Table 4 Relationship Between the Perineural Invasion and the Radial and Ductal Margins

		Perineural invasion		*p* value
		Negative	Positive	
Radial margin	Negative	12	48	0.045*
	Positive	0	17	
Ductal margin	Negative or mucosal positive	12	46	0.03*
	Submucosal positive	0	19	

*$p<0.05$

invasion. This strong relationship between perineural invasion and the extent of cancer invasion has been reported by other authors.[8,20,27] Bhuiya et al.[27] reported that perineural invasion was an independent prognostic factor that was not influenced by the site, size of the tumor, or lymph node metastasis. These results may suggest that the presence of perineural invasion represents a malignant behavior of the tumor and that this type of tumor infiltrates the adjacent structures in the hepatoduodenal ligament via neural pathways and also exhibits replacement growth along the ductal wall, resulting in positive radial or submucosal ductal margins. This importance of perineural invasion on a negative prognosis has been reported not only in patients with cholangiocarcinoma[2,4,8,20,27] but also in patient with gallbladder cancer.[28,29]

Ikeyama et al. reported that an extended right hemihepatectomy is essential for nodular and infiltrating (as classified according to the gross type on a cholangiogram) Bismuth type I or II hilar cholangiocarcinomas but that EHBD with or without limited hepatectomy is adequate in patients with papillary tumors.[21] In our series, 28 patients with macroscopically nodular and infiltrating Bismuth type I or II tumors underwent surgical resection (detailed data not shown). The median survival of the 14 patients who underwent extended right hemihepatectomy or right trisectionectomy was 2.0 years, while that of the 14 patients who underwent other procedures (EHBD, PD, or other types of hepatectomies) was 3.3 years ($p=0.38$). Therefore, we cannot conclude that extended right hemihepatectomy is necessary for Bismuth type I or II hilar cholangiocarcinoma. The 5-year survival rate of the patients undergoing extended right hemihepatectomy in Ikeyama's series was favorable (63%) probably because negative surgical margins were obtained in 18 patients who underwent extended right hemihepatectomy.

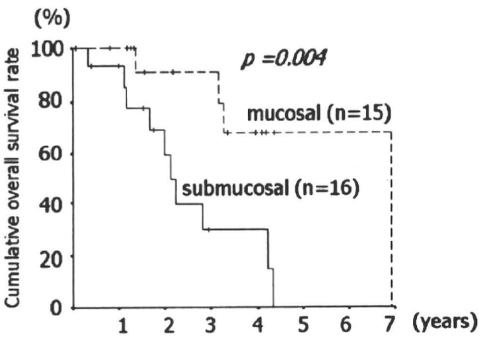

Figure 2 Cumulative overall survival of 31 patients with positive hepatic-side ductal margins. The survival of patients with positive mucosal infiltration (*dotted line*, $n=16$) was significantly better than that of patients with positive submucosal infiltration (*solid line*, $n=15$; $p=0.004$).

MHx combined with EHBR enables the resection of the hilar bile duct including the biliary trees in the caudate process and should be one of the ideal surgical procedures for hilar cholangiocarcinoma.[1–7] The introduction of preoperative adequate biliary drainage and portal vein embolization of the future resectable liver[30] has dramatically increased the safety of the extensive hepatectomy. We previously reported a zero mortality rate for major hepatectomy for hilar cholangiocarcinoma between 2000 and 2004.[7] When the duodenal-side ductal margin is positive, additional resection of the intrapancreatic bile duct can be performed as far as the confluence with the main pancreatic duct. We have examined the ductal margins using frozen sections, and the positive and negative predictive values of frozen section were 0.97 and 0.86. We believe that these predictive values are acceptable and frozen sections are useful for intraoperative decision making. Nevertheless, the hepatic and duodenal sides of the ductal margins are positive in 41% and 44% of cases, respectively. These percentages illustrate the difficulty in obtaining a negative ductal margin against the ductal spread of cholangiocarcinoma.

Historically, we have been increasingly performed PD rather than other resections since 2001 mainly because of the improved safety of this procedure. The most troublesome drawback of PD for biliary cancer is the high incidence of pancreatic fistula from normal and soft pancreas tissue. HPD enables a broad resection of the biliary system, but is associated with a considerable risk of major pancreatic leakage and subsequent hepatic insufficiency. A two-staged PD may be an alternative approach that could enable life-threatening complications associated with pancreatic fistula to be avoided,[31] but we have only performed a one-stage pancreatic reconstruction using mucosa-to-mucosa anastomosis of the pancreatic duct. Although the incidence of pancreatic fistula was very high in our series (79%), no deaths occurred among the 34

patients undergoing PD. This outcome may be partly due to the adequate postoperative drainage of pancreatic juice and the prevention of bleeding by wrapping the stump of the gastroduodenal artery with the falciform ligament.[32] PD guaranteed a negative duodenal-side ductal margin, but the overall incidence of a positive surgical margin was comparable between the PD group and the non-PD group (50% vs. 60%, $p=0.36$). These results indicate the difficulty in achieving a complete resection of cholangiocarcinoma arising in the hepatoduodenal ligament and also suggest that HPD should be indicated in highly select patients with widespread cholangiocarcinoma who have an acceptable general condition for surgery. All of the six patients undergoing HPD for widespread cholangiocarcinoma had perineural invasion, and five of them had nodal metastases, but the 5-year survival rate was 44%, which was relatively favorable. HPD could be indicated in patients with positive submucosal ductal margins and negative radial margins following MHx or PD because the submucosal infiltration on the hepatic-side ductal margin was a possible prognostic factor, associated with the presence of perineural invasion (Fig. 2; Table 4). In this sense, aggressive and safe application of HPD in selected patients might improve the curability and the survival.

Blood transfusion is reportedly associated with a poor outcome in several other malignancies, such as colorectal,[33] breast,[34] gastric,[35] periampullary,[36] and hepatocellular carcinoma.[37] The immunosuppressive effect of transfusion has been proposed as a possible reason.[38] Blood transfusion might also reflect the extensiveness of the operation, and extensive surgery, rather than blood transfusion itself, may be related to a poor outcome. Surgeons should make all possible efforts to prevent blood loss during the surgery and to avoid blood transfusion.

In summary, in the surgical management of infrahilar/suprapancreatic cholangiocarcinoma, no significant difference was found in the incidence of positive overall surgical margins or long-term survivals among the four groups of patients undergoing four types of surgical procedures if applied within our intention-to-treat strategy. Perineural invasion, rather than the type of surgical procedure, had a significant impact on surgical curability and survival.

Acknowledgments This study was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare of Japan.

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Clinical Cancer Research

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Clinical Cancer Research

Human Cancer Biology

Combined Functional Genome Survey of Therapeutic Targets for Hepatocellular Carcinoma

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Abstract

Purpose: The outcome of patients with advanced hepatocellular carcinoma (HCC) has remained unsatisfactory. Patients with HCC suffer from chronic hepatitis or liver cirrhosis, and their reserve liver function is often limited.

Experimental Design: To develop new therapeutic agents that act specifically on HCC but interfere only minimally with residual liver function, we searched for genes that were upregulated in 20 cases of HCC [namely, discovery sets 1 (n = 10) and 2 (n = 10)] in comparison with corresponding nontumorous liver and a panel representing normal organs using high-density microarrays capable of detecting all exons in the human genome.

Results: Eleven transcripts whose expression was significantly increased in HCC were subjected to siRNA-based secondary screening of genes required for HCC cell proliferation as well as quantitative reverse transcription-PCR analysis [validation sets 1 (n = 20) and 2 (n = 44)] and immunohistochemistry (n = 19). We finally extracted four genes, *AKR1B10*, *HCAP-G*, *RRM2*, and *TPX2*, as candidate therapeutic targets for HCC. siRNA-mediated knockdown of these candidate genes inhibited the proliferation of HCC cells and the growth of HCC xenografts transplanted into immunodeficient mice.

Conclusions: The four genes we identified were highly expressed in HCC, and HCC cells are highly dependent on these genes for proliferation. Although many important genes must have been overlooked, the selected genes were biologically relevant. The combination of genome-wide expression and functional screening described here is a rapid and comprehensive approach that could be applied in the identification of therapeutic targets in any type of human malignancy. *Clin Cancer Res; 16(9); 2518–28. ©2010 AACR.*

Liver cancer is the fifth most common human cancer worldwide and the third most common cause of cancer mortality. Hepatocellular carcinoma (HCC) is the most common histologic subtype of liver cancer and is highly endemic in Southeast Asia and sub-Saharan Africa (1). HCC develops mainly in liver affected by chronic hepatitis or cirrhosis caused by persistent infection with hepatitis B or C virus; however, the precise molecular mechanisms that drive the transition from the background liver conditions to cancer are largely unknown. Liver resection, ethanol injection, radiofrequency ablation, and chemoembolization have been used successfully for the local management of HCC; however, no single cytotoxic chemotherapeutic agent has been proven effective for the systemic treatment of HCC; thus, the outcome for patients with locally advanced, multicentric, and/or metastatic HCC who are not eligible for these local treatments has remained unsatisfactory.

