Expression Profiling in Multistage Hepatocarcinogenesis: Identification of HSP70 as a Molecular Marker of Early Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) associated with chronic liver disease evolves from precancerous lesions and early HCC to a progressed form. Nodule-in-nodule–type HCC (progressed HCC within early HCC) represents the transition from early to progressed HCC and, therefore, is useful in molecular genetic analysis of HCC progression during multistage carcinogenesis. We compared expression profiles among 7 early components and 7 progressed components of nodule-in-nodule–type HCCs and their corresponding noncancerous liver tissues with oligonucleotide array. Of the approximately 12,600 genes that were analyzed, a set of 95 genes provided a molecular signature that distinguished between early HCC components and their noncancerous liver tissues, and a set of 92 genes distinguished between progressed and early HCC components. Of these genes, the most abundantly up-regulated gene in early HCC components ($P < .001$) was heat-shock protein 70 (HSP70). Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed this finding. Further immunohistochemical examination of HSP70 revealed its significant overexpression in early HCC compared with precancerous lesions ($P < .001$) and in progressed HCC compared with early HCC ($P < .001$). In conclusion, molecular signatures were clearly different in noncancerous liver tissue as compared with the early and progressed components of nodule-in-nodule–type HCC. Moreover, HSP70 could be a sensitive marker for the differential diagnosis of early HCC from precancerous lesion or noncancerous liver, a difficult distinction for pathologists due to very well differentiated histology with little atypia in early HCC. (HEPATOLOGY 2003;37:198-207.)

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and one of the leading causes of cancer death in Japan.[1] Despite remarkable advances in diagnostic and therapeutic techniques, the incidence of HCC is still on the increase. HCC occurs mainly in livers that are chronically diseased as a result of hepatitis virus infection.[2] Progression often leads to vascular invasion and intrahepatic metastasis. As is known for other cancers,[3] HCC is also characterized by an obvious multistage process of tumor progression. Histopathologic and molecular biological studies have revealed the multistep development of human HCCs.[4-7] It has been shown that small nodular hypercellular lesions known as adenomatous hyperplasia (AH) or atypical AH (AAH: AH with focally increased atypia but too indefinite for a diagnosis of HCC) appear in damaged liver infected with hepatitis virus B or C. These lesions develop into early HCC, which corresponds to *in situ* or microinvasive carcinoma, in which the portal tracts within the nodule are preserved, and then into progressed HCC through the stage of nodule-in-nodule–type HCC (progressed HCC within early HCC), which indicates a transition from early to progressed HCC. These pathologic findings are also supported by radiologic findings.[8,9]

Abbreviations: HCC, hepatocellular carcinoma; AH, adenomatous hyperplasia; AAH, atypical adenomatous hyperplasia; HSP70, heat-shock protein 70; HCV, hepatitis C virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction; mRNA, messenger RNA; HBV, hepatitis B virus.

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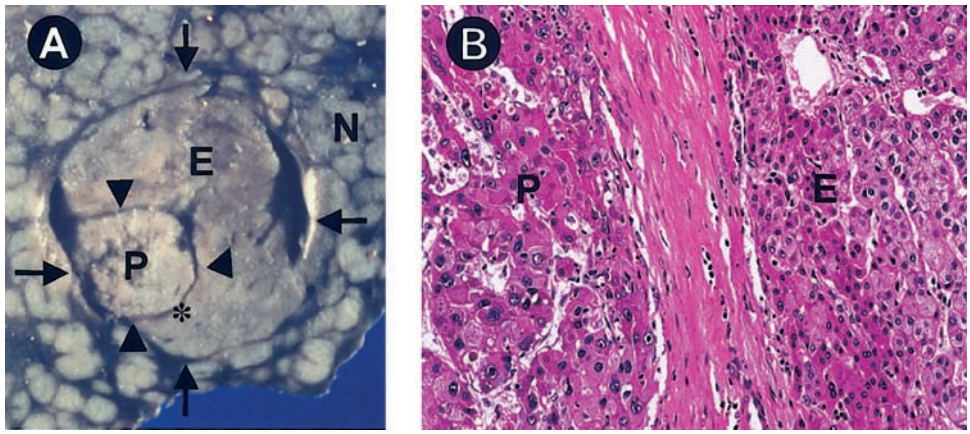

Fig. 1. Nodule-in-nodule–type HCC. (A) Grossly, a small nodular mass (P, progressed component) is seen inside an early HCC (E, early component) appearing as a nodule-in-nodule lesion. N, noncancerous liver. Arrows and arrowheads indicate borders between early component and noncancerous liver, and between progressed component and early component, respectively. (B) Histology of the border between progressed component and early component shown by the asterisk in (A). The progressed component (P), composed of Edmondson grade II-III HCC, shows expansive growth against the surrounding early component (E), composed of grade I HCC. Fibrous septa are seen between the 2 components. (Hematoxylin-eosin stain; original magnification, ×20.)

On the basis of this current knowledge of multistage hepatocarcinogenesis, high-risk patients are closely followed up, and increasing numbers of small equivocal lesions are detected by imaging diagnosis. Ultrasound-guided needle biopsy is performed on such lesions, and if they are diagnosed histologically as cancer they are treated. However, as is the case with other early cancers, such as those of the lung,[10] stomach,[11] and colon,[12] early HCC shows minimum atypia, and lacks definite invasive or destructive growth.[6] Therefore, it is often difficult, even for the hepatopathologist, to distinguish regenerative nodules, precancerous lesions, and early HCC. For these reasons, the discovery of an objective molecular marker that will help to standardize histologic diagnosis of early HCC and lead to appropriate treatment is eagerly anticipated.

