Identification and Characterization of Genes Associated with Human Hepatocellular Carcinogenesis

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ABSTRACT

Eight cDNAs encoding galectin 4 (Gal-4), UGT2B4 (UDP-glucurono-syltransferase), ribosomal phosphoprotein P0 (rpP0), dek, insulin-like growth factor binding protein (IGFBP) 1, vitronectin, retinoic acid-induced gene E (RIG-E), and CYP3A4 (cytochrome P450 nifedipine oxidase) were identified as differentially expressed genes between human hepatocellular carcinoma (HCC) and matched nontumorous liver tissues. Higher levels of UGT2B4, rpP0, dek, vitronectin, Gal-4, and IGFBP-1 mRNAs combined with a lower level of RIG-E mRNA were observed in at least four of five primary HCCs compared to matched nontumorous liver tissues. Furthermore, a pathological study suggested that the levels of UGT2B4, rpP0, dek, and vitronectin increased and the level of RIG-E decreased with the histological grading. On the other hand, the expression of CYP3A4 mRNA and CYP3A7 (F-450 Fla) mRNA, a transcript found in the fetus and highly homologous to CYP3A4, was higher in all nontumorous liver and some of the carcinoma tissues from five HCC patients, whereas it was significantly lower in normal liver tissues from two non-HCC patients. The examination using HCC cell lines HuH-7 and HepG2 under different growth conditions suggested that the expression of dek mRNA was growth-associated. In contrast, the expression of Gal-4, UGT2B4, IGFBP-1, and RIG-E mRNAs was regulated in a cell density-dependent manner: the levels of Gal-4, UGT2B4, and IGFBP-1 were undetectably low, whereas the level of RIG-E was high in rapidly proliferating, subconfluent HCC cells in 10% serum; however, the expression levels were reversed in dense, overcrowded cultures. In addition, IGFBP-1 and Gal-4 mRNAs were also induced by reducing the serum concentration to 0.1%. We also demonstrated that sodium butyrate, an inducer of differentiation, up-regulated and down-regulated RIG-E and dek mRNAs, respectively, in a dose-dependent manner in HuH-7 cells, supporting, in part, our pathological observation. In summary, therefore, high expression of Gal-4, UGT2B4, rpP0, dek, IGFBP-1, and vitronectin, together with low expression of RIG-E, was correlated with the malignant potential of HCC. CYP3A4 and CYP3A7 could be induced in HCC-bearing livers. These transcripts are differentially regulated depending on cell-cell contact, serum growth factors, growth and differentiation status, and/or other mechanisms in premalignant and malignant liver cells.

INTRODUCTION

The development of cancer in the liver is a long, chronic process. Many steps in this process are attributable to hepatitis and cirrhosis caused, for the most part, by virus infection (1–3). These steps include the development of hyperplastic nodules. In the adult liver, cell growth can be induced both in compensatory regeneration and direct hyperplasia. The former is a feature of the normal liver as seen after partial resection or cell necrosis, in which hepatocytes undergo a process of differentiation as they return to a stable, original size. In contrast to this, the latter can be induced without any cell loss and results in the formation of tightly compacted, persistent nodules in which genetically committed hepatocytes occasionally undergo a stepwise promo-