An increasing number of therapeutic agents targeting molecular components essential for cancer cell growth have begun to be incorporated into oncological practice: Imatinib, which blocks the Bcr-Abl fusion kinase of chronic myeloid leukemia (CML), is currently the first-line therapy for CML (2). The epidermal growth factor receptor inhibitors gefitinib and erlotinib have been used in the treatment of advanced non–small cell lung cancer (3). Recently, it was shown in a phase III study that sorafenib (BAY 43-9006), a multikinase inhibitor, significantly improved the overall survival of patients with advanced HCC (4, 5), and, consequently, sorafenib has since been approved for the treatment of patients with unresectable HCC by the American Food and Drug Administration. However, most patients enrolled in those studies retained relatively well-compensated

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Microarray data from this study have been submitted to the Gene Expression Omnibus database (accession no. GSE12941).

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Translational Relevance

Liver cancer is the fifth most common human cancer worldwide and the third most common cause of cancer mortality. Recently, a multikinase inhibitor, sorafenib, has been approved as a systemic chemotherapeutic drug for advanced hepatocellular carcinoma (HCC); however, further improvement seems to be necessary. To identify an "Achilles heel" of HCC cells and develop new therapeutic agents that act specifically on HCC but interfere only minimally with residual liver function, we performed an unbiased survey of the whole genome. We finally identified four genes as candidates. siRNA-mediated knockdown of these candidate genes inhibited the proliferation of HCC cells and the growth of HCC xenografts transplanted into immunodeficient mice, confirming their feasibility as therapy targets.

liver function. In reality, the reserve liver function of HCC patients is often limited due to underlying liver conditions. Therefore, the safety and tolerability of sorafenib remain to be determined in HCC patients with compromised liver function. Therapeutic targeting molecules other than protein kinases have also been developed against various tumors of other organs (6–8). To identify a molecule essential for HCC cell growth and develop new therapeutic agents that would act specifically on HCC and only minimally interfere with residual liver function, a survey of the whole genome would be necessary.

In this study, we adopted a combined functional approach. We first searched for genes that were upregulated in HCC in comparison with the background nontumorous liver tissue. This was followed by siRNA-based screening of genes required for HCC cell proliferation. Recently, whole-genome RNA interference (RNAi)–based functional screening has been reported to successfully identify genes that sensitize lung cancer cells to a chemotherapeutic drug and genes required for proliferation and survival of several cancer cell lines; however, in those studies, the expressional specificity of the identified targets was not taken into consideration (9–12). Here, we report the identification of possible therapeutic target molecules of HCC through a combination of genome-wide expression and functional screening.

Materials and Methods

Patients and microarray analysis. Samples of HCC and surrounding nontumorous liver tissue were collected from 84 patients who underwent liver resection for HCC at the National Cancer Center Hospital (Tokyo, Japan) with informed consent. The clinical and histologic data for these patients are summarized in Supplementary Table S1. Total RNA of normal human organs was obtained from a commercial source (FirstChoice Human Total RNA Survey Panel, Ambion).

One microgram of total RNA was converted to end-labeled cRNA using a Whole Transcript Sense Target Labeling kit (Affymetrix). The fluorescent cRNA probes were hybridized to Human Exon 1.0 ST arrays (Affymetrix), as instructed by the supplier. Data analysis was carried out using the ArrayAssist software package (version 5.5.1, Stratagene). A GC content-based background correction followed by quantile normalization was done with an exonRMA algorithm available in the package. Multiple exonic expression data were also summarized into a single value using the same algorithm, as instructed by the supplier (http://www.stratagene.com/manuals/ArrayAssist.pdf).

The protocol of this study was reviewed and approved by the ethics committee of the National Cancer Center (Tokyo, Japan).

Cell lines. Three human cell lines derived from HCC were used in this study. KIM-1 was kindly provided by Dr. Masamichi Kojiro (Kurume University, Kurume, Japan). Hep3B was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). HLE was obtained from the Health Science Research Resources Bank (Osaka, Japan). KIM-1 and Hep3B were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. HLE was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum.

siRNA-based functional screening. The day before siRNA transfection, cells were seeded at 5×10^3 per well in 96-well plates to obtain 50% to 60% confluency. They were then transfected with siRNA using Lipofectamine 2000 (Invitrogen) at a concentration of 10, 20, or 50 nmol/L in KIM1, Hep3B, or HLE cells, respectively. Three days later, the relative proportion of living cells was assessed using a Premix WST-1 Cell Proliferation Assay System (Takara Bio) in accordance with the manufacturer's instructions. The siRNA was synthesized by Ambion, and the identification (ID) numbers of siRNAs used in this study are listed in Supplementary Table S4. Silencer Negative Control #1 siRNA (Ambion) was used as a nontargeting control. siRNA targeting *TOP2A* was described previously (13).

Real-time PCR. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript reverse transcriptase (Invitrogen). Real-time PCR was done as described previously (14). Primers and probes sets were obtained from Applied Biosystems, and their Assay IDs are provided in Supplementary Table S5. The amplification reaction was done according to the manufacturer's instructions (95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 50°C for 2 minutes, and 60°C for 1 minute).

Immunohistochemistry and immunoblot analysis. Anti-AKR1B10 (clone 1A6) and anti–HCAP-G (clone 4B1) monoclonal antibodies were purchased from Abnova. Anti-RRM2 antibody (E-16) was purchased from Santa

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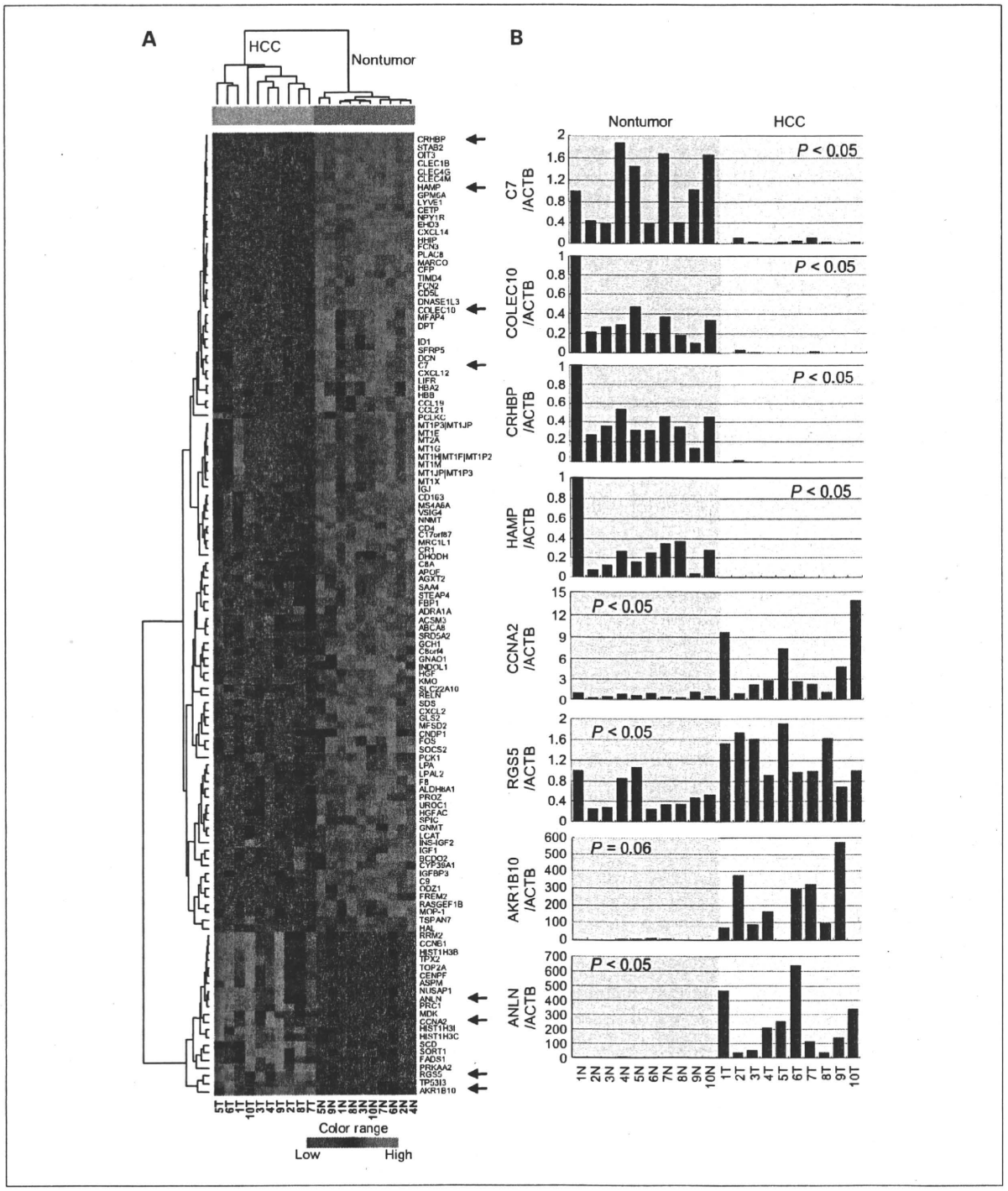

