Decreased Expression of UK114 Is Related to the Differentiation Status of Human Hepatocellular Carcinoma

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Abstract

Previous studies have identified that the expression of UK114 is tissue specific and the protein has been found to be most abundant in liver and kidney. However, the expression of UK114 in human hepatocellular carcinoma and its relationship to differentiation and transformation of hepatocellular carcinoma have not been studied. In this study, the expression of UK114 in human hepatocellular carcinoma was examined by Northern and Western blot analyses. We found that UK114 was significantly down-regulated in most of hepatocellular carcinoma tissues compared with adjacent nontumor tissues (72.7%) at both mRNA and protein levels. We looked into the possibility that this decreased expression of UK114 in the hepatocellular carcinoma tissues may play a role in the differentiation or tumorigenicity of hepatocellular carcinoma. Immunohistochemical staining showed that the reduced expression of UK114 in hepatocellular carcinoma tissues was correlated with the tumor differentiation status as graded by the Edmondson-Steiner classification. On the other hand, overexpression of UK114 was not able to suppress the proliferation of human hepatoma cells and tumorigenicity in nude mice. These results suggest that UK114 does not seem to act as a tumor suppressor gene; however, it may useful as a biomarker that will assist in the grading of the differentiation status of hepatocellular carcinoma samples. (Cancer Epidemiol Biomarkers Prev 2008;17(3):535–42)

Introduction

Hepatocellular carcinoma is one of the most common malignancies in the world, is the third most important cause of cancer death, and causes an estimated 600,000 deaths worldwide in 2002 (1). A marked difference has been found in the worldwide distribution of hepatocellular carcinoma, with significantly higher incidence rates in Southeast Asia and Africa (2). The development of hepatocellular carcinoma is strongly associated with chronic liver disease, particularly cirrhosis, which occurs as a result of viral infection (hepatitis B or C virus), alcohol abuse, metabolic disorders, and aflatoxin exposure (3). Despite the use of various screening and treatment protocols, the overall prognosis of hepatocellular carcinoma patients remains very poor (4-7). In addition, to better understand the molecular mechanism and the possible application of novel therapies, another area of research interest is the search for reliable hepatocellular carcinoma–associated biomarkers that will have a great value in diagnosis and developing therapeutic strategies. Furthermore, prognosis and clinical management of hepatocellular carcinoma is closely related to the differentiation status of the hepatocellular carcinoma as measured by its pathologic grading. At the present time, the grading of hepatocellular carcinoma is still determined by experienced pathologists using a popular system based on the Edmondson-Steiner classification (8). Therefore, the discovery of a new biomarker that correlates with the present pathologic classification of hepatocellular carcinoma will be helpful for diagnosis and therapy.

In our effort to find a new biomarker for hepatocellular carcinoma, we have screened genes differentially expressed in paired hepatocellular carcinoma and normal liver tissues using microarray analysis. Several genes that were differentially expressed in hepatocellular carcinoma tissues have been identified. Among them, UK114 was significantly down-regulated. UK114 (also called translational inhibitor protein p14.5) has been purified from the perchloric acid–soluble fraction of goat liver (9). It shares a high degree of homology with other perchloric acid–soluble proteins (PSP) extracted from the rat liver and kidney (10, 11) and shows similarity to the new hypothetical protein family, YER057c/YJGFs, which is highly conserved across the world range from prokaryotes to eukaryotes (12-14). The evolutionary conservation of these proteins

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A

	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebellum, left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain	yeast total RNA
B	cerebral cortex	cerebellum, right	nucleus accumbens	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney	E.coli rRNA
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver	poly r(A)
E	occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen	human C0t-1 DNA
F	temporal lobe	hippo-campus		ventricle, right	ileocecum		lymph node	testis	mammary gland	Burkitt's lymphoma, Daudi	fetal thymus	human DNA 100 ng
G	p.g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone marrow	ovary		colorectal adeno-carcinoma SW480	fetal lung	human DNA 500 ng
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549		

*paracentral gyrus

B

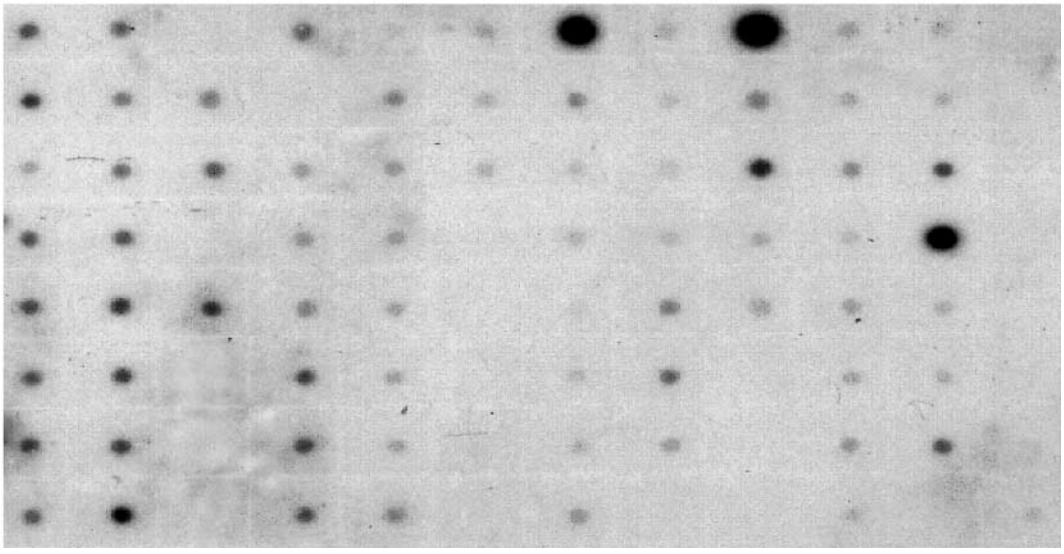


Figure 1. A. Detection of UK114 mRNA in human tissues. Representative human tissue and the internal control in the Human Multiple Tissue Expression (MTE) Array (Clontech), which in essence is an array of poly(A)$^+$ for quickly profiling gene expression. **B.** Human Multiple Tissue Expression (MTE) Array was hybridized with a human UK114-specific cDNA probe to detect the expression of UK114 in the various normal human tissues. Hybridization signals were visualized by autoradiography.