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2 The abbreviations used are: HCC, hepatocellular carcinoma; UGT, UDP-glucuronosyltransferase; RIG-E, retinoic acid-induced gene E; Gal-4, galectin-4; rpP0, ribosomal phosphoprotein P0; IGFBP, insulin-like growth factor-binding protein; IGF-I, insulin-like growth factor-1; FBS, fetal bovine serum; RT, reverse transcription; AFP, α-fetoprotein; RAR, retinoic acid receptor; AT, ataxia-telangiectasia; H.L., HCC-nontumorous liver; HCV, hepatitis C virus; HBV, hepatitis B virus; GAP, glycogen synthase-phosphatidylinositol.
cDNA Probes. The probes used for the Northern hybridization were generated by RT-PCR amplification using the following primer pairs: (a) 5’-CTATGTGCTCCACAGG-3’ and 5’-TGGGTAACACCTTTGAAGGC-3’ for Gal-4; (b) 5’-ACGTCTAGGCTGAAGAC-3’ and 5’-TTCTCATTGA-CAATTTGTTA-3’ for UGT2B4; (c) 5’-GTTATCCGTGTTGTCG-3’ and 5’-TTTTGTCAGAGCACAGTCC-3’ for dek; (d) 5’-CTTCTCAAAAGCTCCTG-3’ and 5’-CAACTATACCCACAGGTAGC-3’ for IGFBP-1; (e) 5’-GGC-CGCAGGCAAAGAACC-3’ and 5’-AGGGCTCCACAGTGCACC-3’ for vitronectin; (f) 5’-GTGGTGACCTCAGGACG-3’ and 5’-CTTGGCTTTCTCTG-3’ for RIG-E; (g) 5’-CTTCAACAGTTGCTCAG-3’ and 5’-CTACTCCACACCAAGCTC-3’ for Afp; and (h) 5’-AGTCAAGGGTGTCACG-3’ and 5’-CTCATACGTATTTGTGTCACC-3’ for CYP3A4. PCR was performed using 1.5 μl of the RT products from HCC or liver tissues using a Taq polymerase amplification system (Pharmacia) with a denaturing temperature of 96°C (30 s), an annealing temperature of 54°C (1 min), and an extension temperature of 72°C (1 min) for 25 cycles. A cDNA fragment encompassing from bp 728–891 of human rp0 (GenBank accession number M17885) was isolated by the differential display method. A cDNA encoding full-length human cyclin A was a gift from Dr. Steven I. Reed (The Scripps Research Institute, La Jolla, CA).

RESULTS

Isolation of Differential cDNA Clones. To identify genes that were differentially expressed in HCC and nontumorous liver tissues, we analyzed sequence tags and constructed gene expression profiles representing HCC and the adjacent liver tissue mRNAs from a patient (patient 1 in Fig. 1). Among 50,515 and 50,472 transcripts, which represented 20,534 and 15,163 different genes derived from HCC and nontumorous liver tissues, respectively, we picked 150 known transcripts that occurred at least six times in either of the profiles and showed differences more than five times between the two profiles. Among them, 28 were reproducibly overexpressed in HCC, whereas 2 were in nontumorous liver in the RT-PCR analysis using samples from patient 1. However, of the 28 transcripts mentioned above, 22 were excluded from further analysis because they were expressed more abundantly in liver tissue than in tumor tissues from other HCC patients (data not shown). Accordingly, we focused on the following eight transcripts: Gal-4, UGT2B4, IGFBP-1, vitronectin, rp0, and dek, which were more abundant in HCC than in nontumorous liver

Table 1 Differentially expressed genes in HCC

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* Matched GenBank accession number.

* The number of each cDNA tag in 50,515 transcripts is indicated.

* The number of each cDNA tag in 50,472 transcripts is indicated.
tissue, and CYP3A4 and RIG-E, of which the reverse was true (Table 1).

The Expression of mRNAs in Surgically Resected Liver Tissues. To confirm differential expression of mRNAs, Northern blot analysis using total RNAs from five HCC patients was performed. Among the six mRNAs that were more abundant in HCC than in matched nontumorous liver tissue, the expression of rpP0 (1.2 kb), UGT2B4 (2.5 kb), and dek (2.7 kb) was significantly higher in all five HCC tissues than in adjacent liver tissues. The mRNA expression of Gal-4 (1.2 kb), IGFBP-1 (1.5 kb), and vitronectin (1.6 kb) was higher in four of five HCCs than in the adjacent liver tissues (Fig. 1). On the other hand, the expression of RIG-E mRNA (1.3 kb) was higher in all five nontumorous liver tissues than in the matched HCC tissues.