Fig. 1. Genes differentially expressed between HCC and nontumorous liver. A, hierarchical clustering of 124 genes whose expression differed significantly ($P < 0.001$ and >3-fold change) between HCC and adjacent nontumorous liver. Transcriptional signal intensity is shown as a heat map. Red indicates higher signals, whereas blue indicates lower signals. Arrows indicate eight genes selected for validation by real-time PCR (B). B, validation of the microarray data by real-time RT-PCR. The expression levels of eight representative genes whose expression differed significantly between adjacent nontumorous liver (left) and HCC (right) were validated by real-time RT-PCR (shown in arbitrary units). Significant correlation between array (discovery set 1) and real-time RT-PCR data was confirmed by calculating correlation coefficient values in eight randomly selected genes (indicated by arrows in A): *C7*, 0.96; *COLEC10*, 0.97; *CRHBP*, 0.98; *HAMP*, 0.98; *CCNA2*, 0.82; *RGS5*, 0.80; *AKR1B10*, 0.98; *ANLA*, 0.92. The significance of differential expression between HCC and adjacent nontumorous liver tissue was assessed using a permutation paired *t* test, and Bonferroni-corrected *P* values are provided.

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Cruz Biotechnology. Anti-TPX2 antibody was purchased from Novus Biologicals.

Formalin-fixed and paraffin-embedded liver tissues containing HCC were obtained from the National Cancer Center Hospital, and stained as described previously (15, 16). Immunoblot analysis of the KIM-1 cell lysate was done as described previously (15).

Animal experiments. Eight million KIM-1 cells suspended in 0.1 mL of PBS were s.c. inoculated into the flanks of 5-week-old female BALB/c nu/nu nude mice (SLC). Eight days later, the tumor-bearing mice were treated with siRNA together with atelocollagen (Koken Co., Ltd.), as described previously (17, 18). The final concentration of siRNA and atelocollagen was 11 μmol/L and 0.5%, respectively, and 200 μL of the siRNA solution were injected directly into each tumor. Tumor volume was determined every 3 days using the formula $V = 1/2\ (A \times B^2)$, where A and B represent the largest and smallest dimensions of the tumor, respectively.

Animal experiments were reviewed by the institutional ethics committee and performed in compliance with the

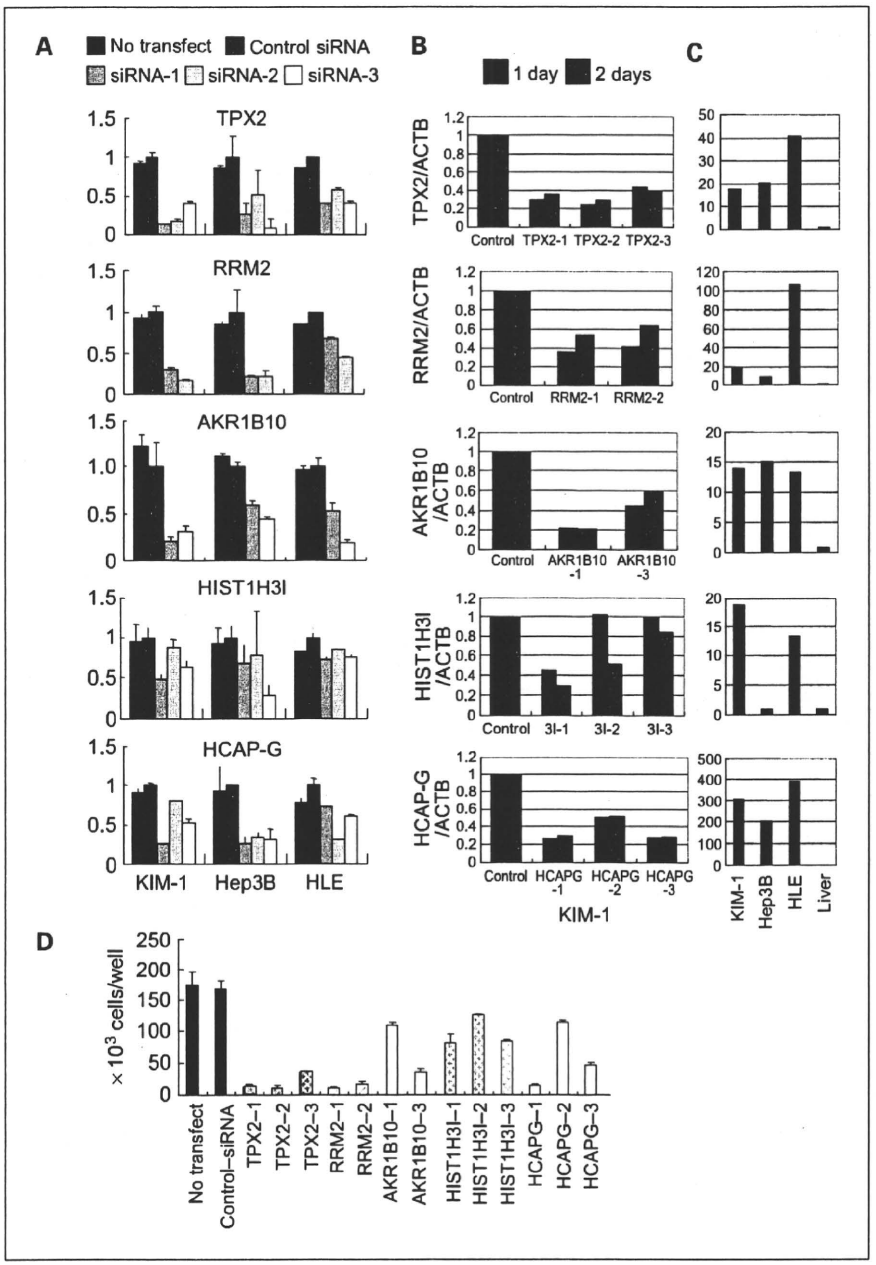

Fig. 2. siRNA-based functional screening. A, siRNA-mediated screening of genes required for proliferation of HCC cells. Three HCC cell lines (KIM-1, Hep3B, and HLE) were transfected with the indicated siRNAs, and the relative proportion of living cells was assessed 3 days later by measuring the mitochondrial succinate-tetrazolium reductase activity. Values for control siRNA were set at 1. B, reduction of the level of mRNA for each gene was determined by real-time PCR 1 and 2 days after transfection of KIM-1 cells with the indicated siRNAs. Values for control siRNA were set at 1. C, expression of each gene in HCC cell lines (KIM-1, Hep3B, and HLE) and normal liver tissue. D, confirmation of siRNA-mediated inhibition of HCC cell proliferation. KIM-1 cells were transfected with the indicated siRNAs, and the number of living cells was counted 3 days later by trypan blue dye exclusion using a hemocytometer.

