Expression of HNFs and C/EBPα is correlated with immunocytochemical differentiation of cell lines derived from human hepatocellular carcinomas, hepatoblastomas and immortalized hepatocytes

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(Received May 12, 2003/Revised June 27, 2003/Accepted June 30, 2003)

Objective assessment of the differentiation grade of hepatocellular carcinomas (HCCs) is important for evaluation of the pathological diagnosis, prognosis and therapeutic treatment. Differentiation of hepatocytes is reflected by their expression of hepatic functional proteins in the mouse embryo, and liver-enriched transcription factors (LETFs) have been shown to regulate hepatic functional genes strictly. Previous reports demonstrated that the level of LETF expression is altered in HCC or preneoplastic nodules compared with noncancerous tissues. Therefore, LETF expression levels might be useful as a measure of HCC maturation. In this study, to clarify the correlation between the expression of LETFs and the differentiation grade of HCCs, we performed a quantitative analysis of the mRNA expressions of HNFs and C/EBPα using real-time reverse-transcription PCR and immunocytochemical analysis for hepatic functional proteins in twelve cell lines. Furthermore, we examined orthotopic transplantations of the HCC cell lines in C.B-17/Icrj-scid/scid mice and characterized the histologic and cytologic differentiation of the tumors that developed. Our results showed that comprehensive expressions of HNF-3β, HNF-4α, HNF-1α, and C/EBPα were specific to HCCs with well-differentiated function and morphology. Furthermore, among these four transcription factors, HNF-4α and HNF-1α expressions showed synchronism and had a close relation with HCC differentiation. These *in vitro* results were confirmed in tumors developed in SCID mice *in vivo*. These findings suggested that HNF-4α and HNF-1α are useful markers to assess the degree of HCC differentiation, which we suggest could be evaluated objectively by the quantitative analysis of HNFs and C/EBPα in HCCs. (Cancer Sci 2003; 94: 757–763)

Hepatocellular carcinoma (HCC) is a highly malignant tumor found throughout the world, but predominantly in Asia, Africa, and southern Europe.[1)] At the present time, histological diagnosis is primary and essential for evaluation of the prognosis and for the choice of appropriate treatment. The World Health Organization (WHO) classifies HCCs into the trabecular type, pseudoglandular type, compact type and scirrhous type based on histology. The WHO further classifies HCCs into well-, moderately and poorly differentiated types. Clinicopathological studies have shown that poorly and moderately differentiated HCCs are associated with poorer convalescence as compared with highly differentiated HCCs, and that a higher differentiation grade of HCCs is related to a lower recurrence rate.[2)] Thus, evaluation of the differentiation grade of HCCs is important for deciding treatment methodology and for evaluating prognosis.

Previous studies using mouse embryonic cells have demonstrated that the degree of differentiation of hepatocytes is reflected by their expression of hepatic functional proteins.[3)] Concomitantly with the growth of the embryo, hepatocytes gradually mature, and express hepatic functional proteins in a stepwise fashion. At the earliest embryonic stage, hepatoblasts (cells constituting the early embryonic liver) express α-fetoprotein (AFP) as a hepatic functional protein. As they mature, their expression pattern changes and they express albumin (ALB), transferrin (Tf), α1-antitrypsin (AAT) and so on, while AFP expression decreases and becomes undetectable after birth. Moreover, mature hepatocytes of neonatal mouse start to express functional proteins of hepatic detoxification, such as cytochrome P450, Subfamily 1 and Polypeptide 2 (CYP1A2). Thus, it is expected that the degree of cell differentiation of HCCs can be estimated by measuring the expression of hepatic functional proteins.

On the other hand, it is well understood that expressions these functional proteins are regulated strictly by multiple transcription factors, including liver-enriched transcription factors (LETFs) such as hepatocyte nuclear factors (HNFs) and CCAAT/enhancer-binding protein (C/EBP), which have been identified to bind to the promoter or enhancer regions of various hepatic functional genes.[4–7)] Hepatic cytodifferentiation is thought to be controlled by the combined action of these factors. Furthermore, transcriptional alterations of these factors were observed between normal tissues and preneoplastic nodules or cancerous tissue taken from the same subject, both in clinical samples[8)] and in chemically induced tumors of mice.[9, 10)] These reports suggest that changes of LETFs expression occur in hepatocarcinogenesis and their pattern and extent reflect the characteristics of HCCs, especially the differentiation grade. However, little is known about the correlation between histological differentiation and HNF-1α and HNF-1β,[11)] and no study has as yet clarified the correlation between the expression of LETFs mRNAs and differentiation at the level of functional protein expression in HCCs.