In addition to this diagnostic problem, the molecular mechanisms of hepatocarcinogenesis are far from clear. A molecular understanding of multistep hepatocarcinogenesis is an important step toward the identification of additional biomarkers and new therapeutic targets with increased specificity for HCC development. The establishment of microarray methods for large-scale analysis of gene expression[13,14] has made it possible to seek molecular markers for cancer classification and outcome prediction and to identify molecules involved in carcinogenesis in a variety of tumor types.[15-17] We compared expression profiles among 7 early components and 7 progressed components of nodule-in-nodule–type HCCs and their corresponding noncancerous liver tissues with oligonucleotide array to identify genes generally involved in multistep hepatocarcinogenesis. We showed that molecular signatures distinguished between early HCC components and their noncancerous liver tissues and between progressed and early HCC components. We also found that heat-shock protein 70 (HSP70) is involved in multistep hepatocarcinogenesis and could be a sensitive marker for the differential diagnosis of early HCC from precancerous lesions or from noncancerous liver tissues.

Materials and Methods

Tissue Samples. Seven pairs of nodule-in-nodule–type HCCs and corresponding noncancerous liver tissues were obtained from patients who underwent surgical resection at the National Cancer Center Hospital, Japan. For each nodule-in-nodule–type HCC, the early components, in which the underlying liver structure within the nodule was well preserved, and the progressed components, which showed definite destructive or expansive growth (Fig. 1), were separated macroscopically. This classification was confirmed histologically by 2 pathologists. Specimens were immediately cut into small pieces, snap-frozen in liquid nitrogen, and stored until use. Histologically, early components were all well differentiated HCCs and progressed components were 2 well, and 5 moderately differentiated HCCs. Serologically, 1 case was positive for hepatitis-B-surface-antigen, 5 cases were hepatitis C virus (HCV)–positive, and 1 was negative for both. For immunohistochemical analysis, 18 AHs, 15 AAHs, 63 early HCCs, 41 nodule-in-nodule–type HCCs, and 78 progressed HCCs were analyzed. Sections were prepared from formalin-fixed, paraffin-embedded tissues of samples resected surgically between 1988 and 2002. Histologic diagnosis was made according to the World Health Organization criteria.[7,18]

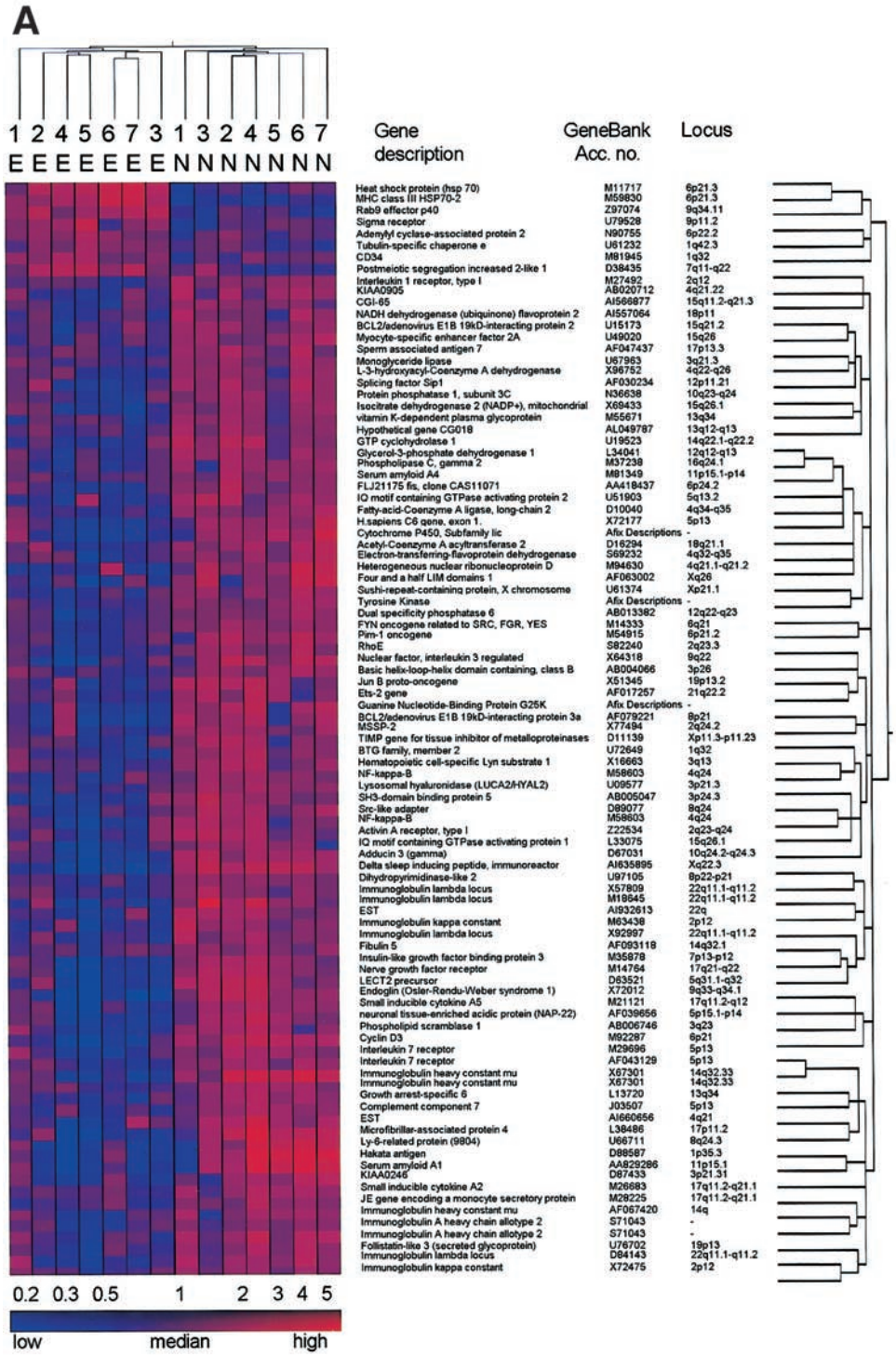

Fig. 2. A 2-way hierarchical clustering algorithm. Cluster map and phylogenetic tree resulting from a 2-way, pairwise, average-linkage cluster analysis. Each color patch in the resulting visual map represents the expression level of the associated gene in that tissue sample, with a continuum of expression levels from blue (lowest) to bright red (highest). A 2-way hierarchical clustering algorithm successfully distinguished between early HCC components and their noncancerous liver tissues (A), and between progressed and early HCC components (B). The scale bar reflects the fold increase (red) or decrease (blue) for any given gene relative to the median level of expression across all samples.