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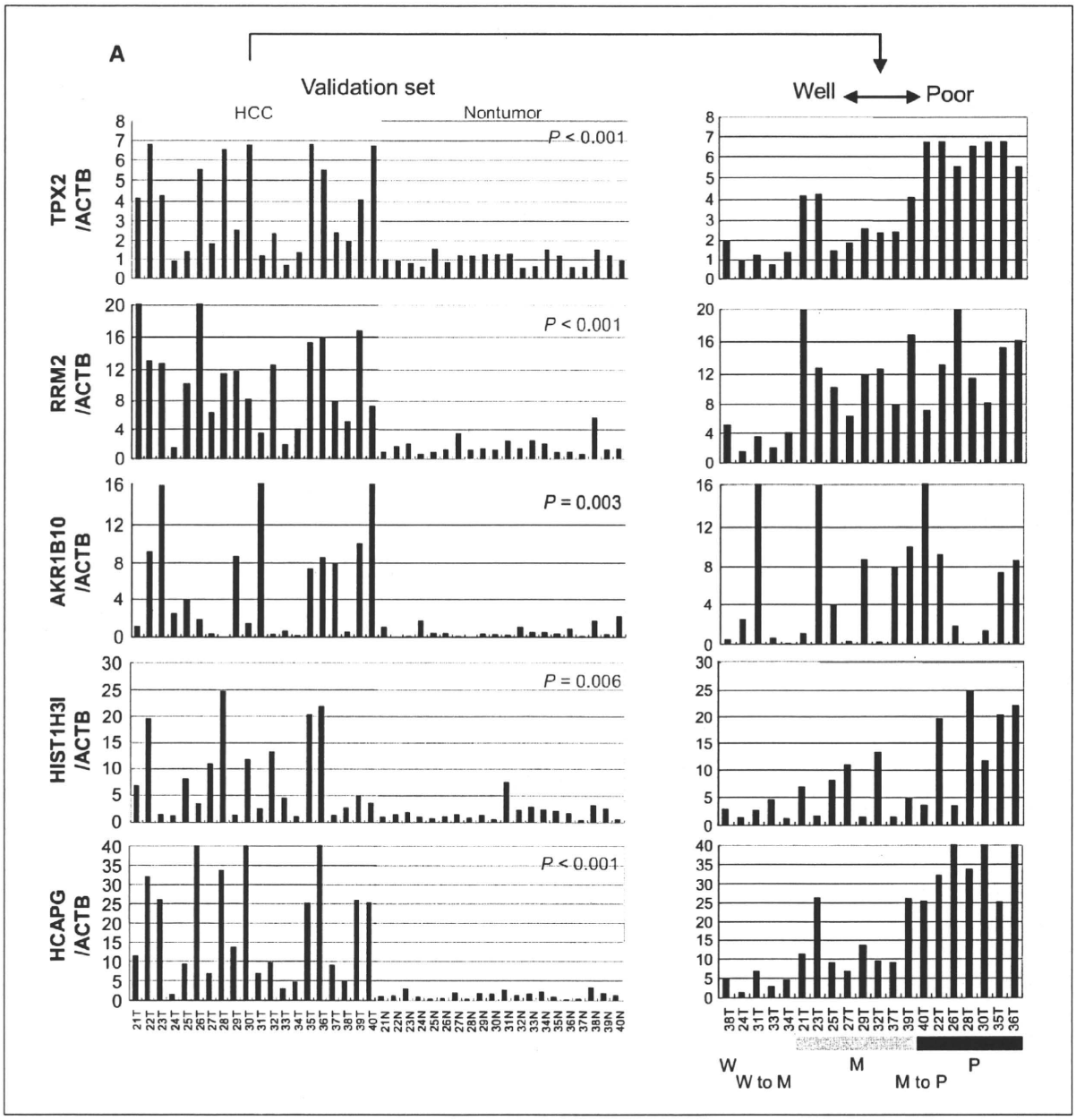

Fig. 3. Validation of differential expression. A, mRNA expression levels of selected genes in 20 independent pairs of HCC (21-40T) and adjacent nontumorous liver tissue (21-40N; validation set 1) determined by real-time PCR (left). The expression levels in HCC were realigned according to histologic differentiation (right). W, well differentiated; W to M, well to moderately differentiated; M, moderately differentiated; M to P, moderately to poorly differentiated, P, poorly differentiated.

guidelines for Laboratory Animal Research of the National Cancer Center Research Institute (Tokyo, Japan).

Statistical analysis. To extract differentially expressed genes from the array data, a paired *t* test with no correction was done (19) with asymptotic distribution to determine the *P* value. Correlations between array data and real-time PCR measurements were assessed using the Pearson correlation coefficient. The significance of differential gene expression between HCC and adjacent nontumorous liver tissue was assessed using the permutation paired *t* test followed by Bonferroni correction.

The weights and volumes of tumors are given as means (+SE). To evaluate the chronological effect of siRNAs on the growth of xenografts in comparison with control siRNA,

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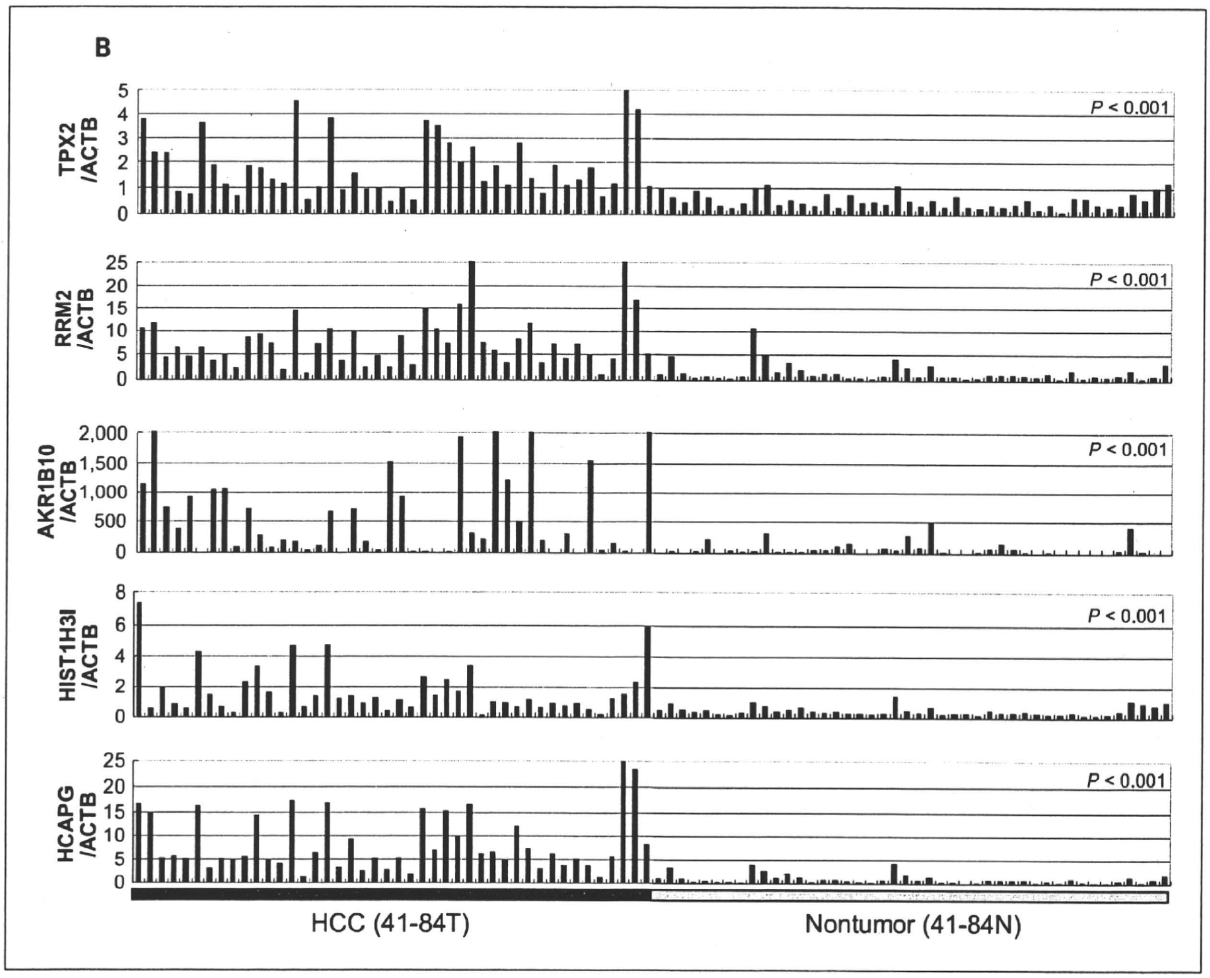

Fig. 3. ***Continued.*** B, expression levels of mRNAs for selected genes in 44 independent pairs of HCC (41-84T) and adjacent nontumorous liver tissue (41-84N; validation set 2) determined by real-time PCR.

a generalized linear mixed-effects model was used (20). The volume of the xenograft was modeled using γ-error distribution and log link function. This model considers each siRNA treatment as a fixed effect with control siRNA as an intercept and the number of days after implantation as a random effect. Estimates of variance components were obtained using the Laplacian approximation method, and the model fit was assessed using deviances. The significance of effects was estimated from the degree of freedom and t statistics followed by Bonferroni correction. Analysis was done using the lmer function for fitting generalized linear mixed-effects models, in the R statistical software package (version 2.6.0).