It should be useful for evaluation of the prognosis and for the choice of appropriate treatment of HCCs to clarify the correlation between the expression of LETFs and the differentiation grade of HCCs, and to obtain information allowing a more objective assessment of the differentiation grade of HCCs based on molecular biology. Moreover, information is needed about the molecular mechanisms of hepatocarcinogenesis and the dedifferentiation of HCCs.

In this study, to clarify the correlation between the expression of LETFs and the differentiation grade of HCCs, we performed a quantitative analysis of the expression of HNFs and C/EBPα mRNAs using real-time reverse-transcription PCR (RT-PCR) and an immunocytochemical analysis of the expressions of hepatic functional proteins in twelve HCC cell lines. Furthermore, we examined the histological and immunohistochemical differ-

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entiation grade of the HCC cell lines transplanted orthotopically in C.B-17/Icrj-scid/scid mice, to confirm the *in vitro* correlation.

Materials and Methods

Cell lines. We used nine established human cell lines derived from HCCs, HuH7,[12, 13] Alexander,[14] KYN-1,[15, 16] KYN-2,[16, 17] KYN-3,[16, 18] Li7NM,[19, 20] Li7HM,[19, 20] Li21[21] and tPH5T,[22] two cell lines derived from hepatoblastoma, HepG2[23] and Li24,[21] and an immortalized human non-neoplastic hepatocyte cell line, tPH5CH.[22] tPH5CH was established from non-cancerous liver tissue of a chronic hepatitis patient by transfection with SV-40 large T antigen gene, while tPH5T was established in the same manner from the cancerous tissue of the same patient. HepG2, HuH7, Alexander, KYN-1, KYN-2, KYN-3, Li7NM, Li7HM, Li21 and Li24 were maintained in RPMI1640 medium (Invitrogen Corp., Carlsbad, CA). tPH5T and tPH5CH were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (Invitrogen Corp.) with 120 ng/ml hydrocortisone (WAKO, Inc., Osaka), 25 ng/ml epidermal growth factor (EGF) (Sigma Chemical Co., St. Louis, MO), 10 μg/ml transferrin (Sigma), 10 ng/ml selenium (Sigma) and 500 ng/ml linoleic acid (Sigma). HepG2, HuH7, tPH5T and tPH5CH were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Corp.) and 5% fetal bovine serum (Invitrogen Corp.), and the others were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum.

Animals. Female scid mice, C.B-17/Icrj-scid/scid (5 weeks of age) were obtained from CLEA Japan, Inc. (Tokyo) and maintained in a specific pathogen-free environment. Before the experiments, mice were subjected to an adaptation period of at least 1 week. All animals received humane care, and the studies were conducted in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals."

Preparation of nuclear extracts. Total RNA was extracted from cells growing confluently in 100 mm tissue culture dishes (Iwaki, Tokyo) using TRIzol reagent (Invitrogen Corp.) and extracted total RNA was dissolved in 100 μl of DEPC-treated water. To assess the quality of the total RNA, 1 μl was directly analyzed on an RNA LabChip (Agilent, Wilmington, DE) according to the manufacturer's instructions. cDNA synthesis reaction was performed in a 20 μl reaction mixture containing 250 m*M* Tris acetate (pH 8.4), 375 m*M* potassium acetate, 40 m*M* magnesium acetate, stabilizer, 0.1 *M* DTT, 10 m*M* dNTP mixture, 40 U of "RNASEOUT," 15 U of THERMOSCRIPT transcriptase and the reaction was started with 1 μg of total RNA, using the "ThermoScript" RT-PCR System (Invitrogen Corp.). The reaction was performed for 10 min at 25°C, followed by incubation steps of 65°C for 50 min and 85°C for 5 min.