Radiointensity of each mRNA was measured, and the H:L ratios from five patients were plotted against histological grades in Fig. 2. The mean H:L ratios for UGT2B4, rpP0, dek, IGFBP-1, and vitronectin mRNAs, respectively, were lowest in a well to moderately differentiated (Edmondson I or II) HCC from patient 5, intermediate in moderately differentiated HCCs (Edmondson II) from patients 1 and 4, and highest in the moderate to poorly differentiated (Edmondson II or III) samples from patients 2 and 3. Conversely, the mean H:L ratio for RIG-E mRNA was higher in patient 5, and lower in patients 2 and 3. However, the H:L ratios for IGFBP-1 and Gal-4 in moderately differentiated samples fluctuated widely, hence no correlation with the histological grading was observed.

Although substantial expression of a mRNA (2.2 kb) that hybridized with the CYP3A4 cDNA probe was observed in all nontumorous liver samples of HCC patients, the level of expression in matched HCC tissues was highly variable; i.e., in patients 1, 2, and 3, the expression was significantly lower in the HCC, whereas in patient 4, it was much higher in the HCC. Because pathological examinations showed that all patients in Fig. 1 suffered from HCV infection or liver cirrhosis (see “Materials and Methods”), nontumorous liver tissues in HCC patients are not normal but rather may be precancerous. Therefore, in such tissues, gene expression may not represent the normal status. Therefore mRNA expression was examined using virus-negative, normal liver samples from patients who died of pancreatic carcinoma (patient 6) and subarachnoid bleeding (patient 7). The levels of Gal-4, UGT2B4, rpP0, dek, IGFBP-1, vitronectin, and RIG-E mRNAs in normal liver were similar to those in nontumorous liver tissue from five HCC-bearing patients. In contrast, CYP3A4 expression was undetectably low in normal liver tissues from two non-HCC patients.

Because the nucleotide sequence of CYP3A4 is highly similar to but distinct from that of CYP3A7 (23), we may not be able to distinguish between these messages in the same Northern blot using a probe encoding CYP3A4 cDNA. Therefore, as an additional experiment, we designed primers specific for both CYP3A4 and CYP3A7 and performed RT-PCR analysis using the same RNA samples as used for Northern analysis. As shown in Fig. 3, the levels of CYP3A4 relative to β2-microglobulin, an internal control, were higher in both nontumorous liver and carcinoma tissues of HCC patients than in normal liver from a non-HCC patient. The overall expression pattern was quite similar to that obtained from the Northern blot analysis in Fig. 1, suggesting that CYP3A4 is the major species represented by the hybridization signal with the CYP3A4 probe. On the other hand, the expression of CYP3A7 was generally much lower. Although its levels were also higher in liver tissues of HCC patients than in normal liver, the pattern of expression was distinct from that of CYP3A4. We confirmed by direct sequencing analysis that these selective primer pairs successfully amplified the predicted portions of the CYP3A4 and CYP3A7 cDNAs (data not shown). From these results, we concluded that the expression of both CYP3A4 and CYP3A7 was higher in liver tissues of HCC patients than in normal liver of non-HCC patients.

These results suggest that up-regulation of Gal-4, UGT2B4, rpP0, dek, IGFBP-1, and vitronectin, combined with suppression of RIG-E mRNA expression, was associated with HCC progression. Among them, levels of UGT2B4, rpP0, dek, vitronectin, and RIG-E mRNAs seemed to be associated with histological grades. On the other hand,

Fig. 2. H:L ratios of mRNA levels were compared with histological grades. W/M, well to moderately differentiated HCC tissues; M, moderately differentiated HCC tissues; M/P, moderate to poorly differentiated HCC tissues. Each symbol represents a patient. Patient 1, ○; patient 2, ; patient 3, △; patient 4, ▲; patient 5, ■.