Results

Exon-based array analysis of HCC. Twenty paired samples of HCC and adjacent nontumorous liver tissue were subjected to genome-wide expression analysis using two different batches of the GeneChip Human Exon 1.0 ST arrays [discovery sets 1 (10 pairs) and 2 (10 pairs)]. Statistical analysis was done separately, and genes expressed differentially in the two sets were selected to eliminate any experimental bias caused by batch-to-batch variations. The exon array can detect mRNAs with low abundance as well as alternatively polyadenylated and spliced mRNA because the probes are designed to hybridize with the entire sequences of the transcripts (21). We identified 124 annotated genes that were differentially expressed between the background (nontumorous) liver tissue and HCC [at least a 3-fold change in transcription signal; $P < 0.001$ (paired t test with no correction)] in discovery set 1 (Supplementary Tables S2 and S3). The genes were clustered according to the similarity of their expression profiles (Fig. 1A), and the differential expression of representative genes was confirmed by real-time PCR (Fig. 1B). It was noteworthy that although 103 genes were found to be significantly downregulated, only 21 were apparently upregulated.

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We selected 9 genes (*AKR1B10*, *ANLN*, *CCNB1*, *HIST1H3B*, *HIST1H3C*, *HIST1H3I*, *RRM2*, *TOP2A*, and *TPX2*) whose expression was upregulated in HCC (≥3-fold change in transcription signal; $P < 0.001$, t test) in both discovery sets 1 and 2. Furthermore, two additional genes (*HCAP-G* and *DEPDC1*) were selected using a different criterion (>2.5-fold change across all of the 20 cases in discovery sets 1 and 2, and a raw signal of <50 in all 20 of the nontumorous liver tissues; $P < 0.05$, t test).

RNAi-based screening of genes required for HCC cell proliferation. To identify genes that are essential for HCC cell proliferation, siRNA-based screening was done for the 11 genes that were upregulated in HCC. Two or three constructs of siRNA were designed for each gene. Relative cell viability was evaluated by the mitochondrial succinate-tetrazolium reductase activity-based assay 3 days after transfection (Fig. 2A). We selected five genes (*TPX2*, *RRM2*, *HCAP-G*, *HIST1H3I*, and *AKR1B10*) based on the criterion that at least two siRNAs per gene reproducibly suppressed cell proliferation by >20% in all of three cell lines (KIM-1, Hep3B, and HLE). Representative data are shown in Fig. 2A and B. The baseline expression of these genes was determined in the three cell lines by real-time reverse transcription-PCR (RT-PCR; Fig. 2C). We confirmed the cell proliferation–inhibitory activity of the siRNA by counting the numbers of cells (Fig. 2D).

Validation of differential gene expression in additional cases of HCC. The increased expression of the five genes selected using the siRNA-based screen was validated in 20 cases of HCC (validation set 1) by real-time PCR (Fig. 3A). The expression of all five genes was confirmed to be increased in HCC. The expression of *TPX2*, *RRM2*, *HCAP-G*, and *HIST1H3* was associated with loss of histologic differentiation (Fig. 3A, right). The expression of *AKR1B10* was upregulated in HCC regardless of differentiation. We further confirmed the differential expression of these genes between HCC and nontumorous liver tissues in 44 additional independent cases of HCC (validation set 2) by real-time PCR (Fig. 3B).

In the 18 normal organs examined, no significant expression of *TPX2*, *RRM2*, or *HCAP-G* was observed, except for the thymus (Fig. 4, left), which is largely involuted in nonjuvenile adults. No organs showed higher expression of *AKR1B10* than was the case in HCC. We did not select *HIST1H3I*, as this gene showed high expression in several vital organs (Fig. 4).

Protein expression analysis. Expression of the products of four candidate genes, *TPX2*, *HCAP-G*, *RRM2*, and *AKR1B10*, was examined immunohistochemically in 19 independent cases of HCC (Fig. 5). In 84% (16 of 19) of the cases, AKR1B10 protein was detected in the cancer but was hardly evident in the adjacent nontumorous liver tissue. The nuclear staining of HCAP-G and TPX2 was stronger in HCC than in the adjacent nontumorous liver in 42% (8 of 19) and 58% (11 of 19) of cases, respectively. Patchy staining of RRM2 was observed in 84% (16 of 19) of the HCCs.

***Inhibition of tumor growth* in vivo.** Finally, we performed an *in vivo* experiment to evaluate the feasibility of the four selected genes as therapeutic targets. siRNA against *AKR1B10*, *HCAP-G*, *RRM2*, and *TPX2* mixed with atelocollagen was injected into tumors (31.5 ± 1.9 mm^3) established by xenografting KIM-1 cells into the flank of nude mice (Fig. 6). Atelocollagen forms a complex with siRNA, thus enhancing its stability and allowing sustained release of siRNA *in vivo* (17, 18). The silencing of the target genes by each relevant siRNA was confirmed by real-time PCR (Fig. 6A). Treatments with siRNA against *AKR1B10*, *HCAP-G*, *RRM2*, or *TPX2* given twice, 1 week apart, significantly suppressed tumor growth (Fig. 6B; Supplementary Table S6), and the growth-inhibitory effects of siRNA were confirmed by weighing the excised tumors (Fig. 6C).

Discussion

There is now strong epidemiologic evidence that persistent infection with hepatitis B or C virus is a major cause of HCC. However, the precise molecular mechanism behind the development of HCC is still unclear. Mutation in the tumor suppressor gene *TP53* is most frequently observed in HCC associated with aflatoxin B exposure as well as chronic infection with hepatitis B and C viruses (22–24); however, it seems to be a late event during multistep carcinogenesis (22). Deregulation of the Wnt as well as other signaling pathways has been reported in HCC (22, 25). Therefore, a therapeutic method that can normalize these aberrantly activated oncogenic signals would be clinically valuable. In an attempt to discover therapeutic targets with high specificity for HCC, we searched for genes that are specifically upregulated in HCC in comparison with nontumorous liver tissue and normal vital organs using high-density microarrays designed to detect all the exons in the human genome (Figs. 1 and 4). This was followed by siRNA-based screening of genes required for HCC cell proliferation (Fig. 2) as well as quantitative RT-PCR analysis and immunohistochemistry of additional cases (Figs. 3 and 5). We finally identified four candidate genes and confirmed their functional involvement in the tumor growth of HCC xenografts (Fig. 6). These genes, *AKR1B10*, *HCAP-G*, *RRM2*, and *TPX2*, were expressed strongly and specifically in HCC, which is highly dependent on these genes for proliferation, and their feasibility as therapy targets also seems to be supported by the literature.

RRM2 is a subunit of ribonucleotide reductase that catalyzes the conversion of ribonucleoside 5′-diphosphates into their corresponding 2′-deoxyribonucleotides. Because this reaction is the rate-limiting step of DNA synthesis, and inhibition of ribonucleotide reductase stops DNA synthesis and cell proliferation, RRM2 has been considered a promising target for cancer therapy (26).

TPX2 (C20ORF1) is a microtubule-associated protein whose expression is restricted to the S, G_2, and M phases of the cell cycle. Suppression of TPX2 expression by RNAi causes defects in microtubule organization during mitosis,

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leading to the formation of two microtubule asters that do not form a spindle (27). TPX2 is necessary for maintaining aurora A kinase in an active conformation (28, 29). Aurora kinases are essential for the regulation of chromosome segregation and cytokinesis during mitosis and have been reported to be overexpressed in a wide range of human tumors. Several aurora kinase inhibitors, such as VX-680/MK-0457, have been showed to have anticancer effects *in vitro* and *in vivo* (30, 31). The binding of TPX2 modulates the conformation of aurora A and reduces its affinity