Real-time RT-PCR analysis. Expression of HNF-3β, HNF-4α, HNF-1α and C/EBPα mRNAs was evaluated by real-time RT-PCR based on *Taq*Man methodology. In brief, PCR was performed in an ABI PRISM 5700 sequence detector (Perkin Elmer/Applied Biosystems, Foster City, CA) in a final volume of 50 μl. The PCR mixture contained 10 m*M* Tris-HCl buffer, pH 8.3 (Perkin Elmer/Applied Biosystems), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP mixture, 1.25 U of "Ampli*Taq* Gold" (Perkin Elmer/Applied Biosystems), 0.5 μ*M* primers and probe. The primer and probe sequences for gene amplification were as follows: (i) *HNF-3β*; 5′-AGTTAATATGCTGGGAGCGGTG-3′ (forward primer (FP)), 5′-TGTACGTGTTCATGCCGTTCA-3′ (reverse primer (RP)) and 5′-CCCTGGCGGCAATCCTGACTTTC-3(probe), (ii) *HNF-4α*; 5′-CACCTGATGCAGGAACATATGG-3′ (FP), 5′-CTGTCCGTTGCTGAGGTGAGT-3′(RP) and 5′-CGTCATCGTTGCCAACACAATGCC-3′ (probe), (iii) *HNF-1α*; 5′-GCAGCCTGGTGCTGTACCA-3′ (FP), 3′-GGGAGGAAGAGGCCATCTG-5′ (RP) and 5′-CAGAGCCACCTGCTGCCATCCAAC-3′ (probe), (iv) *C/EBPα*; 5′-GGGCCAGGTCACATTTGTAAA-3′ (FP), 5′-AGTAAGTCACCCCCTTAGGGTAAGA-3′ (RP) and 5′-CCCTGGCGGCAATCCTGACTTTC-3′ (probe), (v) glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): the housekeeping gene; 5′-AATTCCATGGCACCGTCAA-3′(FP), 5′-CCAGCATCGCCCCACTT-3′ (RP) and 5′-CCATCACCATCTTCCAGGAGCGAGA-3′ (probe).

The "*Taq*man" probes carried a 5′ TET reporter label and a 3′ TAMRA quencher group and were synthesized by PE-Applied Biosystems. The Ampli*Taq* Gold enzyme was activated by heating for 10 min at 95°C. All genes were amplified by a first step of 15 s at 95°C followed by 1 min at 60°C for 50 cycles. We initially performed 60 cycles, but as samples never showed positivity at a cycle threshold higher than 50, the maximum number of cycles was determined to be 50.

Quantification. For the construction of standard curves of positive controls, the total RNA of the cell line HepG2 was reverse-transcribed into cDNA and serially diluted in five or six log steps into water to give 4-fold serial dilutions of cDNA from 83.33 ng to 81.38 pg. This cDNA serial dilution was prepared once for all examinations performed in this study and stored at −20°C. The coefficient of linear regression (r) for each standard curve was calculated. When the cycle threshold (CT) value of a sample was substituted into the formula for each standard curve, the relative concentration of HNF-3β, HNF-4α, HNF-1α, C/EBPα, or GAPDH could be calculated. To normalize for differences in the amount of total RNA added to each reaction mixture, GAPDH was selected as an endogenous RNA control. The data represent the averages of the targeted gene:GAPDH ratio±SD from three independent cultures.

Immunocytochemistry. Cells cultured on type I collagen-coated dishes (Iwaki) were fixed with 4% neutral buffered paraformaldehyde, for subsequent examination for ALB, AFP, Tf, AAT, CYP1A2 and E-cadherin, or with absolute ethanol at −30°C for detection of cytokeratin (CK) 7 and CK19. Immunocytochemistry was performed using the standard streptavidin-biotin-peroxidase complex (Dako LSAB Kit, Dako Japan Co., Kyoto) methods with the primary antibodies listed in Table 1, according to the manufacturer's instructions. Tumor samples of HCCs

Table 1. Antibodies and reaction conditions employed in the present study

Antigen	Antibody type	Dilution	Primary antibody reaction time	Fixing solution
α-Fetoprotein	Polyclonal	1:300	4°C, overnight	Formalin
Albumin	Polyclonal	1:100	4°C, overnight	Formalin
Transferrin	Polyclonal	1:200	4°C, overnight	Formalin
α1-Antitrypsin	Polyclonal	1:1	4°C, overnight	Formalin
Cytochrome P450	Polyclonal	1:200	4°C, overnight	Formalin
Cytokeratin7	Monoclonal	1:40	Room temperature, 1 h	Ethanol
Cytokeratin19	Monoclonal	1:1	Room temperature, 1 h	Ethanol
E-Cadherin	Monoclonal	1:30	4°C, overnight	Formalin

formed in SCID mice were similarly examined for AFP, ALB, Tf, and AAT.