RNA Preparation and Oligonucleotide Array. Total RNA was extracted from the bulk tissues with Trizol reagent (Invitrogen Corp., Carlsbad, CA). Biotin-labeled complementary RNA was synthesized from 10 μg of total RNA derived from each sample using a Super Script Choice System (Gibco-BRL, Rockville, MD) and BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) accord-

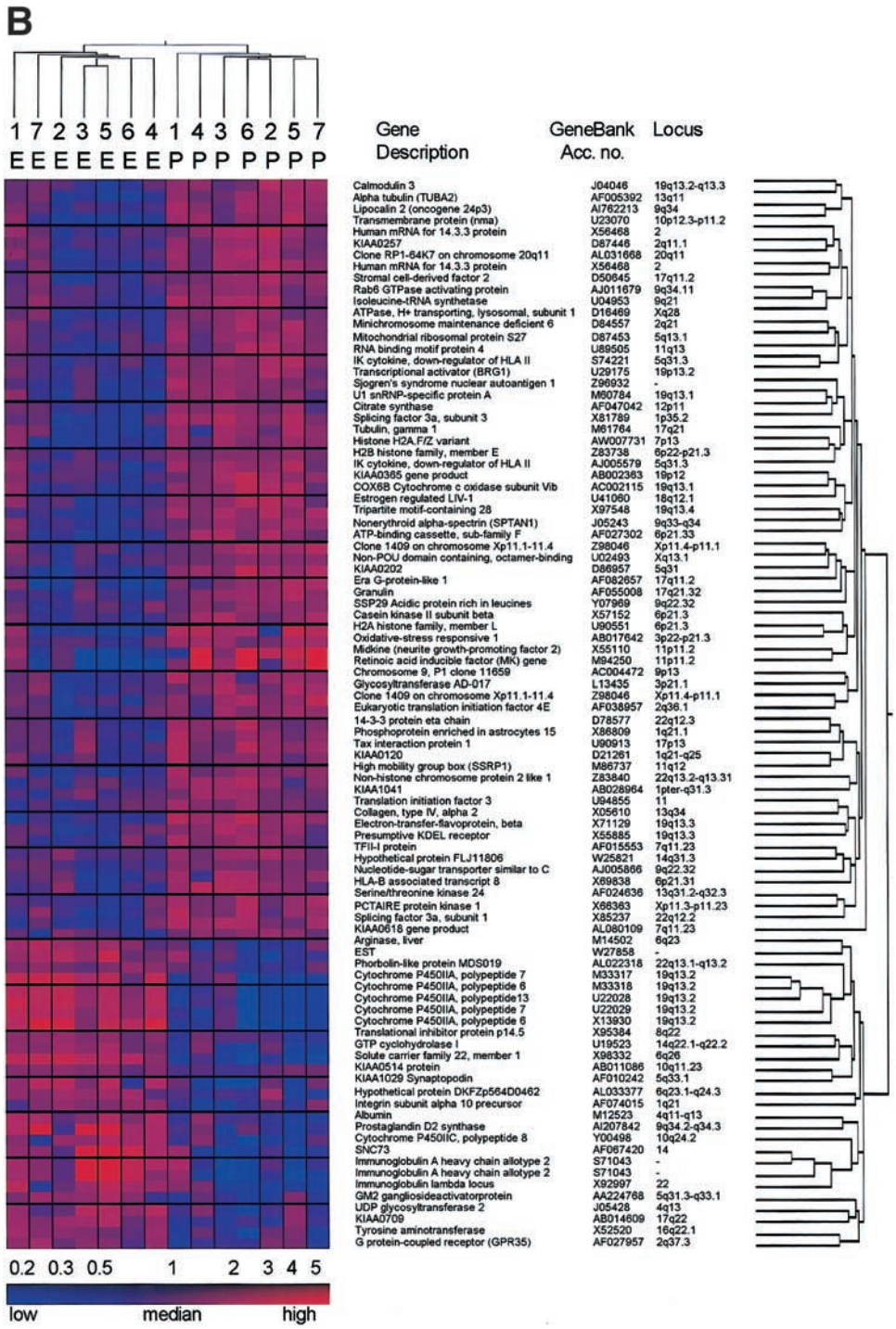

Fig. 2. (Cont'd.)

ing to the manufacturers' instructions. Hybridization of each complementary RNA to the probe array, HG-U95Av2 (Affymetrix, Santa Clara, CA), and detection of the signals were performed as instructed by the manufacturer.

Data Analysis. Using the Microarray Suite 4.0 software package (Affymetrix), the hybridization intensity data were normalized to 1,000 of total signal intensities of each array and transformed for each probe set into average difference representing the mean difference of signal intensity between the match and mismatch probe pairs and, thus, the expression level of the target gene. Two-way hierarchical clustering algorithm[19] of the genes and individual samples was performed by the software, GeneSpring (Silicon Genetics, Redwood City, CA), to arrive at a Pearson correlation coefficient .[19]

Real-Time Quantitative RT-PCR Analysis. For reverse transcription polymerase chain reaction (RT-PCR) analysis, all RNA samples were treated with DNase I (Promega Corp., Madison, WI) to remove genomic DNA. Real-time quantitative RT-PCR analysis was performed as reported previously.[20] The primer set 5′-AGGCCGACAAGAAGAAGGTGCT-3′ (forward) and 5′-TGGTACAGTCCGCTGATGATGG-3′ (reverse) was designed against the 3′ untranslated region of HSP70. For standardization of the amount of RNA, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample was quantified by using the primer set 5′-GAAGGTGAAGGTCGGAGTC-3′ (forward) and 5′-CCCGAATCACATTCTCCAAGAA-3′ (reverse). All PCR reactions were performed with the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems, Foster City, CA) under the following conditions: 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Real-time detection of the emission intensity of SYBR Green was performed with an ABI prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems), as reported previously.[21] Quantitative RT-PCR was performed at least 3 times, including a no-template control as a negative control. Statistical analyses were performed by the paired *t* test.