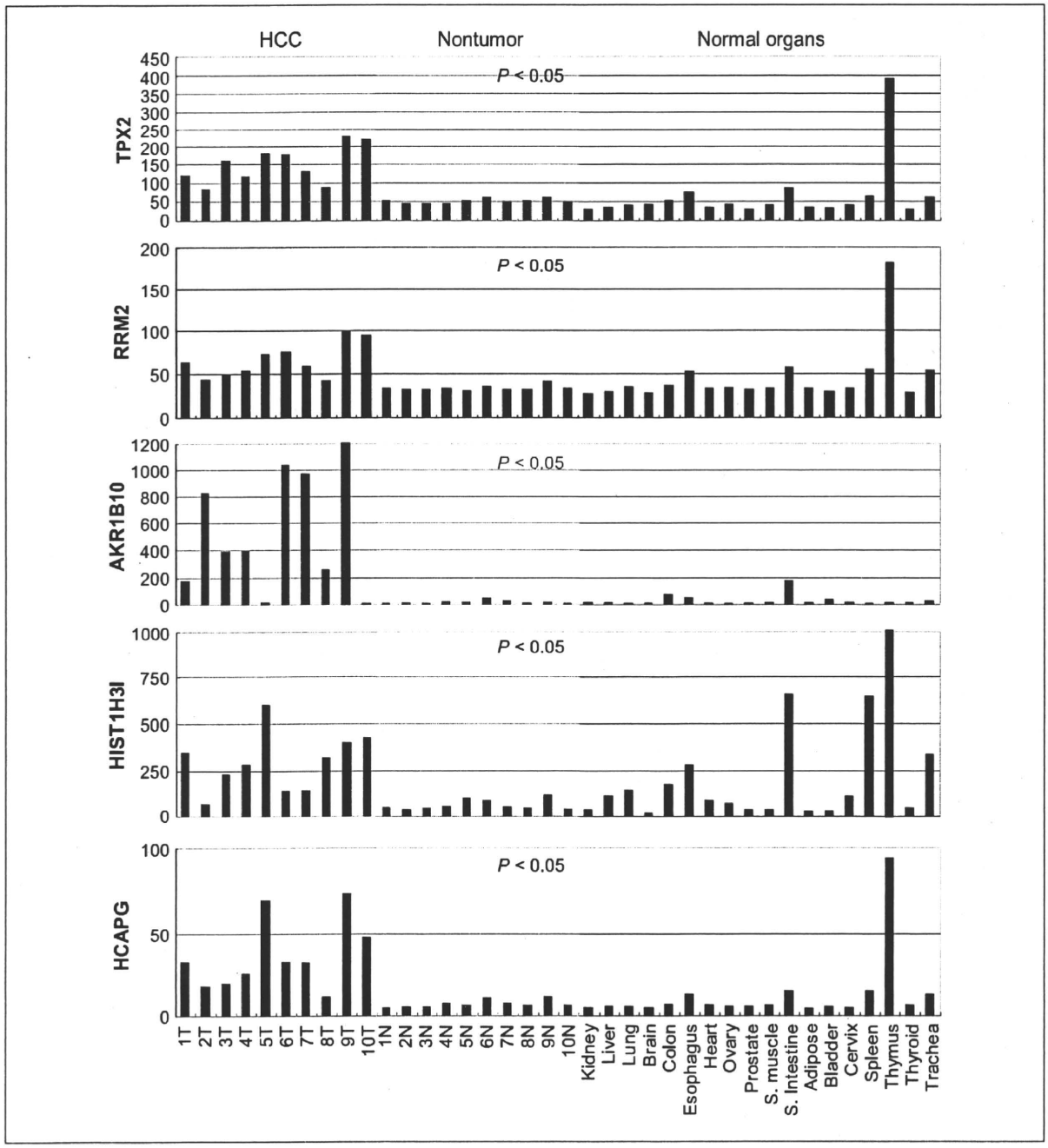

Fig. 4. Expression in normal organs. Expression levels of mRNAs for selected genes in 10 pairs of HCC (1-10T) and adjacent nontumorous liver tissue (1-10N; discovery set 1) and 18 normal organs determined by Human Exon 1.0 ST arrays (shown in arbitrary units). The significance of differential expression between HCC and adjacent nontumorous liver tissue was assessed using permutation paired *t* test, and Bonferroni-corrected *P* values are provided. S. muscle, skeletal muscle; S. intestine, small intestine.

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for VX-680 (32). Inhibition of TPX2 may increase the efficacy of this class of aurora kinase inhibitors.

HCAP-G is a component of the condensin complex that organizes the coiling topology of individual chromatids. Condensin also contributes to mitosis-specific chromosome compaction and is required for proper chromosome segregation, although the functional significance of HCAP-G in the condensing complex is largely unknown (33, 34).

AKR1B10 (ARL1, aldose reductase-like 1) was originally isolated as a new member of the aldo-keto reductase superfamily overexpressed in HCC and is reportedly related to the histologic differentiation of HCC (35, 36). AKR1B10 was also overexpressed in squamous cell carcinoma of the lung and its precursor conditions (37). Because the expression of AKR1B10 was highly specific to HCC and its inhibition suppressed tumor growth (Fig. 6), chemicals that specifically inhibit AKR1B10 activity may be useful anticancer drugs with minimal side effects.

It cannot be denied that many important genes were probably overlooked at every step of the present screen,

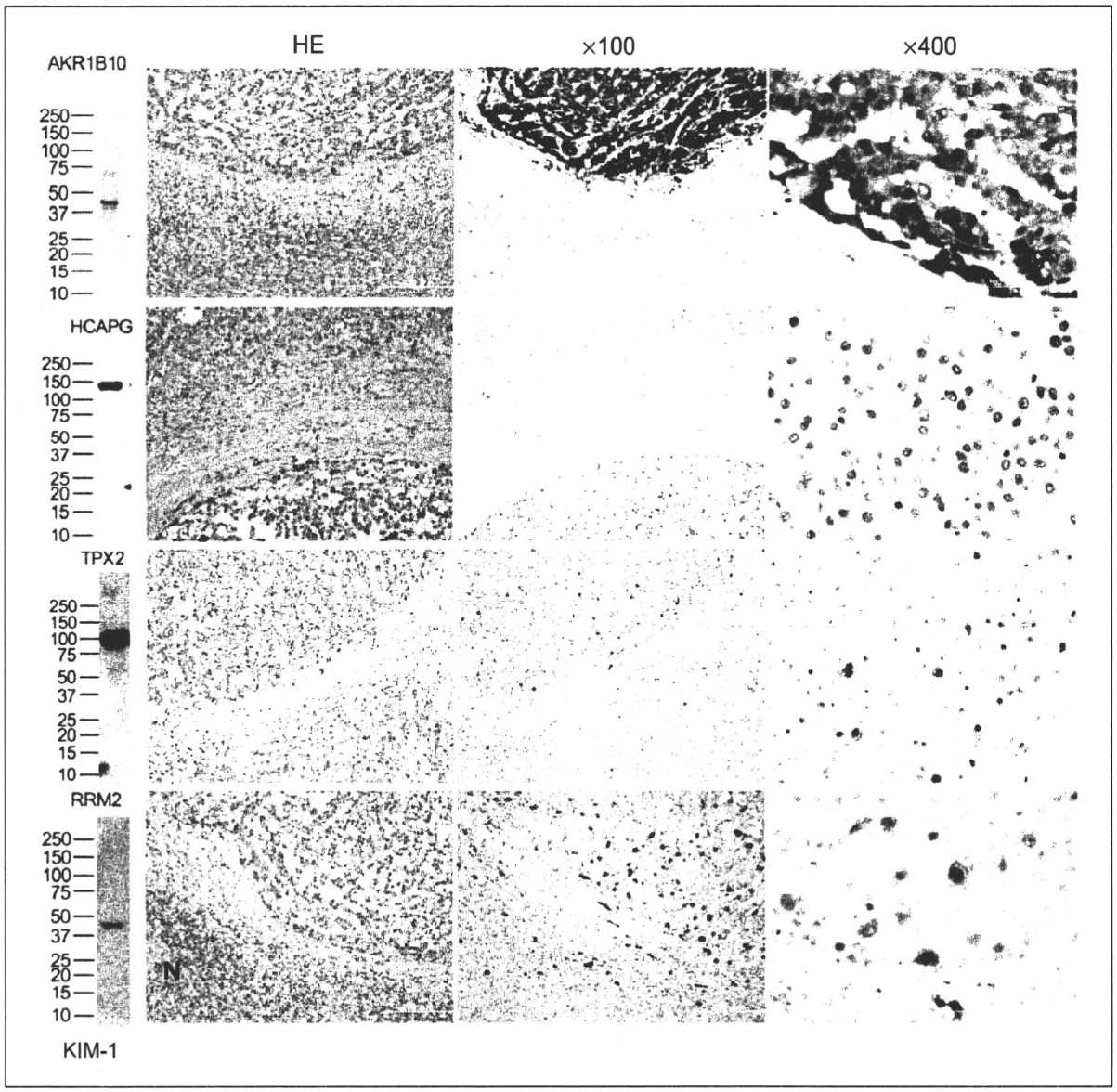

Fig. 5. Protein expression in HCC. Hematoxylin and eosin (HE) staining (original magnification, × 100) and immunoperoxidase staining (original magnifications, × 100 and × 400) of AKR1B10, HCAP-G, RRM2, and TPX2 proteins in HCC and adjacent nontumorous liver tissue. The specificity of antibodies was determined by immunoblotting of the KIM-1 cell lysate (left). N, nontumorous liver.