Transplantation. Six-week-old female SCID mice were used to examine the tumorigenicity and the tumor differentiation of HCCs. The transplanted cell lines were KYN-2 and KYN-1 as well-differentiated cell lines, tPH5T and Li24 as moderately differentiated cell lines, and Li21 as a poorly differentiated cell line. Mice were anesthetized with 2.5% 2,2,2-tribromoethanol (Aldrich Chemical Co., Milwaukee, WI) in 25 µl/ml *t*-amyl alcohol (WAKO) ("Avertin"). With a 27-gauge needle, 2×10^6 cells suspended in 50 µl of sterile RPMI1640 medium were injected into the hepatic intermediate lobe. Abdominal palpation was performed every day. The mice were killed between the 4th week and the 12th week after the intrahepatic injection (IH), when tumors were palpated, and the liver was excised for histological and functional analyses.

Transmission electron microscopy. Fresh tissues resected from the HCC tumors in SCID mice were fixed with 2.5% glutaraldehyde in 0.1 mol/liter cacodylate buffer (pH 7.4) at room temperature for 30 min, postfixed with 2% OsO_4 in the buffer, and embedded in Epon 812. Semithin and ultrathin sections were cut on a Bromma 2088 ultratome V (LKB, Schweden, Germany). The semithin sections were stained with 1% toluidine blue and examined with a light microscope. The adjacent thin sections were stained with uranyl acetate, followed by lead citrate and examined at 60 kV with a Hitachi H-7000 transmission electron microscope (Hitachi, Ltd., Tokyo).

Results

Real-time RT-PCR analysis. The gene expressions of HNFs and C/EBPα in the cell lines were examined by real-time RT-PCR. Fig. 1 shows the CT values plotted versus the relative input amount (total starting RNA) to produce the standard curves for quantification of target genes and GAPDH. From these standard curves, the relative concentrations of HNF-3β, HNF-4α, HNF-1α, and C/EBPα with respect to GAPDH could be calculated. The results are summarized in Fig. 2 and Table 2. According to the expression levels of four genes, we divided the twelve cell lines into three groups (Table 2). HepG2, KYN-2, HuH7, and KYN-1 retained full expression of all four genes and they comprised group 1. Li7HM, Li7NM, and Li21 did not express any of the four genes, and comprised group 3. The remaining 5 cell lines, Alexander, tPH5T, Li24, tPH5CH, and KYN-3 all expressed HNF-3β, but not C/EBPα; one of them expressed both of the other two genes, one expressed one of them, and the others expressed neither (group 2).

Since HNFs and C/EBPα are thought to be expressed in more matured hepatocytes, the cell lines of group 1 were con-

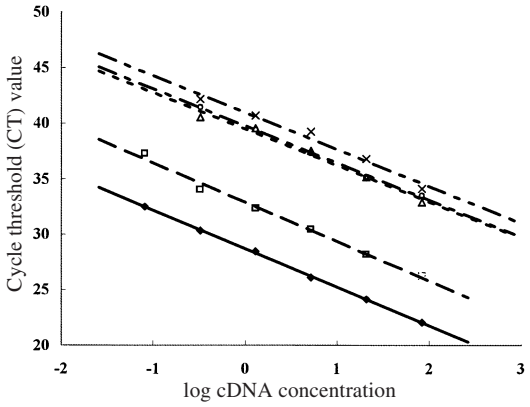

Fig. 1. Representative standard curves for targeted genes and GAPDH, constructed using several concentrations of standard samples (HepG2). Note that as the sample concentration increased, the CT values decreased. CT values (*y*-axis) showed an inverse linear correlation with the log of sample concentration (*x*-axis). × C/EBPα, ○ HNF-1β, △ HNF-3β, □ HNF-4α, ◆ GAPDH.