Immunohistochemistry. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections by an immunoperoxidase method, as described previously.[22] Briefly, each section was deparaffinized, rehydrated, and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature and then washed in phosphate-buffered saline. Normal swine serum (DAKO, Glostrup, Denmark) was applied for 30 minutes and removed. The sections were incubated with mouse monoclonal antibodies for HSP70 (SC-24; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500 overnight at 4°C, washed 3 times in phosphate-buffered saline, and incubated with secondary antibody for 30 minutes at room temperature. For detection, a Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, CA) was used.

Staining Evaluation. Staining was evaluated by 2 independent observers. Equal or more intense nuclear and cytoplasmic staining compared with bile duct was considered positive. The positivity index was expressed as the percentage of positive cells in each lesion. Statistical analyses were performed by the unpaired *t* test.

Results

Two-Way Hierarchical Clustering Algorithm. To identify genes generally involved in multistep hepatocarcinogenesis, we compared expression profiles between 7 early HCC components and their noncancerous liver counterparts and between 7 progressed and 7 early HCC components by using oligonucleotide array. We filtered all genes, with the following limits: 1. presence (*i.e.,* exactly expressed in the sample); 2. average difference of more than 1,000 in at least 7 of 14 samples in each of the 2 groups; 3. Mann-Whitney *U* test with significance set at $P < .05$ to identify genes expressed differently between the 2 groups. In the 95 and 92 genes selected under the above criteria, a 2-way hierarchical clustering algorithm successfully distinguished between early components and their noncancerous liver tissues (Fig. 2A), and between progressed components and early components (Fig. 2B).

Next, we listed genes that met an additional limit: 4. a more than 2- fold increase of average difference between 2 groups, to identify genes with more significant difference of expression in noncancerous liver tissues, early components, or progressed components (Tables 1 and 2). Six genes were up-regulated and 18 genes were down-regulated in early components compared with in noncancerous liver tissues (Table 1). Thirteen genes were up-regulated and 15 were down-regulated in progressed components compared with in early components (Table 2).

HSP70 mRNA Expression in HCC. Of the genes listed in Table 1, the most abundantly up-regulated gene in early components (Mann-Whitney *U* test, $P < .001$) was HSP70. To confirm our findings, we next analyzed the level of HSP70 messenger RNA (mRNA) by real-time quantitative RT-PCR. In 5 of 7 cases, the expression level in the early component was up-regulated compared with that in the corresponding noncancerous liver tissue, and in 3 of 7 cases, the expression level in the progressed component was up-regulated compared with that in the corresponding early component (Fig. 3A). The average level of expression of HSP70 mRNA in each component of nodule-in-nodule–type HCC was up-regulated in a stepwise manner: noncancerous liver (0.16 ± 0.09) versus early component (0.30 ± 0.18), $P = .018$; early component versus progressed component (0.48 ± 0.29), $P = .056$ (Fig. 3B).

Protein Expression of HSP70. To determine whether HSP70 is also overexpressed at the protein level and increasingly expressed according to the stepwise progression of hepatocarcinogenesis, we employed an antibody against HSP70 in an immunohistochemical study (Fig. 4) and counted the percentage of positive cells in each lesion (Fig. 5). Hepatocytes in noncancerous liver tissue with chronic hepatitis or cirrhosis showed no immunostaining or only focal and faint staining in the nucleus. However, the bile duct epithelium always stained strongly and thus served as an internal control of positive

Table 1. Genes Up-regulated and Down-regulated in Early Components of "Nodule-in-Nodule"–Type HCC

GeneBank Acc. No.	Gene Symbol	Gene Description	
Up-regulated			Early/Noncancer
Stress response			
M59830	MHHSP2	MHC class III HSP70-2	3.7
M11717	HSP70D	Heat-shock protein (HSP70)	3.0
Signal transduction			
N90755	CAP2	Adenylyl cyclase-associated protein 2	2.5
U79528	SR-BP1	Sigma receptor	2.0
Z97074	RAB9P40	Rab9 effector p40	2.4
DNA repair			
D38435	PMS2L1	Postmeiotic segregation increased 2-like 1	2.1
Down-regulated			Noncancer/Early
Immune system			
X67301	IGHM	Immunoglobulin heavy constant mu	8.5
U66711		Ly-6-related protein (9804) gene	4.8
S71043	Ig alpha 2	Immunoglobulin A heavy chain allotype 2	4.0
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D84143	IGL	Immunoglobulin lambda locus	3.9
AF067420	IGHM	Immunoglobulin heavy constant mu	3.6
J03507	C7	Complement component 7	3.3
X72475	IGKC	Immunoglobulin kappa constant	3.1
X67301	IGHM	Immunoglobulin heavy constant mu	2.8
M28225	JE	JE gene encoding a monocyte secretory protein	2.7
X92997	IGL	Immunoglobulin lambda locus	2.3
M26683	SCYA2	Small inducible cytokine A2	2.2
M63438	IGKC	Immunoglobulin kappa constant	2.1
ECM and cell adhesion			
L38486	MFAP4	Microfibrillar-associated protein 4	5.0
D88587	FCN3	Hakata antigen	5.4
Amyloid related			
AA829286	SAA1	Serum amyloid A1	5.5
Unknown function			
A1660656		EST	4.1
D87433		K1AA0246	2.5