implies that they may have an important cellular function. Several studies indicate that UK114 may be a multifunctional protein. Rat liver PSP has endoribonucleolytic activity and can inhibit protein synthesis *in vitro* in rabbit reticulocyte lysate (15). A natural calpain activator protein has been isolated from the bovine brain and when characterized was found to be very similar to goat liver UK114 (16). Biological studies of its function suggest that it may play a role in cellular differentiation and development because levels are significantly increased during differentiation from human monocytes to macrophages (17) and during the development process of rat kidney, rat brain, pig liver, and chick liver (11, 18-20). Furthermore, expression of PSP depends on the proliferation states of the cell and overexpression of PSP reduces cell proliferation of normal rat kidney epithelial NRK-52E cells (21, 22).

Many previous studies have indicated that the expression of UK114 is tissue specific and the protein has been found to be most abundant in liver and kidney. However, the expression of UK114 in human hepatocellular carcinoma and its relationship to the differentiation and transformation of human hepatocellular carcinoma have not been reported. In this study, we examined the expression level of UK114 by Northern and Western blot analyses and immunohistochemistry and found that UK114 was significantly down-regulated in most hepatocellular carcinoma tumor tissue as well as human

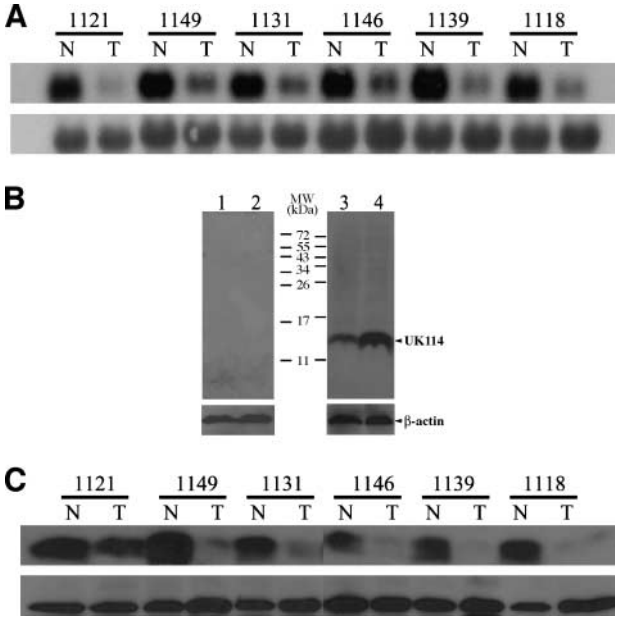


Figure 2. Expression of UK114 in human hepatocellular carcinoma tumor tissues versus its adjacent normal tissues. **A.** Expression of UK114 mRNA in six representative pairs of hepatocellular carcinoma tumor tissues (*T*) and their adjacent normal tissues (*N*) was detected by Northern blot analysis with a human UK114-specific cDNA probe (*top*). The level of 18S rRNA was used as an internal control for the adjustment of the RNA loading (*bottom*). **B.** Specificity of polyclonal antibody against UK114 was examined by Western blot analysis. The human hepatoma HuH7 cells were transfected with control vector (*lanes 1* and *3*) or UK114 expression vector (*lanes 2* and *4*). The cell lysates were prepared and detected by preimmune serum (*lanes 1* and *2*) or polyclonal antibody against UK114 (*lanes 3* and *4*). The level of β-actin was used as an internal control for the adjustment of protein loading. **C.** Expression of UK114 protein in the same batch of hepatocellular carcinoma specimens was detected by Western blot analysis using the anti-UK114 polyclonal antibody (*top*). The level of β-actin was used as an internal control for the adjustment of protein loading (*bottom*).

hepatoma cell lines. In addition, the expression pattern of UK114 was closely correlated with the differentiation status of the hepatocellular carcinoma tissue according to the Edmonson-Steiner classification. However, overexpression of UK114 was not able to suppress cellular proliferation of human hepatoma cells and tumorigenicity in rude mice. These results indicated that UK114 probably does play a role in the process of hepatocyte differentiation and may serve as a biomarker for the staging of hepatocellular carcinoma.

Materials and Methods

Tissue Samples. Twenty-two pairs of frozen hepatocellular carcinoma specimens made up of paired tumor tissue and their adjacent noncancerous tissue were obtained from the tissue bank of surgical department of Taipei Veterans General Hospital. All the specimens had been immediately frozen after surgery and stored in liquid nitrogen until used. In addition, paraffin sections were prepared from 73 formalin-fixed hepatocellular carcinoma specimens, which had been obtained from the Department of Pathology of Chang Gung Memorial Hospital. The histologic diagnosis and tumor grade of all the samples were confirmed by an experienced pathologist (S.F.H.). The tumor grading was determined based on the Edmondson-Steiner classification.

Cell Culture. Various human hepatoma cell lines, including HuH7 (23), HepG2 (24), HA22T (25), Mahlavu (26), Tong (27), and SNU-387 (28), were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2.