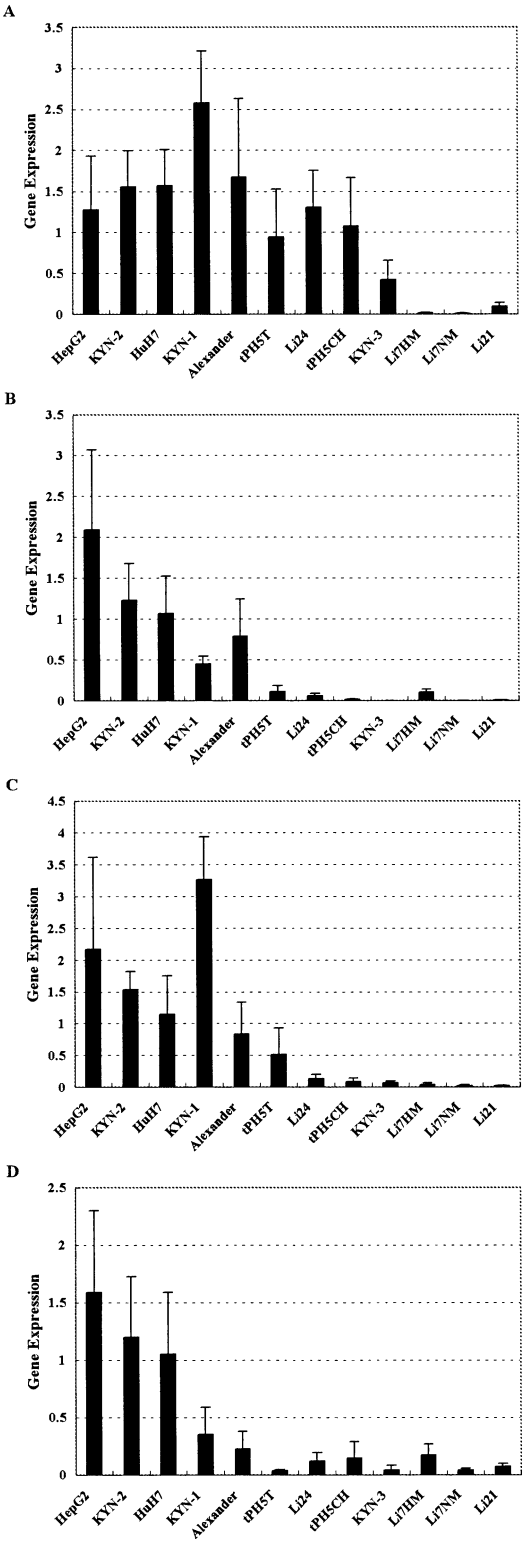

Fig. 2. Expressions of HNF-3β (A), HNF-4α (B), HNF-1α (C), C/EBPα (D) mRNA in 12 cell lines. Gene expression (*y*-axis) represents the relative expression of the target transcription factors compared to the standard sample (HepG2) in each cell line, normalized with respect to GAPDH expression. Data are mean±SD.

sidered most mature. All the cell lines of group 2 expressed HNF-3β (the basic transcriptional factor of hepatocytes), but the levels of expression of HNF-4α and HNF-1α varied. The cell lines of group 3 did not express any HNF or C/EBPα, and were thought to be the most poorly differentiated.

Immunocytochemistry. To assess the cytodifferentiation grade of cell lines in terms of the expression of hepatic functional and structural proteins, we performed immunostaining for AFP, ALB, Tf, AAT, CYP1A2, and E-cadherin. CK7 and CK19 were also immunostained as markers of bile duct epithelial cells. The results are summarized in Table 3 and representative staining is shown in Fig. 3. The group 1 cell lines expressed ALB, Tf and CYP1A2, and these cell lines were confirmed to be functionally well-differentiated. On the contrary, the cell lines in group 3 were suggested to be functionally poorly differentiated because they lacked this expression. All cell lines of group 2 expressed the functional proteins at various levels. Neither CK7 nor CK19 expression had any relation to the expression of other functional proteins. All cell lines except for Li7HM and Li7NM were positive for E-cadherin.

Table 2. Classification of 12 cell lines according to amounts of LETFs expression

Group	Cell line	Gene			
		HNF-3β	*HNF-4α*	*HNF-1α*	*C/EBPα*
Group 1	HepG2	3	4	4	4
	KYN-2	3	4	4	4
	HuH7	4	4	4	4
	KYN-1	4	1	4	1
Group 2	Alexander	4	2	3	–
	tPH5T	3	–	1	–
	Li24	3	–	–	–
	tPH5CH	2	–	–	–
	KYN-3	2	–	–	–
Group 3	Li7HM	–	–	–	–
	Li7NM	–	–	–	–
	Li21	–	–	–	–

We characterized the expression level of each targeted gene as follows, compared to the expression level in HuH7 cells: 4, >100%; 3, 75–100%; 2, 50–75%; 1, 25–50%; –, <25% or undetectable. We further classified these 12 cell lines into 3 groups by the number of genes expressed.

Orthotopic transplantations. To investigate the correlation between functional differentiation grade assessed in terms of the expression level of LETFs *in vitro* and the histological differentiation of tumors, we selected cell lines KYN-2 and KYN-1 from group 1, tPH5T and Li24 from group 2, and Li21 from group 3, and orthotopically transplanted them into C.B-17/SCID mice. In terms of both gene and functional protein expressions, KYN-2 and KYN-1, tPH5T and Li24, and Li21 represented well-differentiated, moderately differentiated, and poorly differentiated cell lines, respectively. The results are summarized in Table 4. Three of the five inoculated cells, KYN-2, KYN-1, and Li21, exhibited tumorigenicity.