staining (Fig. 4B and D). Some sinusoidal cells also showed moderate staining. All cases of AH were negative for HSP70 (Fig. 4B), with the exception of 1 case that showed 5% positive staining. Sixty percent positivity was observed in one case of AAH, and 20% in another. In contrast, we detected immunoreactivity ranging up to 80% in most cases of early HCC (Fig. 4D) and in early components of nodule-in-nodule–type HCC (Fig. 4F). The progressed components and progressed HCCs showed more widespread staining than the early components (Fig. 4F). In progressed HCC, no specific correlation was found between immunostaining for HSP70 and clinicopathologic features such as differentiation or presence of vascular invasion (data not shown). As shown in Fig. 4, strong immunoreactivity was observed in both the nucleus and cytoplasm, and, in most cases, the intensity of staining of the cytoplasm corresponded to that of the nucleus. The percentage of positive cells increased gradually according to the stepwise progression of hepatocarcinogenesis, and was significantly different between the precancerous lesions AH + AAH (3.49 ± 10.9) and early HCC (32.9 ± 24.3) ($P < .001$), and between early HCC and progressed HCC (60.8 ± 24.6) ($P < .001$).

Discussion

The recent development of complementary DNA microarray or oligonucleotide array technology, a high-throughput method of monitoring gene expression, has made it possible to analyze the expression of thousands of genes at once.[13,14] Consequently, new classifications of cancers now can be proposed on the basis of the altered expression of multiple genes in tumor tissues.[15-17,23] For example, molecularly distinct subtypes, with significant differences in clinical behavior, have been recognized on the basis of differences in gene expression patterns for morphologically indistinguishable diffuse large B cell lymphoma.[15] To elucidate the characteristic changes associated with multistep hepatocarcinogenesis, we globally analyzed the gene expression of 7 early components and 7 progressed components of nodule-in-nodule–type HCC representing a transition from early to progressed HCC and their corresponding noncancerous liver tissues. We

Table 2. Genes Up-regulated and Down-regulated in Progressed Components of "Nodule-in-Nodule"–Type HCC

GeneBank Acc. No.	Gene Symbol	Gene Description	
Up-regulated			Progressed/Early
Growth factor			
M94250	MKXX	Retinoic acid inducible factor (MK) gene	9.8
X55110	MDK	Midkine (neurite growth-promoting factor 2)	6.5
AF055008	GRN	Granulin	2.4
Transcription			
U90551	H2AFL	H2A histone family, member L	3.2
Z83738	H2BFE	H2B histone family, member E	2.1
X97548	TRIM28	Tripartite motif-containing 28	2.0
Translation			
AF027302	ABCF1	ATP-binding cassette, sub-family F	2.0
Tumor associated			
AI762213	LCN2	Lipocalin 2 (oncogene 24p3)	2.8
U23070	NMA	Transmembrane protein (nma)	2.2
DNA maintenance			
D84557	MCM6	Minichromosome maintenance deficient 6	2.2
ECM			
X05610	COL4A2	Collagen, type IV, alpha 2	2.0
Metabolism			
AF082657	ERAL1	Era G-protein-like 1	2.4
Unknown function			
D86957		KIAA0202	2.5
Down-regulated			Early/Progressed
Detoxification			
X13930	CYP2A6	Cytochrome P450IIA, polypeptide 6	7.4
U22029	CYP2A7	Cytochrome P450IIA, polypeptide 7	4.7
M33317	CYP2A7	Cytochrome P450IIA, polypeptide 7	4.6
M33318	CYP2A6	Cytochrome P450IIA, polypeptide 6	4.2
U22028	CYP2A13	Cytochrome P450IIA, polypeptide 13	3.8
Y00498	CYP2C8	Cytochrome P450IIA, polypeptide 8	2.2
Immune system			
S71043	Ig alpha 2	Immunoglobulin A heavy chain allotype 2	7.4
S71043	Ig alpha 2	Immunoglobulin A heavy chain allotype 2	5.4
AF067420	IGHM	Immunoglobulin heavy constant mu	5.4
X92997	IGL	Immunoglobulin lambda locus	2.7
Metabolism			
J05428	UGT2B9	UDP glycosyltransferase 2	2.0
M14502	ARG1	Arginase, liver	2.0
AI207842	PTGDS	Prostaglandin D2 synthase	8.9
Liver specific			
U22961		Albumin	2.0
Unknown function			
W27858		EST	2.2

showed that a 2-way hierarchical clustering algorithm successfully distinguished between noncancerous liver tissues, early components, and progressed components. These results indicate that there is a clear difference in molecular signatures between each step in the progression of HCC. Our present approach was able to show genes involved in early hepatocarcinogenesis commonly seen in HCV-related cases. Another approach for revealing differences between hepatitis B virus (HBV)- and HCV-related hepatocarcinogenesis is expected. We further showed that HSP70 could be a sensitive marker for the differential diagnosis of early HCC from precancerous lesions or noncancerous liver tissue, a diagnosis that is difficult even for pathologists because of the very well differentiated histology with minimum atypia seen in early HCC. Although early HCC is defined on the basis of histopathologic[4-7] and clinical studies,[8,9] the molecular changes that occur in early HCC are not well understood. Early HCC is characterized by an increase of cell density and growth,[6] but no positive molecular marker has yet been identified. The situation is the same in other types of early cancer, and this is the first example that shows that gene expression profiling is a promising method for finding molecular markers useful for the diagnosis of early cancer. We expect that similar efforts would facilitate standardization of histologic diagnosis of early cancer in many organs.