Fig. 3. RT-PCR assay analyzing the expression of CYP3A4 and CYP3A7 messages in nontumorous liver (L), HCC (H), and normal liver (N). β2-Microglobulin (β2m) primers were used in a parallel reaction as a control.
the expression of \textit{CYP3A4} and \textit{CYP3A7} mRNAs may be induced in premalignant liver cells of HCC patients and maintained at variable levels in carcinoma tissues.

**Expression of mRNAs in HCC Cell Lines under Different Growth Conditions.** The levels of cyclin A mRNA, as an indicator of proliferating cells in primary liver cancer (24), were significantly higher in all five HCC samples than in matched nontumorous liver tissue (Fig. 1), suggesting the high proliferating activity of HCC tissues. Therefore, the differences in the levels of mRNA expression observed between HCC and nontumorous liver may simply reflect the growth states of cells in these tissues. The expression was therefore examined in HCC cell lines under different growth conditions. As indicated in Fig. 4A, HuH-7 and HepG2 cells grew rapidly in 10% FBS until they formed a confluent monolayer (approximately $3.5 \times 10^6$ cells). The growth rate was then markedly reduced when the cells started piling up. In subconfluent cultures grown in medium containing 0.1% serum, both cell lines also showed slower growth, but the growth rate was not as slow as that of the overcrowded cells. Cell cycle analysis demonstrated that the proportion of $G_0/G_1$-phase cells increased whereas that of $S$-phase cells decreased when rapidly growing cells became overcrowded or were seeded in 0.1% serum, respectively (Fig. 4B). Thus, cell cycle distribution agreed well with the growth rate of these cell lines.

Northern blot analysis using total RNAs indicated that the levels of cyclin A mRNA were high in rapidly growing HuH-7 and HepG2 cells in 10% serum (Fig.4C), whereas they were significantly suppressed in growth-inhibited cells at higher cell density (Lane 3) and undetectable in normal liver tissue (Lane N). However, cyclin A mRNA was not markedly reduced in 0.1% serum (Lane 4). On the other hand, the levels of \textit{dek} mRNA were higher in both rapidly growing and growth-inhibited cells in 10% and 0.1% serum, respectively (Lanes 1, 2, and 4), than in overcrowded cells (Lane 3). The expression was barely detectable in normal liver tissue (Lane N). These expression levels were analyzed quantitatively using the BAS-2000 to compare radiointensity, revealing an intimate correlation with the levels of cyclin A ($P < 0.025$, F test; data not shown).
were also unaffected by butyrate in confluent HuH-7 and HepG2 cells (data not shown). These results suggest that the high levels of dek and the low levels of RIG-E mRNAs are correlated with dedifferentiation of HCC cells, whereas IGFBP-1 may be preferentially expressed in moderately differentiated HCC cells rather than in poorly or well-differentiated HCC and liver cells.

**DISCUSSION**

We have demonstrated that HCC cells differentially express several mRNAs originally identified from gene expression profiles. The expression of Gal-4, UGT2B4, rpP0, IGFBP-1, vitronectin, and dek mRNAs was higher and that of RIG-E mRNA was lower in HCC than in nontumorous liver. Although the number of samples were limited, up-regulation of UGT2B4, rpP0, dek, and vitronectin mRNAs, together with down-regulation of RIG-E mRNAs, seemed to be correlated with histological grading. Furthermore, the expression of dek was down-regulated and that of RIG-E was up-regulated in differentiated HuH-7 cells treated with sodium butyrate. These facts suggest that up-regulation of dek and down-regulation of RIG-E mRNAs are linked to the dedifferentiation of HCC cells. However, inconsistent with the pathological observation, sodium butyrate did not affect the levels of rpP0, Gal-4, or UGT2B4 mRNAs, suggesting that it may be a possible agent inducing some, but not all, of the differentiating properties.