KYN-2 formed Burmese ruby or black tumors in all transplanted mice on the 4th week after IH and showed many intrahepatic metastases and lymph node metastases (Fig. 4A). Microscopically, they exhibited random thick trabeculae which were solidly arranged, surrounded by unclear spaces containing blood with hemorrhagic foci (Fig. 4B). These findings demonstrated that the KYN-2-mediated mouse tumor was a relatively differentiated hepatocellular carcinoma. Electron microscopy demonstrated a pseudoglandular structure, well-developed microvilli, and tight junctions (data not shown).

KYN-1 formed white tumors in an inoculated lobe in only one of seven transplanted mice on the 12th week after IH. Microscopically, they exhibited random thin trabeculae and frequently had low-grade atypia of nuclei and steatosis in comparison with KYN-2. Electron microscopically, they exhibited a solid growth pattern with pseudoglandular structures and tight junctions. These findings demonstrated that the KYN-1 mediated mouse tumor was also a relatively differentiated hepatocellular carcinoma.

Li21 formed white tumors with no intrahepatic metastasis, but with many extrahepatic metastases larger than the primary tumors. Microscopically, they were spindle cell carcinomas, exhibited no trabeculae and formed a solid arrangement surrounded by slit-like blood spaces with hemorrhagic foci (Fig. 4D). Moreover, they had a large nuclear/cytoplasm ratio and low-grade nuclear atypia. Electron microscopy demonstrated

Table 3. Immunohistochemical findings

Group	Cell line	Antigen							
		AFP	ALB	TF	AAT	CYP1A2	CK7	CK19	E-Cadherin
Group 1	HepG2	+	+	+	+	+	–	±	+
	KYN-2	+	+	+	+	p+	–	–	+
	HuH7	+	p+	p+	+	+	–	+	+
	KYN-1	+	+	+	+	+	p+	+	+
Group 2	Alexander	+	p+	–	+	+	–	–	+
	tPH5T	+	–	–	+	±	–	+	+
	Li24	p+	–	–	+	–	+	+	+
	tPH5CH	–	–	–	–	+	±	+	p+
	KYN-3	+	p+	–	+	–	+	–	+
Group 3	Li7HM	+	+	–	+	–	–	–	–
	Li7NM	+	–	–	+	–	+	–	–
	Li21	+	–	–	p+	–	–	–	±
	Property	fetal protein → functional protein					a marker of bile duct epithelial cells		a morphological marker of mature hepatocyte

ALB, albumin; AFP, α-fetoprotein; CK7, cytokeratin 7; TF, transferrin; AAT, α1-antitrypsin; CK19, cytokeratin 19; CYP1A2, cytochrome P450, subfamily I, polypeptide 2. The results of immunostaining are symbolized by the positive rate of all stained cells: +, over 80%; p+, 80–10%; –, less than 10% or negative; ±, focally positive. The groups correspond to those in Table 2.