Cloning of the UK114 Gene. The prokaryotic expression vector for UK114 was constructed as follows. A cDNA fragment containing the human UK114 gene was amplified by reverse transcription-PCR using the primer set 5′-GGAATTCCATATGTCGTCCTTGATCAGAAGGG-3′ for the sense strand and 5′-CGCGGATCCCACTTATAGTGATGCCGTTGTCAG-3′ for the antisense strand. The fragment was then ligated into pET-15b vector (Novagen) at the *Nde*I/*Bam*HI site in frame with the His tag for prokaryotic expression; the latter allowed the preparation of polyclonal antibody against UK114 (pET-15b/UK114). For eukaryotic expression vector, the cDNA fragment of human UK114 gene was amplified using a second another primer set: 5′-CCGGAATTCCATATGTCGTCCTTGATCAGAAGG-3′ for the sense strand and 5′-CGCGGATCCCACTTATAGTGATGCCGTTGTCAG-3′ for the antisense strand and the fragment cloned into pcDNA3.1(-) vector (Invitrogen) in frame at the *Eco*RI/*Bam*HI site (pcDNA/UK114).

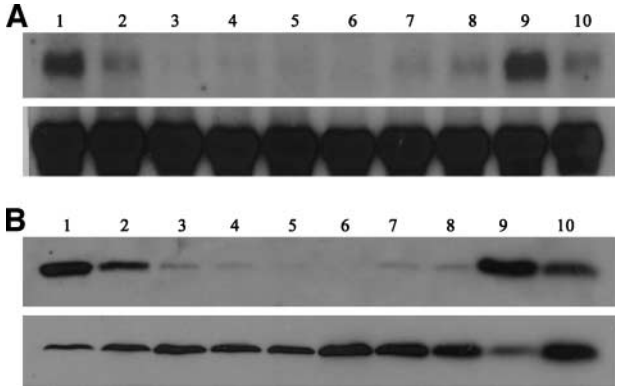


Figure 3. Expression of UK114 in human hepatoma cell lines. **A.** Expression of UK114 mRNA in hepatocellular carcinoma tissues and cell lines was detected by Northern blot analysis with a human UK114-specific cDNA probe (*top*). The level of 18S rRNA was used as an internal control for the adjustment of the RNA loading (*bottom*). **B.** Expression of UK114 protein in the same specimens was detected by Western blot analysis with a rabbit anti-UK114 polyclonal antibody (*top*). The level of β-actin was used as an internal control for the adjustment of protein loading (*bottom*). 1, adjacent normal tissue of sample 1148; 2, tumor tissue of sample 1148; 3, HA22T; 4, Mahlavu; 5, Tong; 6, SNU-387; 7, HuH7; 8, HepG2; 9, adjacent normal tissue of sample 1139; 10, tumor tissue of sample 1139.