The expression of dek was growth-regulated because it was well synchronized with that of cyclin A, an S phase-specific cyclin. In contrast, the expression of Gal-4, UGT2B4, IGFBP-1, and RIG-E was cell density dependent. The expression of Gal-4, UGT2B4, and IGFBP-1 was induced and the expression of RIG-E was suppressed in dense, overcrowded conditions. Furthermore, high expression of IGFBP-1 and Gal-4 mRNAs was also induced by reducing the serum concentration. Similar density-dependent induction of the MN oncoprotein has been reported in HeLa cells (26). In persistent nodules, focal proliferation could cause an increase of cell-cell interaction or growth factor shortage due to poor supply or increased consumption. Under such circumstances, a set of cell density-regulated genes may have critical roles in the manifestation of HCC cell phenotypes.

Galactolectins are a family of β-galactoside-binding lectins with related amino acid sequences. They are soluble proteins and are generally localized in the cytosol. However, they can accumulate on the cell surface under certain conditions to play an important role in cell-cell and cell-matrix interactions. Gal-1 mRNA expression is elevated in fibrosarcoma cells (27) and squamous cell carcinoma (28), whereas Gal-3 is overexpressed in thyroid tumors (29), colon cancer (30), and squamous cell carcinoma (28). Furthermore, the introduction of a Gal-1 cDNA expression vector transforms BALB/3T3 fibroblasts (31), and the recombinant Gal-3 product stimulates the growth of lung fibroblasts (32). However, there has been no previous report about the association between Gal-4 and liver malignancy. In contrast to our results in liver, Gal-4 expression is down-regulated in colon carcinoma (33), suggesting that the tissue-specific background is important in determining the functional significance of this molecule for tumor development.

UGTs play a major role in the detoxification of chemicals including carcinogens, and are separated in two families, designated UGT1 and 2, in which UGT2B4 gene belongs (34). UGT2B4 gene could also have mutated sub-isofoms (35, 36). In contrast to our observation of UGT2B4 expression, down-regulation of several UGT1A genes are demonstrated in HCCs (37). Since UGTs could also render resistance to anticancer drugs (38), differences in the substrate specificity of these isofoms could be important to determine the effects on the liver carcinogenesis.

In line with our results, increased expression of rpP0 has been reported in HCC as well as in colon carcinoma cells (39). Our results further suggest that the expression of this gene may not simply be
correlated with growth states. Meanwhile, rpP0 is thought to interact with eukaryotic elongation factors (40). Because the eukaryotic translational initiation factor 4 transforms NIH3T3 cells when the mRNA is overexpressed (41), rpP0 may also have a causal effect on HCC progression via the translational machinery.

DEK is first identified in a fusion with CAN nucleoporin protein in a subtype of acute myelogenous leukemia (42). The product of the nonrearranged dek gene is a DNA-binding nuclear protein that can recognize a specific element in the HIV-2 enhancer (43). Our results demonstrated that the expression of dek was S-phase dependent, suggesting its involvement in DNA synthesis. It is of particular interest that a G1-S phase cell cycle checkpoint defect in D-type ATM is, in part, complemented by a NH2-terminal-truncated dek product (44). Because the ATM (mutated in the disease AT) gene functions upstream of the p53 antioncogene (45), these observations imply that endogenous DEK may have an oncogenic effect by inhibiting p53.

IGFBPs are present in extracellular fluids and can modulate the binding of IGF to its receptor (46). We observed that IGFBP-1 mRNA was induced in low serum. This is probably consistent with the fact that IGF-I or insulin in serum can suppress IGFBP-1 expression in HCC cells (47). Furthermore, it has been reported that the level of IGF-I mRNA in HCC is lower than that in nontumorous liver (48). These observations, together with our results, suggest that the levels of IGF-I and IGFBP-1 mRNAs are inversely regulated in both HCC and nontumorous liver. Interestingly, butyrate optimally stimulated IGFBP-1 expression at about 1 mM but repressed it at 3 mM. Our observation using tissue samples demonstrated that the highest H:L ratio was observed in moderately differentiated HCC, although the ratios fluctuated widely among the samples. Accordingly, IGFBP-1 mRNA may be preferentially expressed in moderately differentiated HCC cells compared to either poorly differentiated or well-differentiated HCC or nontumorous liver cells. This hypothesis is, in part, supported by the observation that IGFBP-1 mRNA is lower in poorly differentiated rat hepatoma cells than in differentiated cells (49).