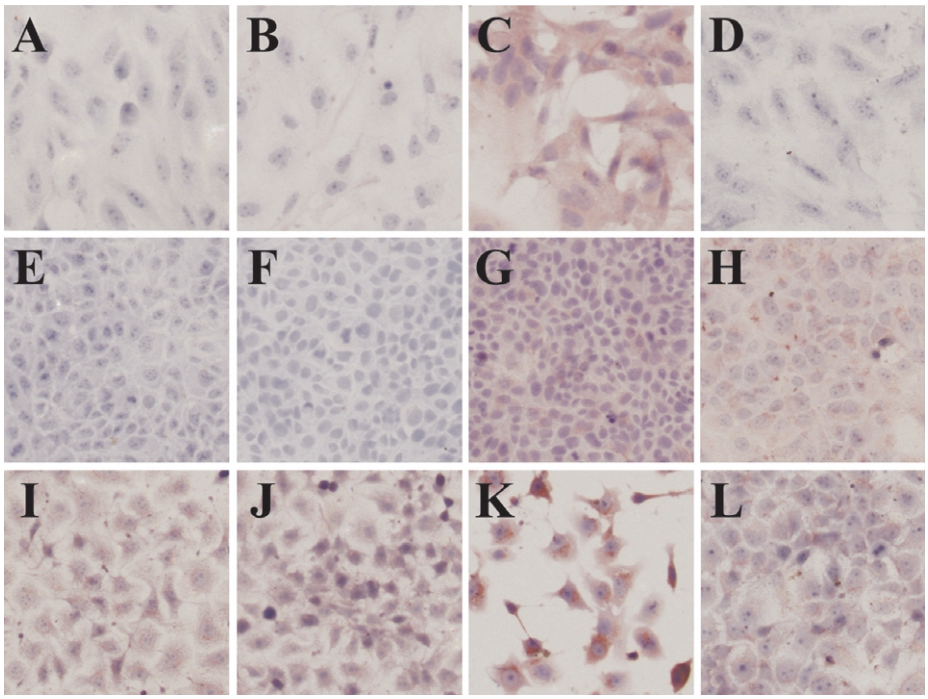

Fig. 3. Immunohistochemistry of cultured cell lines. A–D) Li21. E–H) tPH5T. I–L) KYN-2. Immunohistochemistry for ALB (A, E, I), Tf (B, F, J), AAT (C, G, K), CYP1A2 (D, H, L).

Table 4. Tumorigenicity, metastatic ability in liver and lymph node, and histological grade of tumor of 5 cell lines in SCID mice

Group	Cell line	Tumorigenicity in liver	Intrahepatic metastases	Lymph node metastases	Histological differentiation
Group 1	KYN-2	100% (6/6)	6/6	6/6	moderate
	KYN-1	14% (1/7)	1/6	0/6	moderate
Group 2	tPH5T	0% (0/8)	0/6	0/6	—
	Li24	0% (0/7)	0/6	0/6	—
Group 3	Li21	80% (4/5)	3/6	4/6	poor

high-grade nuclear atypia, few cytoplasmic organelles and tight junctions (data not shown). These findings demonstrated that the Li21 mediated mouse tumor was a relatively poorly differentiated hepatocellular carcinoma.

To confirm the histological differentiation of the mouse tumors, we conducted immunostaining for the functional proteins AFP, ALB, Tf, and AAT (data not shown). KYN-1 and KYN-2 expressed all the antigens examined, suggesting that they were functionally well-differentiated. However, Li21 had lost these expressions, suggesting that it was functionally poorly differentiated.

Discussion

Studies using human HCC tissues and the results of analysis of chemically induced murine liver tumors have previously demonstrated that the expression levels of LETFs in tumor tissues or nodules are different from those in normal liver.[8–10] It has also been recognized that LETFs play important roles in liver differentiation, which is strictly controlled by the appropriate LETF expression pattern at each embryonic developmental stage.

We hypothesized that the transcription factors of LETFs also control the cytodifferentiation of HCC cell lines in the same manner as in embryogenesis. We subdivided the 12 cell lines examined in this study into three groups. Cell lines in group 1 were highly differentiated lines expressing high levels of LETFs. Cell lines in group 3 were less differentiated lines expressing no LETFs. Cell lines in group 2 expressed various levels of LETFs and showed intermediate differentiation between those in groups 1 and 3. As HNF-4α, HNF-1α, and C/EBPα are expressed during late embryogenesis and are related to the functional differentiation of hepatocytes, it is reasonable that their expression was detected mainly in transcriptionally differentiated cell lines in group 1. The expression of HNF-3β however, which is reported to be detected during the earliest stage of hepatogenesis, was maintained not only in the cell lines of group 1, but also in those of group 2. There seem to be two possible explanations for this result. One is that the HCC is a tumor that exhibits dedifferentiation, showing transcriptional factors of early embryogenesis. The other is that HNF-3β is not related only to hepatocyte differentiation, but also has another function. Recent studies suggested that in adults, HNF-3 is related to the regulation of basic amino acid metabolism through aspartate aminotransferase and γ-glutamyltranspeptidase, whose functions are maintained in both normal and tumor cells.[24, 25]

This study revealed that the cell lines in group 1 expressed functional proteins such as CYP1A2, ALB, Tf, and AAT. The cell lines that expressed CYP1A2 tended to maintain ALB and Tf expressions. However, whereas AAT expression is also regarded as a marker of well-differentiated hepatocytes, like CYP1A2, some cell lines that expressed AAT had lost the ex-