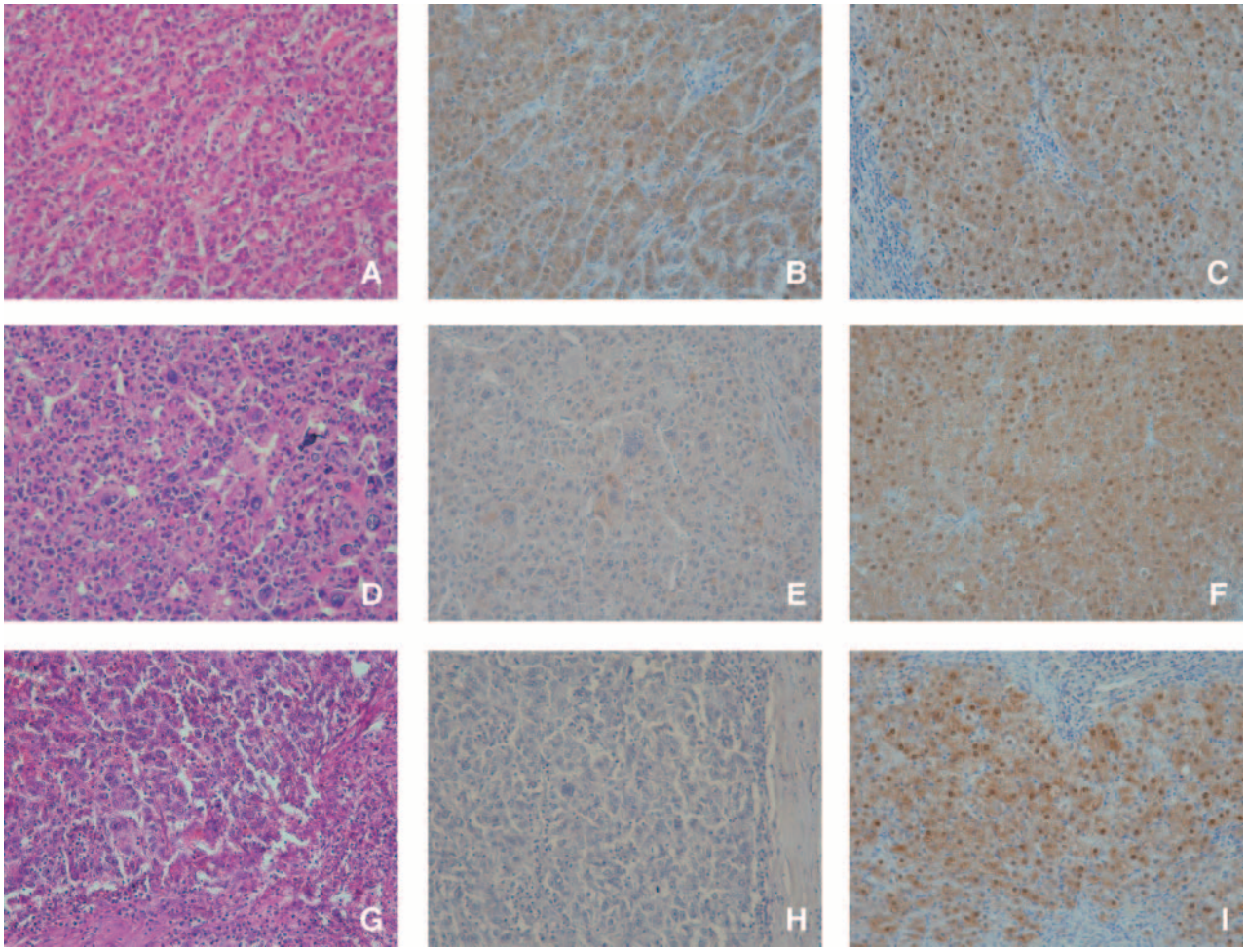


Figure 4. Representative immunohistochemistry of UK114 in human hepatocellular carcinoma tumor tissue and adjacent normal liver tissue (magnification, ×400). Expression level of UK114 was higher in the normal liver tissue compared with tumor tissue. Left, H&E staining of the sample (**A**, **D**, and **G**). Middle (**B**, **E**, and **H**) and right (**C**, **F**, and **I**), immunohistochemistry of UK114 in the adjacent normal liver tissue and tumor liver tissue, respectively. All of the tissue sections were graded according to Edmondson-Steiner classification. Representatives of grade 2 hepatocellular carcinoma (moderate differentiation; **A-C**). Representatives of grade 3 hepatocellular carcinoma (poor differentiation; **D**, **E**, and **H**). Representatives of grade 4 hepatocellular carcinoma (undifferentiation; **G-I**).

RNA Preparation and Northern Blot Analysis. Total RNA was extracted from frozen tissue and cultured cells with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Twenty micrograms of total RNA were fractionated on 1.2% formaldehyde agarose gels and transferred to nylon membranes. The membranes were cross-linked using an ultraviolet cross-linker (Stratagene) and hybridized with α-^{32}P-radiolabeled DNA probes (2×10^8 counts/min/μL) produced by a random prime labeling system (rediprime II) (Amersham Biosciences). Hybridization and washing conditions were the same as previously (29) and the membranes were then exposed to X-ray film at -70°C. To confirm the amounts of total RNA loaded in each lane, the membranes were hybridized afterward with an 18S ribosomal RNA gene, which served as an internal control.

Generation of Polyclonal Antibody against UK114. To express His-UK114 fusion proteins, plasmid pET-15b/UK114 was transformed into *Escherichia coli* BL21. A fresh transformant colony was grown to log phase in Luria-Bertani medium and supplemented with 1 mmol/L IPTG to induce protein expression. The recombinant proteins were then purified by Ni-Sepharose 4B column (Amersham) according to the manufacturer's instructions. For the preparation of polyclonal antibody, 1 mg of the purified fusion proteins was emulsified with Freund's adjuvant (Sigma) for immunization of New Zealand White rabbits according to the standard protocol. Antibody valence and specificity was checked by Western blot analysis.

Protein Preparation and Western Blot Analysis. Tumor tissues were homogenized with lysis buffer containing 1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.0), 2% SDS, 10% glycerol, and 0.2 mmol/L EDTA. The cell lysate was then centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was collected. The protein

samples (100 μg) were electrophoresed on a 16.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore) using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). After blocking the membrane with 5% non-fat milk, the membrane was incubated with rabbit polyclonal antibody against human UK114 at a 1:1,000 dilution overnight at 4°C and then allowed to interact with peroxidase-conjugated anti-rabbit IgG goat antibody. The immunoblot signals were examined by the enhanced chemiluminesence detection system (Western Lighting Chemiluminescence Reagent Plus, Perkin-Elmer Life Sciences; Australia) and exposed to X-ray film.

Immunohistochemical Staining. Immunohistochemical staining was done on the formalin-fixed and paraffin-embedded tissue sections. Each tissue section was pretreated by deparaffinized, rehydrated, and pretreated with 10 mmol/L citrate buffer (pH 6.0) for 15 min in an 800-W microwave oven. The staining procedure was then done with the Universal LSAB 2 Kit for peroxidase (Dako) following the manufacturer's instructions. Briefly, the tissue sections were treated with 3% hydrogen peroxide to block endogenous peroxidase, incubated with rabbit polyclonal antibody against human UK114 at a 1:2,500 dilution for 1 h, and then incubated at room temperature for 10 min with biotinylated linked anti-rabbit antibody. After reaction with benzidine, the tissue sections were weakly counterstained with hematoxylin. The adjacent normal tissue in each sample was used as the positive control for immunostaining of UK114. All of these sections were examined and classified into four categories according to their histologic grades based on Edmondson-Steiner classification (8): well differentiated (grade 1), moderately differentiated (grade 2), poorly differentiated (grade 3), and undifferentiated (grade 4) hepatocellular carcinoma.

Cell Proliferation *In vitro* and Tumorigenicity *In vivo*. For DNA transfection, cells were seeded 1 day before treatment and transfection procedure was done next day with LipofectAMINE reagent (Invitrogen). Stable transfectants, including UK114 expression clones and vector control clones, were obtained with G418 selection for 3 weeks. To assay the effect of UK114 on cell growth, cells were seeded in 24-well plates and the cell numbers counted every day over a period of 5 days. Each point on the growth curve represents the mean value of three independent wells and the cell doubling times were estimated for each transfectant. To assay the effect of UK114 on tumor formation in nude mice, stably transfected cells (1×10^6 or 5×10^6) were inoculated s.c. into the hind back of BALB/c nude mice. The development of tumors was observed and the size of the tumors was measured weekly for 2 months.

