical samples, study of
additional patients is warranted to verify individual variations and to
reveal genetic changes involved in smaller fraction of patients.

Letfs Involved in HCC Carcinogenesis. A significant portion
of genes down-regulated in HCC was regulated by Letfs (19, 20,
21), and most other genes reportedly regulated by Letfs were down-
regulated in HCC, although in a lower frequency. Northern blot
analyses revealed that most Letfs were up-regulated except C/EBPα,
which was significantly reduced in cancer tissue.

Our observation on the relationship between HCC and Letfs was
consistent with previous studies (24–26). Letfs play important roles in
liver development and differentiation (19, 20, 27–32) and form a
complicated hierarchical network regulating transcription of each
other (33–37) and the liver-specific genes. Among them, C/EBPα was
proposed to have an antiproliferative effect, and its expression was

\[ \text{Internet address: http://rana.stanford.edu/software/}. \]
drastically reduced in regenerating hepatocytes (38). A transgenic mouse model suggested that C/EBPβα was likely to influence proliferation, differentiated gene expression, and survival of differentiated hepatocytes (39). Induction of C/EBPβα expression in human hepatoma cell lines Hep3B and HepG2 resulted in reversible arrest of proliferation and delayed tumorigenesis in immunodeficient mice (40). In a rat-resistant hepatocyte model, C/EBPβα was down-regulated in very early nodules but not the transcription or steady-state mRNA levels of C/EBPβ, HNF-1α, and HNF-4 (26). However, it was once reported that HNF-1α protein level was increased in well-differentiated HCC tissues but reduced in moderately and poorly differentiated HCCs, whereas the HNF-1β could be up-regulated, regardless the differentiation status of the tumor (25). The discrepancy between our results and this report is probably attributable to different clinical standards to HCC differentiation classification. On the basis of our results, we would like to propose that a regulatory factor(s) suppresses the expression of C/EBPβα but stimulates HNFs in HCC. The reduction in C/EBPβα further contributed to hepatocarcinogenesis via its effects on proliferation rate.

Other Molecular Mechanisms Affected in Human HCC. Increased expression of ribosomal proteins in HCC and in other cancers has been reported (41, 42). Our observation that 12 of 36 genes up-regulated in HCC were involved in protein translation suggested that the entire machinery for protein synthesis may be enhanced. Also noticeable was that the genes encoding glyceraldehyde-3-phosphate dehydrogenase, phosphogluconate dehydrogenase, and pyruvate kinase were up-regulated in HCC, consistent with the long-observed phenomenon that glycosis in tumor cells was accelerated (43, 44). In addition, two genes related to thyroid hormone, PKM2, and HNF1α, interact with the thyroid hormone receptorβ and are up-regulated in HCC, consistent with the long-observed phenomenon that glycosis in tumor cells was accelerated (43, 44). In a rat-resistant hepatocyte model, C/EBPβα was down-regulated in very early nodules but not the transcription or steady-state mRNA levels of C/EBPβ, HNF-1α, and HNF-4 (26). However, it was once reported that HNF-1α protein level was increased in well-differentiated HCC tissues but reduced in moderately and poorly differentiated HCCs, whereas the HNF-1β could be up-regulated, regardless the differentiation status of the tumor (25). The discrepancy between our results and this report is probably attributable to different clinical standards to HCC differentiation classification. On the basis of our results, we would like to propose that a regulatory factor(s) suppresses the expression of C/EBPβα but stimulates HNFs in HCC. The reduction in C/EBPβα further contributed to hepatocarcinogenesis via its effects on proliferation rate.

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