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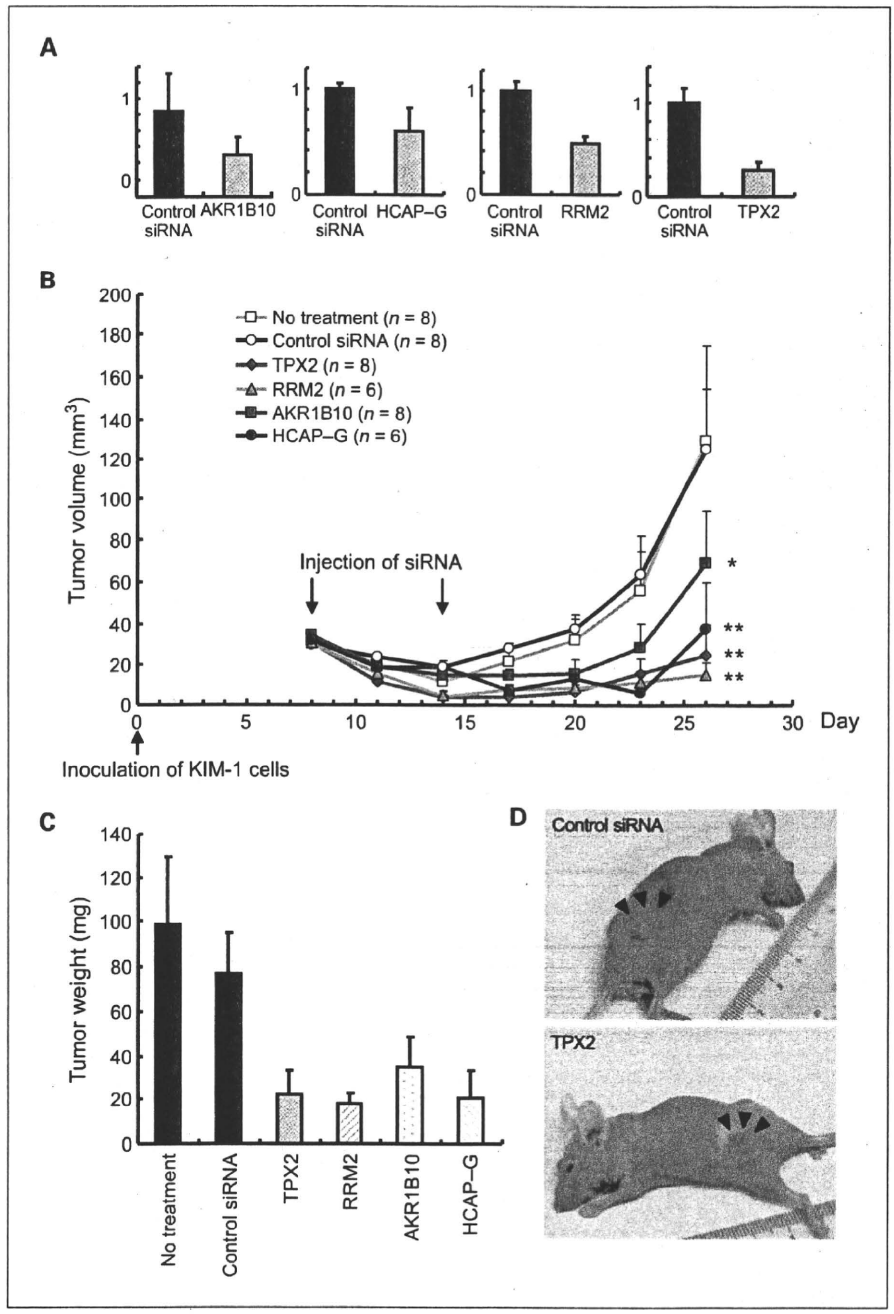

Fig. 6. Suppression of tumor growth by siRNA. A, KIM-1 cells were s.c. inoculated into the flanks of nude mice. Eight days later, control siRNA or siRNA against *AKR1B10*, *HCAP-G*, *RRM2*, or *TPX2* was injected into the developed tumors. The tumors were excised 2 days after the injection, and the expression levels of the indicated genes were determined by real-time PCR. Values of control siRNA were set at 1. B, chronological changes in tumor volume after two injections of the indicated siRNA. Volume of tumors was determined every 3 days as described in Materials and Methods. **, significantly different with a Bonferroni-corrected *P* value of <0.001. *, significantly different with a Bonferroni-corrected *P* value of 0.012. C, weight (mean + SE in mg) of xenografts measured 18 days after the second injection of the indicated siRNA and controls. D, macroscopic appearance of xenografts injected with control siRNA (top) and siRNA against TPX2 (bottom).

although the four selected genes seem to be highly relevant from a biological viewpoint. HCC has been recognized as a single category of disease; however, the overall gene expression patterns seem to differ markedly among individual cases. A search for the genes responsible for the different clinical outcomes of HCC will be the subject of a future study. We used the cell proliferation assay for siRNA-based functional screening. However, the use of other assays capable of evaluating cell motility, migration, drug sensitivity, or cell death may help to identify genes differing in their biological significance. The combination of genome-wide expression and functional screening described here provides a rapid and comprehensive approach that could be applicable for studies of various aspects of human cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Long-term Recurrence-free Survival in a Patient with Primary Hepatic Carcinosarcoma: Case Report with a Literature Review

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A 72-year-old man was found to have a 40 mm mass in liver segment VIII during follow-up abdominal ultrasonography for type C viral hepatitis. Abdominal ultrasound showed a well-defined mass containing a cystic component, and computed tomographic hepatic arteriography showed heterogeneous enhancement except for cystic necrosis. Under a pre-operative diagnosis of atypical hepatocellular carcinoma (HCC), partial resection of liver segment VIII was performed. The encapsulated tumor consisted of a peripheral solid component with a central necrotic area. Histologically, the solid component had a two-layer structure, an HCC component in the external area and a sarcomatous component with neoplastic osteoid formation in the internal area, showing histological transition. Immunohistochemically, the HCC component was positive for hepatocyte antigen and negative for vimentin. The Ki-67 labeling index was found to increase from 5% to 58% with increasing histologic atypia. The sarcomatous component was positive for vimentin and negative for pan-keratin and hepatocyte antigen, with a Ki-67 labeling index of >90%. These findings led to a diagnosis of primary hepatic carcinosarcoma. Although previously reported patients with hepatic carcinosarcoma showed early metastasis with a very poor outcome, this patient has remained free of recurrence for 30 months, which is the longest recurrence-free survival time recorded for this type of cancer. Since relatively early-stage hepatic carcinosarcoma rarely seems to present as a small tumor showing a concentric growth pattern, we report this case with a review of the literature.

Key words: hepatic carcinosarcoma – hepatocellular carcinoma – osteosarcoma – concentric growth pattern

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INTRODUCTION

Primary hepatic carcinosarcoma is defined as a malignant tumor containing an intimate mixture of carcinomatous (either hepatocellular or cholangiocellular) and sarcomatous elements (1). This neoplasm is very rare, and only 20 previous cases have been reported in the English literature (2–17). Here, we describe an additional case, in which it was possible to immunohistochemically demonstrate both the carcinomatous and the sarcomatous components. This neoplasm has a significantly poorer prognosis than hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC), being associated with a high frequency of early metastasis. The present case was encountered a patient who underwent surgical resection for primary hepatic carcinosarcoma. This patient has survived without recurrence for 30 months, which is the longest disease-free period among previously reported cases of hepatic carcinosarcoma. The tumor was composed of an HCC component in its external area and an osteosarcoma component in its internal area, showing histological transition between the two areas. Since the tumor was small in size and showed a concentric growth pattern of carcinoma and sarcoma, which appears to be a very rare presentation of relatively early-stage hepatic carcinosarcoma, and the patient showed prolonged survival, we report this case with a review of the literature.

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CASE REPORT

Clinical Summary

A 72-year-old man was found to have a 40 mm mass in liver segment VIII during follow-up abdominal ultrasonography for type C viral hepatitis at a local hospital, and he was therefore admitted to the National Cancer Central Hospital, Tokyo, Japan. He had no history of blood transfusion or alcoholism. Laboratory data indicated that his serum levels of total protein, total bilirubin, alkaline phosphatase and alpha-fetoprotein were within normal limits, but the serum levels of aspartate aminotransferase (50 IU/ml), alanine aminotransferase (74 IU/ml) and protein induced by the vitamin K absence or antagonist II (46 mAU/ml) were elevated. Serum markers for hepatitis B were negative. Abdominal ultrasound revealed a well-defined, mosaic-patterned mass, 40 mm in diameter, containing a cystic component, in liver segment VIII (Fig. 1A). Abdominal angiography showed a ring-like heterogeneous tumor stain with arterial neovascularity. The tumor appeared as a perfusion defect on computed tomography (CT) during arterial portography and showed marked enhancement at the periphery on CT hepatic arteriography, the central portion being visualized as heterogeneous hypoattenuation (Fig. 1B). Under a preoperative diagnosis of atypical HCC with central necrosis, partial resection of liver segment VIII was performed. At present, 30 months after surgery, the patient remains well without any evidence of tumor recurrence.