IGFBP-1 expression is up-regulated in the liver during postinjury regeneration (50), and autocrine growth regulation of HCC cells involving IGF-I and its receptor has been suggested (51). Thus, IGFBP-1 may be a modulator of liver cell proliferation. There are two isoforms of IGFBP-1: (a) the phosphorylated form has high affinity for IGF and inhibits stimulation of DNA synthesis by IGF; and (b) conversely, the nonphosphorylated form has a low affinity for IGF and enhances the activity of IGF (46). Furthermore, IGFBP-1 binds specifically to the αβ integrin receptor and can stimulate cell migration in the absence of IGF activity (46). These results suggest that this molecule may regulate the metastasis and invasiveness as well as the proliferation of HCC cells.

Vitronectin has been identified as an adhesive protein that promotes the attachment and spreading of normal cells and neoplastic cells in vitro (52). Liver cells are considered to be the principal source of plasma vitronectin. Poorly differentiated HCC is characterized by up-regulation of vitronectin together with other components of the extracellular matrix (53). In line with this, we observed higher levels of vitronectin mRNA in higher-grade HCC than in lower-grade HCC or nontumorous liver tissues. This molecule is accumulated in the interface between the tumor and invaded tissues and within cancerous sinusoids (53), suggesting its stimulatory role in invasion and metastasis of HCC cells.

RIG-E was originally isolated as a transcript induced in promyelocytic leukemia cells by all-trans-retinoic acid (54). Our results demonstrate that this transcript is also up-regulated by sodium butyrate in HCC cells. Retinoic acid induces RAR-β gene expression in hepatoma cells (55), and overexpression of RAR-β results in spontaneous differentiation or antitumor association to facilitate further HCC progression.

The cytochrome enzymes are a superfamily of hemoproteins that are important in the oxidative, peroxidative, and reductive metabolism of endogenous compounds and participate in the chemical carcinogenesis process (59). We observed that CYP3A4 mRNA was highly induced in nontumorous liver and maintained at variable levels in carcinoma tissues from HCC patients but barely detectable in normal liver from non-HCC patients. This expression does not seem to be caused by virus infection, because it was also highly expressed in samples from patient 5, who was HCV and HBV negative. We also detected minor expression of another cytochrome gene, CYP3A7 (23), in liver tissues from HCC patients. This mRNA was originally identified in fetal liver and is developmentally regulated together with CYP3A4 (60), although the expression of CYP3A7 mRNA is not restricted to the fetus (61). Because these two transcripts are very similar (more than 92% nucleotide identity), we used a selective RT-PCR method to distinguish them. CYP3A4 and CYP3A7 mediate the metabolism of hepatocarcinogens, including mycotoxins (62), 2,4-dichlorophenol (63), and aflatoxin B1 (64, 65), and their overexpression increases aflatoxin-induced mutations (66) including those in the p53 tumor suppressor gene (67). In addition, in inflammatory liver disease, a series of CYP transcripts are overexpressed (68). These findings, together with our observations, suggest that CYP3A4 and CYP3A7 may be involved in chronic liver disease and cause the initiation process of liver carcinogenesis. However, their function may not be essential in HCC cells because their expression was maintained at variable levels in HCC tissues and barely detectable in several HCC cell lines (data not shown).

In conclusion, the expression of these mRNAs could be an index for the growth or differentiation of HCC cells or a histological feature of hepatic tumors with compacted tumor cells. Functional analysis of these transcripts should provide a wealth of information with which to further understand liver carcinogenesis.

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