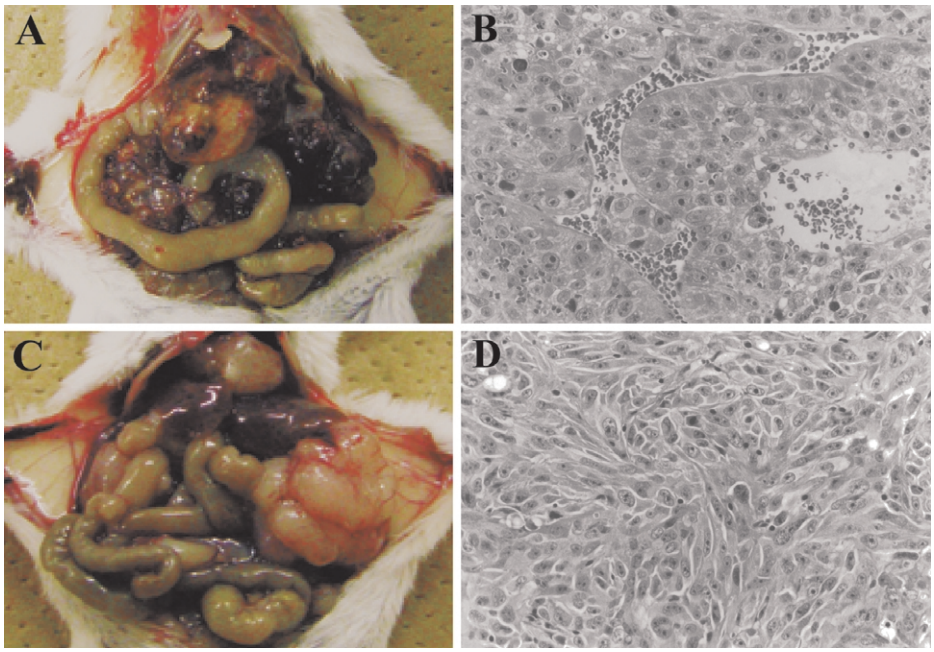

Fig. 4. Macroscopic and microscopic (H.E.) appearance of a developed tumor in a SCID mouse. A) KYN-2: macroscopic appearance showing Burmese ruby or black tumors and a high frequency of intrahepatic metastases. B) Microscopically, they exhibit random thick trabeculae with a solid arrangement of a pleomorphic tumor. C) Li21: white tumors without intrahepatic metastasis, but with a high frequency of intrahepatic metastases, whose size was characteristically larger than that of the primary tumors. D) Microscopically, they are spindle cell carcinomas and do not exhibit any trabeculae, but form a solid arrangement surrounded by slit-like blood spaces with hemorrhagic foci. They further exhibit large masses of nuclei with minimal cytoplasm and low-grade nuclear atypia. Original magnifications, ×200 (B, D).

pression of ALB and Tf. These results indicate that the expression of CYP1A2 is more closely associated with the state of expression of LETFs. On the other hand, the cell lines in group 3 that showed no detectable expression of LETFs tended to have also lost these functional protein expressions. We suggest that the expressions of HNF-4α, HNF-1α, and C/EBPα are closely related to the regulation of hepatic differentiation. The structural proteins CK7 and CK19 are cytoskeletal proteins thought to be markers of bile duct epithelial cells, and co-expression with AFP, ALB, or both is observed in bipotential proliferating hepatic epithelial cells, such as oval cells. Li24, KYN-3, and Li7NM expressed these cytokeratins similarly to AFP and ALB, and they are thought to have bipotentially proliferative activity.

Functional differentiation was also confirmed by the histological examination of the tumors formed in SCID mice. The tumors of KYN-2, a representative cell line in group 1, showed microvilli and tight junctions, and formed a pseudoglandular structure. These morphological characteristics indicated that KYN-2 was more differentiated towards hepatocyte status, compared with Li21, one of the cell lines in group 3.

In conclusion, the comprehensive expression of HNF-3β, HNF-4α, HNF-1α, and C/EBPα corresponded well with differentiated functions and morphology of HCCs. Furthermore, although the functional analyses using knock-down or knock-out models should be done to establish the role of LETFs in cytodifferentiation of hepatocytes, expressions of HNF-4α and HNF-1α among these four transcription factors showed synchronism and had a close relationship with HCC differentiation. A recent study reported that down-regulated C/EBPα was associated with poor survival, tumor diameter and clinical stage of HCC.[26] These findings suggest that HNF-4α and HNF-1α could be useful markers to assess the degree of cell differentiation, and that differentiation of HCCs can be evaluated objectively by the quantitative analysis of HNFs and C/EBPα.

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