Statistical Analysis. The correlation between UK114 expression and the histologic grade of the hepatocellular carcinoma tissues was assessed by Pearson's χ^2 test. The effect of UK114 on tumorigenicity in nude mice was examined by Fisher's exact test. Differences were considered significant if the P value is less than 0.05.

Results

Specific Tissue Distribution of UK114: Predominant Expression in Human Liver and Kidney. The tissue distribution pattern of UK114 was examined by human Multiple Tissue Northern Blots (Clontech) and Northern blot analysis. As shown in Fig. 1, UK114 was predominantly expressed in adult liver and kidney. In addition, the expression level of UK114 was higher in adult liver and kidney when compared with similar fetal tissues. These results agree with the previous studies (17).

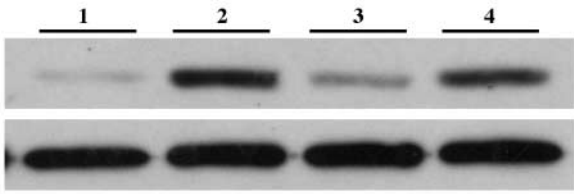
Down-Regulation of UK114 in Human Hepatocellular Carcinoma Tumor Tissues and Hepatoma Cell Lines. Previous studies have shown that UK114 was down-regulated in hepatic adenoma tissue and rat hepatoma cell dRLh 84–bearing tumor by immunostaining (17, 30). To further investigate the expression pattern of UK114 in human hepatocellular carcinoma and normal liver, hepatocellular carcinoma specimen and the adjacent normal tissues were examined by Northern blot analysis (Fig. 2*A*) and Western blot analysis (Fig. 2*C*). The polyclonal antibody against UK114 was prepared from purified overexpressed UK114 (as mentioned in Materials and Methods) and the specificity of this reagent is shown in Fig. 2*B*. The studies on six pairs of hepatocellular carcinoma tissues indicated that the expression of UK114 in the tumor tissue was significantly less than in the paired normal tissue at both RNA and protein levels. In all, we examined 22

Table 1. Correlation between histologic grade and UK114 expression in hepatocellular carcinoma specimens

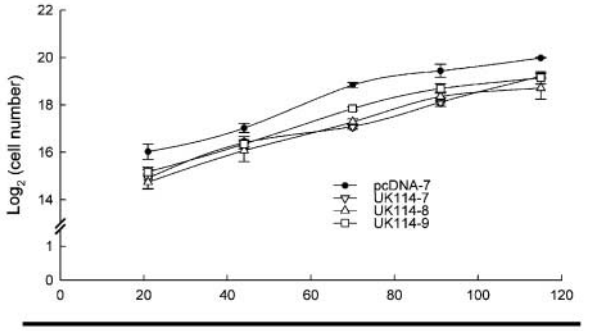
UK114 staining score	Nontumor, *n* (%)	Hepatocellular carcinoma specimens, *n* (%)			
		Grade 1	Grade 2	Grade 3	Grade 4
Class I: ++	73 (100.00)	1 (100.00)	173 (80.95)	23 (57.50)	0 (0.00)
Class I: +	0 (0.00)	0 (0.00)	3 (14.29)	11 (27.50)	1 (9.09)
Class I: ±/-	0 (0.00)	0 (0.00)	1 (4.76)	6 (15.00)	10 (90.91)
Total no. sections	73	1	21	40	11
Nontumor versus			$P < 0.005$	$P < 0.005$	$P < 0.005$
Grade 2 hepatocellular carcinoma versus				$P < 0.005$	$P < 0.005$
Grade 3 hepatocellular carcinoma versus					$P < 0.005$

NOTE: Based on the percentage of positively stained cells in hepatocellular carcinoma tumor tissues, the expression level of UK114 was categorized into three groups as follows: 1, class I staining (++): >20% of tumor cells showed a positive signal; 2, class II staining (+): <20% of tumor cells showed a positive signal; 3, class III staining (±/-): no tumor cells showed a positive signal. All together, 73 specimens were classified according to their differentiation grade by the Edmondson-Steiner method. This was paralleled by analysis of the expression level of UK114 in each sample. The statistical differences between the various grades in terms of UK114 expression was monitor by Pearson χ^2 test.

A



B



Clone	pcDNA-7	UK114-7	UK114-8	UK114-9
Doubling Time (hour±S.D)	22.63±1.35	22.71±1.66	23.02±0.63	22.91±0.92

C

HepG2	pcDNA-7	UK114-7	UK114-9
$1X10^6$	7/10	7/10	8/10
$1X10^6$	8/10	7/10	11/11
Total	15/20	14/20	19/21
pcDNA-7 versus		P>0.05	P>0.05

Figure 5. Effect of the UK114 on cellular proliferation and tumor formation. HepG2 cells were transfected with UK114 expression vector (pcDNA3.1/UK114) or control vector (pcDNA3.1) and selected with G418 for 3 weeks. **A.** Expression of UK114 protein in several transfectants was examined by Western blot analysis using a rabbit anti-UK114 polyclonal antibody (*top*). The level of β-actin was used as an internal control for the adjustment of protein loading (*bottom*). 1, pcDNA7; 2, UK114-7; 3, UK114-8; 4, UK114-9. **B.** Cellular proliferation assay. Cells were seeded in 24-well plates and the cell number was counted daily until day 5. The graph showed uses a logarithmic plot of cell number against time (*top*) and the cell doubling times were calculated based on these results (*bottom*). **C.** Tumor formation in nude mice. Cells were inoculated s.c. into the hind back of BALB/c nude mice. Tumors were observed weekly until 2 months. All transfectants exhibited a similar frequency of tumor formation *in vivo* and were not statistically different when analyzed by Fisher's exact test.

pairs of hepatocellular carcinoma specimen and found that there were 16 cases where the hepatocellular carcinoma tumor tissue (72.7%) showed down-regulation of UK114 expression in the tumor tissue. We also investigated the expression of UK114 in several human hepatoma cell lines, including HA22T, Mahlavu, Tong, SNU-387, HuH7, and HepG2. Similarly, all of the hepatoma cell lines expressed a reduced level of UK114 compared with normal liver tissue (Fig. 3). These results indicated that the expression of UK114 was significantly reduced in most hepatocellular carcinoma tumors and the various human hepatoma cell lines.