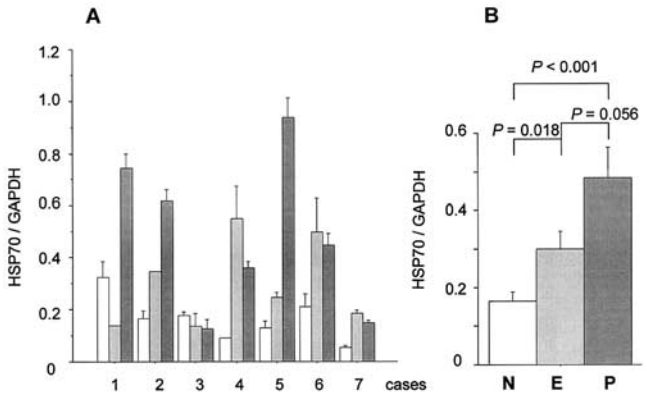

Fig. 3. Real-time quantitative RT-PCR analysis. (A) HSP70 mRNA expression levels in 7 nodule-in-nodule–type HCCs. Noncancerous liver (N, □), early HCC components (E, □), and progressed HCC components (P, ■) in each of the 7 cases. The expression levels are normalized with GAPDH mRNA in each sample. (B) The average expression level in nodule-in-nodule–type HCC was up-regulated stepwise. Noncancerous liver (N, 0.16 ± 0.09) versus early component (E, 0.30 ± 0.18), $P = .018$; early component versus progressed component (P, 0.48 ± 0.29), $P = .056$. Bars, SE.

It is not clear why HSP70 expression increases in early HCC and progressed HCC. HSP70 is a physiologically essential, highly conserved protein in both prokaryotes and eukaryotes that performs a variety of vital intracellular chaperoning functions.[24] Overexpression of HSP70 has been reported in various human tumors.[25-29] Overexpression of HSP70 also has been observed in cultured cells transformed with c-myc[30] or H-ras,[31] or cotransformed with H-ras/p53,[32] suggesting involvement of HSP70 in transformation or progression. Although many reports have described HSP70 expression in hepatoma cell lines,[33] to our knowledge, there have been no descriptions of HSP70 overexpression in human HCC tissues except for recent reports by Iizuka,[34] who listed examples of HSP70 upregulation in human HCC tissues in microarray studies, and by Yin,[35] who described overexpression of HSP70 in HCC. Overexpression of HSP70 in HCC is comparatively novel, and this is the first report of HSP70

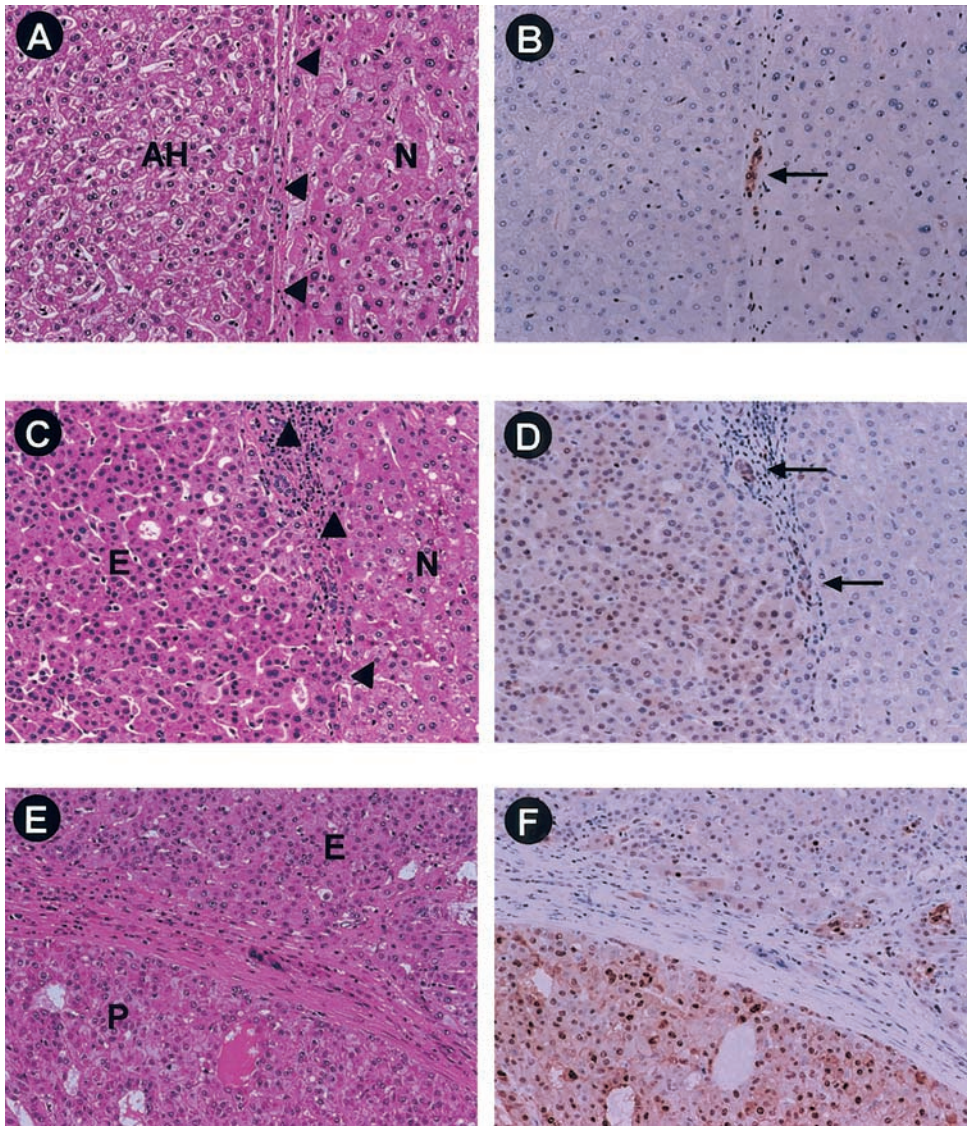

Fig. 4. Immunohistochemical localization of HSP70 in AH and HCC. Representative histology of AH (A), early HCC (C), and nodule-in-nodule–type HCC (E) (hematoxylin-eosin stain, original magnification, ×20), and HSP70 immunostaining of each serial section (B, D, and F). Bile duct epithelium served as the positive control (arrows). The borders between AH (AH) and noncancerous liver (N), and between early HCC (E) and noncancerous liver (N) are indicated by arrowheads. Noncancerous liver in B and D, and AH in B, showed no HSP70 immunoreactivity. Early HCC in D showed diffuse HSP70 immunoreactivity (positive index, 80%). The progressed HCC component (P) in (F) showed stronger HSP70 immunoreactivity (90%) than the early HCC component (E, 50%).