Correlation between UK114 Expression and the Differentiation Grade of Hepatocellular Carcinoma. In a previous study, rat hepatoma cell dRLh 84–bearing tumor have shown less differentiation and reduced expression of PSP (30) raises the possibility that expression of UK114 in liver tissues might be associated with the differentiation process. Currently, the Edmondson-Steiner classification is the most commonly used pathologic method to evaluate the differentiation grades of hepatocellular carcinoma, which is an important reference during the diagnosis, prognosis, and therapeutic treatment of this disease (8). To investigate whether the differentiation grade of hepatocellular carcinoma was related to the expression level of UK114, immunohistochemical analysis was done on paraffin-embedded hepatocellular carcinoma specimens. Among the 73 hepatocellular carcinoma specimens tested, there were only one case of grade 1 but 21 cases of grade 2, 40 cases of grade 3, and 11 cases of grade 4 hepatocellular carcinoma.

All of these specimens were accompanied by their adjacent normal tissue, which was used as the positive control for UK114 staining. The expression level of UK114 was categorized into three groups as follows: class I staining (++) where there was >20% of the tumor cells showed a positive signal; class II staining (+) where <20% of the tumor cells showed a positive signal; and class III staining (±/-) where none of the tumor cells showed a positive signal. Representative staining images are displayed in Fig. 4 and all results are summarized in Table 1. We found that expression of UK114 was reduced in most neoplastic sections. Furthermore, a gradual decrease in the expression of UK114 was observed as one moved from the well-differentiated types of hepatocellular carcinoma toward the poorly differentiated types of hepatocellular carcinoma. For grade 2 hepatocellular carcinoma, 80.95% (17 of 21) of cases had class I staining for UK114 expression, but only 19.05% (4 of 21) of cases showed class II and III staining. For grade 3 hepatocellular carcinoma, 42.5% (17 of 40) of the cases had class II and III staining. For grade 4 hepatocellular carcinoma, 100% (11 of 11) of the cases had class II and III staining.

However, there was only one case of grade 1 hepatocellular carcinoma in our specimens; therefore, it was not possible to perform a valid statistical analysis using the grade 1 hepatocellular carcinoma data. The difference in UK114 expression between normal liver and grade 2, 3, and 4 hepatocellular carcinoma was highly statistically significant ($P < 0.005$). These results indicated that grade 2, 3, and 4 hepatocellular carcinoma clearly show reduced expression of UK114 compared with normal liver tissues. Interestingly, statistical analysis also revealed that the down-regulation of UK114 was more significant in grade 4 versus 3 ($P < 0.001$) and grade 4 versus 2 hepatocellular carcinoma ($P < 0.001$). However, the decreased expression of UK114 was not statistical significant between grade 2 and 3 hepatocellular carcinoma ($P > 0.05$). The reason for this may rest in the fact that grade 2 and 3 hepatocellular carcinoma are sometimes difficult to differentiate and a gray zone existed between these two grades; many hepatocellular

carcinoma tumors are classified as somewhere between grade 2 and 3 according to Edmonson-Steiner classification. Taken together, these results indicated that expression level of UK114 was related to the differentiation grade of hepatocellular carcinoma.

Effect of the UK114 on Transformation Activity. Due to the high degree of evolutionary conservation of UK114 and the down-regulation of UK114 in hepatocellular carcinoma tumors, this raised the possibility that UK114 might serve as a tumor suppressor gene and be involved in the suppression of tumorigenicity. To examine the effect of UK114 on transformation activity, HepG2 cells were transfected with a eukaryotic expression plasmid containing UK114 (pcDNA3.1/UK114) or the empty control vector (pcDNA3.1) and selected by G418 treatment for 3 weeks. Stable transfectants were obtained and the expression level of UK114 was examined. Western blot analysis indicated that the amount of UK114 increased in four of the UK114-stable clones compared with the control groups (Fig. 5*A*). Among these clones, UK114-7 and UK114-9 expressed a high level of UK114; in contrast, clone UK114-8 expressed only a moderate level of UK114. The transfectants UK114-7, UK114-8, and UK114-9 were then used to examine the effect of UK114 on cellular proliferation *in vitro* and on tumorigenicity in nude mice *in vivo*.

As shown in Fig. 5*B*, the growth curve and cell doubling time did not show any significant difference between the UK114 expression clones and the vector-alone clones. Furthermore, there was no statistically significant difference in the tumorigenicity when the clones with a high expression of UK114 were used *in vivo* to study tumorigenicity (Fig. 5*C*). Similar experiments were done with HuH7 cells and this also showed that UK114 did not influence the tumorigenicity (data not shown). These results suggested that overexpression of UK114 alone was not sufficient to suppress the transformation activity of human hepatoma cells.

Discussion

In this study, we have found that UK114 was predominantly expressed in human adult liver and kidney and this agrees with previous reports. Interestingly, we also showed that both UK114 mRNA and its protein product were markedly reduced in the hepatocellular carcinoma tumor tissues and in human hepatoma cell lines compared with the normal liver tissues. Apparently, expression of UK114 is suppressed through transcriptional or post-transcriptional regulation because both the levels of the mRNA and protein were decreased in the cancer cells. The genomic structure, including coding sequence and promoter sequence of UK114, were not altered in these hepatocellular carcinoma samples (data not shown). It is possible that the down-regulation of UK114 during hepatocellular carcinoma carcinogenesis is mediated through epigenetic transcriptional repression similar to the DNA methylation of the promoter region of the human p14.5 gene during cellular differentiation from monocytes to macrophages (31).

The reduced expression of UK114 in hepatocellular carcinoma raised the possibility that UK114 may play a role as a tumor suppressor gene during hepatocellular carcinoma carcinogenesis. Our results did not support this and we found that overexpression of UK114 in HepG2 cells was not able to inhibit cell growth. These results are different from a previous report, in which overexpression of PSP reduced cell proliferation of the normal rat kidney cell line NRK-52E and the rat hepatoma cell line dRLh-84 (22). The reasons for the different results are not clear at present and require further investigation. Furthermore, overexpression of UK114 was not able to reduce the tumorigenicity of the HepG2 and HuH7 cell lines in nude mice. Taken together, these results strongly suggest that UK114 does not function as a tumor suppressor gene during hepatocellular carcinoma carcinogenesis.

This interpretation was also supported by our observation that most of the grade 1/2 hepatocellular carcinoma and 57% of grade 3 hepatocellular carcinoma still expressed UK114 at high levels. Furthermore, to examine in a broader context whether UK114 functions during the process of invasion, we have also measured the effect of UK114 on the invasion activity of human hepatoma cells. However, there is no significant difference between the UK114-expressing cells and the mock control cells when this cell line is used (data not shown). These results suggest that UK114 may not involve in this process.

The reduced expression of UK114 in hepatocellular carcinoma tissue and cell lines might also be related to the differentiation status of hepatocellular carcinoma. In this study, we showed that the expression of UK114 in hepatocellular carcinoma tissues is correlated with hepatocellular carcinoma pathologic grades as measured by the Edmondson-Steiner classification. Significant differences in the expression of UK114 were observed when grade 2, 3, and 4 hepatocellular carcinoma were compared with normal tissue. These results agree with previous studies on other system, which reported that the appearance of UK114 is related to the differentiation process in monocytes and in rat hepatoma cell lines (17).

To improve the diagnostic accuracy of hepatocellular carcinoma, several studies have tried to identify biomarkers for the different pathologic grades of hepatocellular carcinoma. The increased expression of transforming growth factor-α and its receptor and the epidermal growth factor receptor, as measured by immunostaining, have frequently been observed in more differentiated hepatocellular carcinoma cells. However, increased expression of transforming growth factor-α and epidermal growth factor receptor can also be detected in the adjacent nontumorous livers (32). Similarly, α-fetoprotein expression is also increased in hepatocellular carcinoma and may serve as a diagnostic marker; however, it was not a sensitive biomarker for the histologic grading of hepatocellular carcinoma (33). Altered expression of pRB was found to be statistically different in grade 2 versus grade 3 and 4 hepatocellular carcinoma combined (34). Immunostaining of proliferating cell nuclear antigen revealed that its expression in poorly differentiated hepatocellular carcinoma was significantly higher than the well-differentiated and moderately differentiated hepatocellular carcinoma (35).

Recently, a new biomarker, dehydroepiandrosterone sulfotransferase (SULT2A1) was found to exhibit reduced expression in hepatocellular carcinoma. The down-regulation of SULT2A1 was more frequent in

grade 3 versus 1 hepatocellular carcinoma (36). According to our data, the reduced expression of UK114 appears to be better correlated with the histologic grades of hepatocellular carcinoma because the frequency of down-regulation of UK114 is significantly different between grade 2 and 4 hepatocellular carcinoma and grade 3 and 4 hepatocellular carcinoma. Down-regulation of UK114 alone is not sufficient to be used as a sole reference for grading hepatocellular carcinoma because the present criteria of hepatocellular carcinoma grading by a pathologist include nuclear cytoplasmic ratio, cytoplasmic eosinophilia, bile secretion, nuclear chromatism, histologic structure, and cohesiveness of the tumor cells. However, our results suggest that reduced expression of UK114 may be used as a marker to assist pathologists when grading hepatocellular carcinoma.

In summary, our current observation provides evidence that UK114 is down-regulated in hepatocellular carcinoma. Functionally, UK114 does not seem to serve as a tumor suppressor gene during hepatocellular carcinoma carcinogenesis. However, the down-regulation of UK114 correlates well with hepatocellular carcinoma pathologic status and therefore may have a useful role as a biomarker when grading hepatocellular carcinoma.