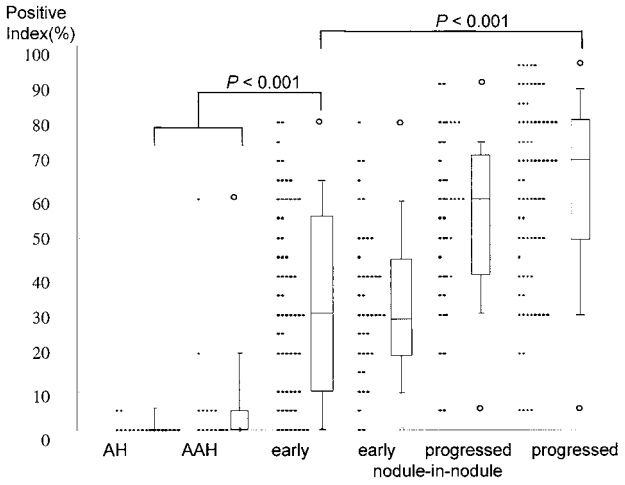

Fig. 5. Relationship between immunohistochemical expression of HSP70 and multistep hepatocarcinogenesis. Scattergram and box plot showing the relation between HSP70 immunostaining and multistage hepatocarcinogenesis. The box encompasses the twenty-fifth through seventy-fifth percentiles of results obtained with the fiftieth percentile (median). The fifth and ninety-fifth percentiles are shown as ○ below and above the tenth and nintieth-percentile whisker caps, respectively. The percentage of positive cells gradually increased according to the stepwise progression of hepatocarcinogenesis.

overexpression in early hepatocarcinogenesis. It is possible that HSP70 expression increases as a result of tumorigenesis. For example, a stressful environment in early HCC (nutrient depletion and hypoxia resulting from insufficient blood supply[8,9]) may stimulate HSP synthesis.

In addition to HSP70, we were able to identify several candidate genes involved in multistep hepatocarcinogenesis. Genes classified in the category of immune system detoxification were down-regulated in cancer cells.[36] Some of the up-regulated genes previously had been correlated with cellular dysplasia and tumor malignancy in several types of cancers. For example, type I sigma receptor[37] and nma,[38] which are transmembrane proteins, are known to be expressed in a variety of human tumor cells. Lipocalin 2, neutrophil-gelatinase-associated lipocalin, is reported to be overexpressed in some carcinomas.[39] Granulin, the glioma-associated growth factor gene, is reported to be overexpressed in brain tumors.[40] Overexpression of the midkine gene[41] and collagen type IV[42] have been described in HCC. The function of adenylyl-cyclase-associated protein 2 is unknown, but its homologue CAP forms a complex with adenylyl cyclase in yeast.[43] CAP overexpressed in early HCC component was confirmed by real-time quantitative RT-PCR (unpublished observation) and might be involved in hepatocarcinogenesis. Further analysis of these genes in multistage hepatocarcinogenesis is important.

In conclusion, the molecular signatures were clearly different between noncancerous liver tissue and the early and progressed components of nodule-in-nodule–type HCC. It was indicated also that HSP70 could be a sensitive marker for the differential diagnosis of early HCC from precancerous lesions such as AH and AAH or noncancerous liver tissue.

References

1. Okuda K. Hepatocellular carcinoma: recent progress. HEPATOLOGY 1992; 15:948-963.
2. Arakawa M, Kage M, Sugihara S, Nakashima T, Suenaga M, Okuda K. Emergence of malignant lesions within an adenomatous hyperplastic nodule in a cirrhotic liver. Observations in five cases. Gastroenterology 1986; 91:198-208.
3. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, et al. Genetic alterations during colorectal tumor development. N Engl J Med 1988;319:525-532.
4. Tsuda H, Hirohashi S, Shimamoto Y, Terada M, Hasegawa H. Clonal origin of atypical adenomatous hyperplasia of the liver and clonal identity with hepatocellular carcinoma. Gastroenterology 1988;95:1664-1666.
5. Takayama T, Makuuchi M, Hirohashi S, Sakaomoto M, Okazaki N, Takayasu K, Kosuge T, et al. Malignant transformation of adenomatous hyperplasia to hepatocellular carcinoma. Lancet 1990;336:1150-1153.
6. Sakamoto M, Hirohashi S, Shimamoto Y. Early stages of multistep hepatocarcinogenesis: adenomatous hyperplasia and early hepatocellular carcinoma. Hum Pathol 1991;22:172-178.
7. Hirohashi S, Ishak KG, Kojiro M, Wanless IR, Theise ND, Tsukuma H, Blum HE, et al. Hepatocellular carcinoma. In: Hamilton SR and LA Aaltonen, eds. Pathology and Genetics of Tumors of the Digestive System. Lyon: IARC Press, 2000:159-172.
8. Matsui O, Kadoya M, Kameyama T, Yoshikawa J, Takashima T, Nakanuma Y, Unoura M, et al. Benign and malignant nodules in cirrhotic livers: Distinction Based on Blood Supply. Radiology 1991;178:493-497.
9. Kudo M. Morphological diagnosis of hepatocellular carcinoma: special emphasis on intranodular hemodynamic imaging. Hepatogastroenterology 1998;45:1226-1231.
10. Noguchi M, Morikawa A, Kawasaki M, Matsuno Y, Yamada T, Hirohashi S, Kondo H, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. Cancer 1995;75:2844-2852.
11. Sclemper RJ, Itabashi M, Kato Y, Lewin KJ, Riddell RH, Shimoda T, Sipponen P, et al. Differences in diagnostic criteria for gastric carcinoma between Japanese and Western pathologists. Lancet 1997;349:1725-1729.
12. Sclemper RJ, Itabashi M, Kato Y, Lewin KJ, Riddell RH, Shimoda T, Sipponen P, et al. Differences in diagnostic criteria for used by Japanese and Western pathologists to diagnose colorectal carcinoma. Cancer 1998; 82:60-69.
13. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995;270:467-470.
14. Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee, MS, Mittmann M, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 1996;14:1675-1680.
15. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000;403:503-511.
16. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999;286:531-537.
17. Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, et al. Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature 2000;406:536-540.
18. Ishak KG, Anthony PP, Sobin LH. Histological typing of tumors of the liver. New York: WHO, Springer-Verlag, 1994.
19. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome wide expression patterns. Proc Natl Acad Sci U S A 1998;95:14863-14868.

20. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986-994.
21. Kanai Y, Ushijima S, Nakanishi Y, Hirohashi S. Reduced mRNA expression of the DNA demethylase, MBD2, in human colorectal and stomach cancers. Biochem Biophys Res Commun 1999;264:962-966.
22. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981;29: 577-580.
23. van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530-536.
24. Bukau B, Horwich AL. The HSP70 and HSP60 chaperone machines. Cell 1998;92:351-366.
25. Kawanishi K, Shinozaki H, Doki Y, Sakita I, Inoue M, Yano M, Tsujinaka T, et al. Prognostic significance of heat shock proteins 27 and 70 in patients with squamous cell carcinoma of the esophagus. Cancer 1999;85:1649-1657.
26. Maehara Y, Oki E, Abe T, Tokunaga E, Shibahara K, Kakeji Y, Sugimachi K. Overexpression of the heat shock protein HSP70 family and p53 protein and prognosis for patients with gastric cancer. Oncology 2000;58: 144-151.
27. Lazaris AC, Theodoropoulos GE, Davaris PS, Panoussopoulos D, Nakopoulou L, Kittas C, Golematis BC. Heat shock protein 70 and HLA-DR molecules tissue expression. Prognostic implications in colorectal cancer. Dis Colon Rectum 1995;38:739-745.
28. Gress TM, Muller-Pillasch F, Weber C, Lerch MM, Friess H, Buchler M, Beger HG, et al. Differential expression of heat shock proteins in pancreatic carcinoma. Cancer Res 1994;54:547-551.
29. Rahlan R, Kaur J. Differential expression of Mr 70,000 Heat shock protein in normal, premalignant, and malignant human uterine cervix. Clin Cancer Res 1995;1:1217-1222.
30. Kingston RE, Baldwin AS, Sharp PA. Regulation of heat shock protein 70 gene expression by c-myc. Nature 1984;312:280-282.
31. Konno A, Sato N, Yagihashi A, Torigoe T, Cho J, Torimoto K, Hara I, et al. Heat or stress-inducible transformation-associated cell-stress antigen on the activated H-ras-oncogene-transfected rat fibroblast. Cancer Res 1989; 49:6578-6582.
32. Pinashi-Kimmhi O, Michalowitz D, Ben-Zeev A, Oren M. Specific interaction between the p53 cellular tumor antigen and major heat shock proteins. Nature 1986;320:182-185.
33. Desiderio MA, Tacchini L, Anzon E, Pogliaghi G, Radice L, Bernelli-Zazzera A. Effect of polyamine imbalance on the induction of stress genes in hepatocarcinoma cells exposed to heat shock. HEPATOLOGY 1996;24: 150-156.
34. Iizuka N, Oka M, Yamada-Okabe H, Mori N, Tamesa T, Okada T, Takemoto N, et al. Comparison of gene expression profiles between hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma by oligonucleotide microarray data on the basis of a supervised learning method. Cancer Res 2002;62:3939-3944.
35. Yin Y, Qin Q, Zhang W, Zhao J, Zhang C, Yu J. Overexpression of heat shock protein 70 and spontaneous cancer cell apoptosis in hepatocellular carcinoma. Zhonghua Gan Zang Bing Za Zhi 2001;9:84-85.
36. Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, et al. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cdna microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 2001;61:2129-2137.
37. Bem WT, Thomas GE, Mamone JY, Homan SM, Levy BK, Johnson FE, Coscia CJ. Overexpression of sigma receptors in nonneural human tumors. Cancer Res 1991;24:6558-6562.
38. Degen WG, Weterman MA, van Groningen JJ, Cornelissen IM, Lemmers JP, Agterbos MA, Geurts van Kessel A, et al. Expression of nma, a novel gene, inversely correlates with the metastatic potential of human melanoma cell lines and xenografts. Int J Cancer 1996;65:460-465.
39. Argani P, Rosty C, Reiter RE, Wilentz RE, Murugesan SR, Leach SD, Ryu B, et al. Discovery of new markers of cancer through serial analysis of gene expression: prostate stem cell antigen is overexpressed in pancreatic adenocarcinoma. Cancer Res 2001;61:4320-4324.
40. Liau LM, Lallone RL, Seitz RS, Buznikov A, Gregg JP, Kornblum HI, Nelson SF, et al. Identification of a human glioma-associated growth factor gene, granulin, using differential immuno-absorption. Cancer Res 2000; 60:1353-1360.
41. Koide N, Hada H, Shinji T, Ujike K, Hirasaki S, Yumoto Y, Hanafusa T, et al. Expression of the midkine gene in human hepatocellular carcinoma. Hepatogastroenterology 1999;46:3189-3196.
42. Grigioni WF, Garbisa S, D'Errico A, Baccarini P, Stetler-Stevenson WG, Liotta LA, Mancini AM. Evaluation of hepatocellular carcinoma aggressiveness by a panel of extracellular matrix antigens. Am J Pathol 1991;138: 647-654.
43. Shima F, Okada T, Kido M, Sen H, Tanaka Y, Tamada M, Hu CD, et al. Association of yeast adenylyl cyclase with cyclase-associated protein CAP forms a second Ras-binding site which mediates its Ras-dependent activation. Mol Cell Biol 2000;20:26-33.