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References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
2. Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. Gastroenterology 2004;127:S5–16.
3. Chen CJ, Yu MW, Liaw YF. Epidemiological characteristics and risk factors of hepatocellular carcinoma. J Gastroenterol Hepatol 1997;12:S294–308.
4. Nagasue N, Kohno H, Chang YC, et al. Liver resection for hepatocellular carcinoma. Results of 229 consecutive patients during 11 years. Ann Surg 1993;217:375–84.
5. Yamamoto J, Kosuge T, Takayama T, et al. Recurrence of hepatocellular carcinoma after surgery. Br J Surg 1996;83:1219–22.
6. Simonetti RG, Liberati A, Angiolini C, Pagliaro L. Treatment of hepatocellular carcinoma: a systematic review of randomized controlled trials. Ann Oncol 1997;8:117–36.
7. Camma C, Schepis F, Orlando A, et al. Transarterial chemoembolization for unresectable hepatocellular carcinoma: meta-analysis of randomized controlled trials. Radiology 2002;224:47–54.
8. Edmondson HA, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. Cancer 1954;7:462–503.
9. Ceciliani F, Faotto L, Negri A, et al. The primary structure of UK114 tumor antigen. FEBS Lett 1996;393:147–50.
10. Levy-Favatier F, Cuisset L, Nedelec B, Tichonicky L, Kruh J, Delpech M. Characterization, purification and cDNA cloning of a rat perchloric-acid-soluble 23-kDa protein present only in liver and kidney. Eur J Biochem 1993;212:665–73.
11. Asagi K, Oka T, Arao K, et al. Purification, characterization and differentiation-dependent expression of a perchloric acid soluble protein from rat kidney. Nephron 1998;79:80–90.
12. Robello C, Dallagiovanna B, Engel JC, Gamarro F, Castanys S. A new member of YER057c family in *Trypanosoma cruzi* is adjacent to an ABC-transporter. Gene 1998;220:1–12.
13. Kim JM, Yoshikawa H, Shirahige K. A member of the YER057c/yjgf/Uk114 family links isoleucine biosynthesis and intact mitochondria maintenance in *Saccharomyces cerevisiae*. Genes Cells 2001;6:507–17.
14. Leitner-Dagan Y, Ovadis M, Zuker A, et al. CHRD, a plant member of the evolutionarily conserved YjgF family, influences photosynthesis and chromoplastogenesis. Planta 2006;225:89–102.
15. Morishita R, Kawagoshi A, Sawasaki T, et al. Ribonuclease activity of rat liver perchloric acid-soluble protein, a potent inhibitor of protein synthesis. J Biol Chem 1999;274:20688–92.
16. Melloni E, Michetti M, Salamino F, Pontremoli S. Molecular and functional properties of a calpain activator protein specific for μ-isoforms. J Biol Chem 1998;273:12827–31.
17. Schmiedeknecht G, Kerkhoff C, Orso E, et al. Isolation and characterization of a 14.5-kDa trichloroacetic-acid-soluble translational inhibitor protein from human monocytes that is upregulated upon cellular differentiation. Eur J Biochem 1996;242:339–51.
18. Suzuki K, Nishida T, Matsumoto M, Kanouchi H, Kaneki K, Oka T. Purification, characterization and developmental expression of rat brain PSP protein. Biochim Biophys Acta 2001;1527:47–53.
19. Kaneki K, Matsumoto M, Suzuki K, Akuzawa M, Oka T. Purification, characterization and developmental expression of pig liver PSP. Comp Biochem Physiol B Biochem Mol Biol 2003;134:571–8.
20. Nordin H, Matsumoto M, Suzuki K, et al. Purification, characterization and developmental expression of chick (*Gallus domesticus*) liver PSP protein. Comp Biochem Physiol B Biochem Mol Biol 2001;128:135–43.
21. Kanouchi H, Oka T, Asagi K, Tachibana H, Yamada K. Expression and cellular distribution of perchloric acid-soluble protein is dependent on the cell-proliferating states of NRK-52E cells. Cell Mol Life Sci 2000;57:1103–8.
22. Kanouchi H, Tachibana H, Oka T, Yamada K. Recombinant expression of perchloric acid-soluble protein reduces cell proliferation. Cell Mol Life Sci 2001;58:1340–3.
23. Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res 1982;42:3858–63.
24. Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980;209:497–9.
25. Chang C, Lin Y, TW OL, et al. Induction of plasma protein secretion in a newly established human hepatoma cell line. Mol Cell Biol 1983;3:1133–7.
26. Oefinger PE, Bronson DL, Dreesman GR. Induction of hepatitis B surface antigen in human hepatoma-derived cell lines. J Gen Virol 1981;53:105–13.
27. Stevenson D, Lin JH, Tong MJ, Marshall GJ. Characteristics of a cell line (Tong/HCC) established from a human hepatocellular carcinoma. Hepatology 1987;7:1291–5.
28. Park JG, Lee JH, Kang MS, et al. Characterization of cell lines established from human hepatocellular carcinoma. Int J Cancer 1995;62:276–82.
29. Chou YC, Jeng KS, Chen ML, et al. Evaluation of transcriptional efficiency of hepatitis B virus covalently closed circular DNA by reverse transcription-PCR combined with the restriction enzyme digestion method. J Virol 2005;79:1813–23.
30. Kaneki K, Kanouchi H, Matsumoto M, Kawasaki Y, Akuzawa M, Oka T. Down regulation of a novel protein, PSP, in rat hepatoma cdRLh 84-beared tumor. J Vet Med Sci 2003;65:781–5.
31. Pozdniakovaite N, Popendikyte V. DNA methylation differences in human p14.5 gene promoter region in normal and proliferating cells. Dev Growth Differ 2005;47:493–9.
32. Morimitsu Y, Hsia CC, Kojiro M, Tabor E. Nodules of less-differentiated tumor within or adjacent to hepatocellular carcinoma: relative expression of transforming growth factor-α and its receptor in the different areas of tumor. Hum Pathol 1995;26:1126–32.
33. Brumm C, Schulze C, Charels K, Morohoshi T, Kloppel G. The significance of α-fetoprotein and other tumour markers in differential immunocytochemistry of primary liver tumours. Histopathology 1989;14:503–13.
34. Hsia CC, Di Bisceglie AM, Kleiner DE, Jr., Farshid M, Tabor E. RB tumor suppressor gene expression in hepatocellular carcinomas from patients infected with the hepatitis B virus. J Med Virol 1994;44:67–73.
35. Saegusa M, Takano Y, Kishimoto H, Wakabayashi G, Nohga K, Okudaira M. Comparative analysis of p53 and c-myc expression and cell proliferation in human hepatocellular carcinomas—an enhanced immunohistochemical approach. J Cancer Res Clin Oncol 1993;119:737–44.
36. Huang LR, Coughtrie MW, Hsu HC. Down-regulation of dehydroepiandrosterone sulfotransferase gene in human hepatocellular carcinoma. Mol Cell Endocrinol 2005;231:87–94.