Pathological Findings

Macroscopically, the resected tumor measured 4.5 × 3.8 × 3.5 cm. Its cut surface showed a yellowish-white, elastic-hard solid component in the peripheral area, and a cavity structure in the central area with degenerated and necrotic tissue containing dark brown and serous material. The tumor was completely surrounded by a fibrous capsule (Fig. 2A).

For pathological examination, the surgically resected specimen was fixed in 10% formalin for 3 days at room temperature. The entire tumor was then cut into slices at intervals of 0.7–1.0 cm, and all the tumor-containing sections were routinely processed and embedded in paraffin to examine their histologic characteristics. Immunohistochemical studies were carried out on formalin-fixed, paraffin-embedded tissues. The antibodies employed included those against pan-keratin (clone AE1/AE3; Dako Cytomation, Glostrup, Denmark; dilution 1:100), cytokeratin 7 (clone OV-TL12/30; Dako; dilution 1:500), hepatocyte antigen (clone OCH1E5; Dako; dilution 1:100), vimentin (clone V9; Dako; dilution 1:200), alpha-smooth muscle actin (α-SMA) (clone 1A4; Dako; dilution 1:100), desmin (clone D33; Dako; dilution 1:100), h-caldesmon (clone hDD; Dako; dilution 1:100), chromogranin A (polyclonal antibody; Dako; dilution 1:500), synaptophysin (polyclonal antibody; Dako; dilution 1:100), CD31 (clone JC/70A; Dako; dilution 1:50), CD34 (clone MY10; Becton–Dickinson; dilution 1:100) and Ki-67 antigen (clone MIB-1; Dako; dilution 1:100). After deparaffinization and rehydration, the sections were developed using the labeled streptavidin-biotinylated antibody technique and visualized with diaminobenzidine using conventional methods.

Histologically, the tumor was covered with a fibrous capsule, and the solid component had a two-layer structure

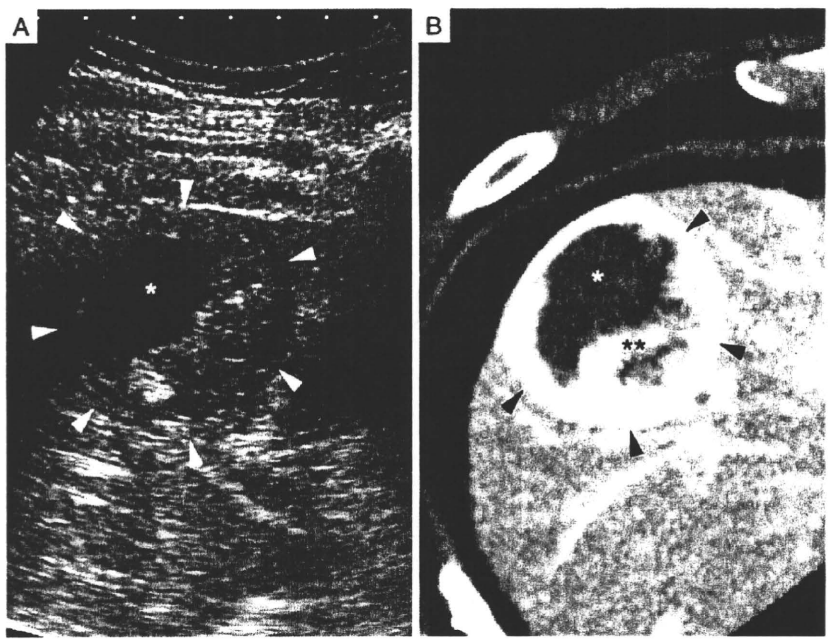

Figure 1. (A) Abdominal ultrasound image shows a well-defined mass with a cystic component (*) in segment VIII of the liver. The solid component shows a heterogeneous area with a mosaic pattern (white arrowhead). (B) Computed tomography hepatic arteriography shows a well-defined tumor consisting of a marginal well-enhanced area (black arrowhead) with an internal poorly enhanced area (**) and a cystic area (*).

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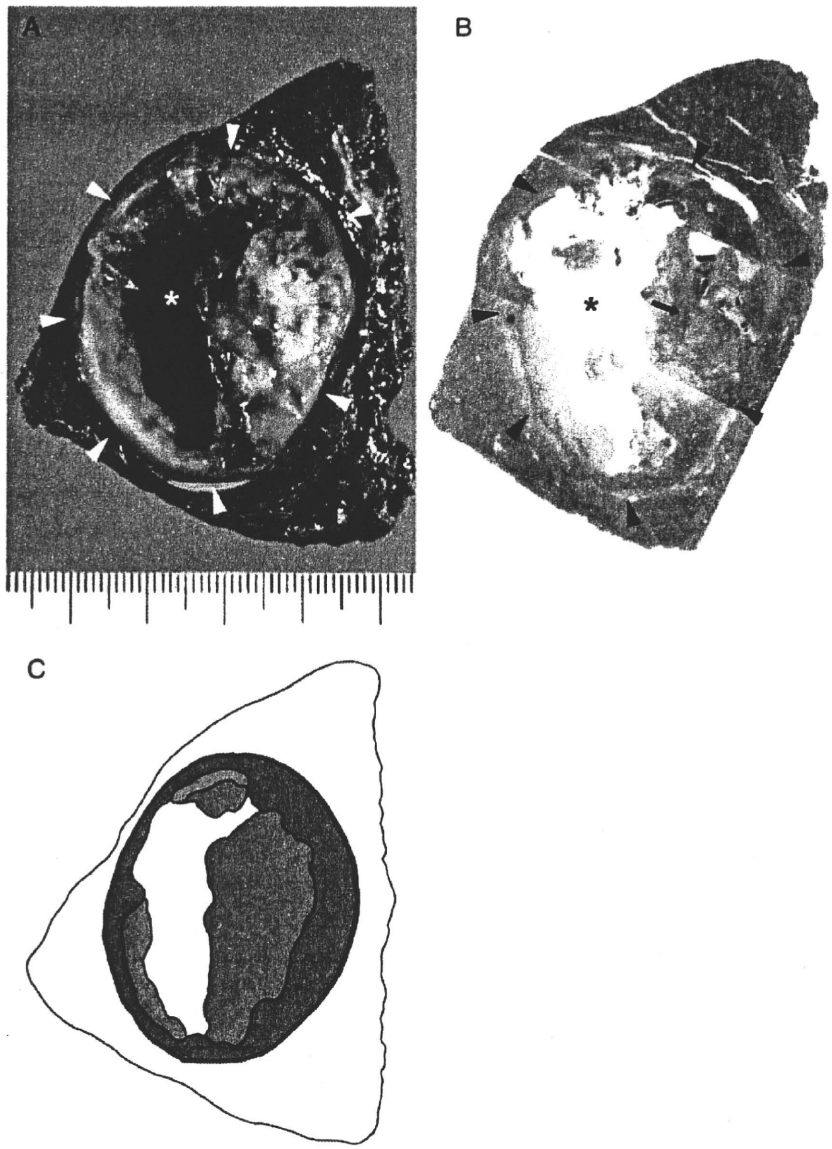

Figure 2. (A) Gross view shows a marginal yellowish viable solid tumor (white arrowhead) and a central cavity with necrotic and degenerative tissue (*). (B) Low-magnification view of the tumor (corresponding to A) shows a cavity area (*) and a solid area (black arrowhead) with neoplastic osteoid tissue (black arrow). (C) Schematic illustration showing the cut surface of the resected specimen. The red area represents the moderately to well-differentiated HCC component, the green area the transitional component and the blue area the sarcoma component. HCC, hepatocellular carcinoma.

with different characteristics in the external and internal areas (Fig. 2B). The external area of the solid component comprised mainly moderately to well-differentiated HCC (Edmondson grades II > I) growing with a thin trabecular and pseudo-glandular pattern in the area adjacent to the capsule and with a thick trabecular pattern in the inner area (Fig. 3A). The internal area of the solid component contained a sarcomatous element consisting of fusiform cells, occasional bizarre cells and scattered foci of neoplastic osteoid tissue (Fig. 3B–D). In part of the boundary area between the carcinomatous and the sarcomatous components, there was a transitional component comprising tumor cells with a pleomorphic appearance admixed with anaplastic giant cells, which represented poorly differentiated HCC and undifferentiated carcinoma components (Edmondson grades III and IV) (Fig. 3B). In other parts of the boundary area, the sarcomatous component infiltrated the sinusoidal spaces of HCC (Fig. 3E). At some sites in the marginal area of the tumor, portal vein tumor thrombi were composed of only the HCC component (Fig. 3F). However, none of the tumor cells appeared to invade the tumor vessels, and there was no tumor thrombus in the area of high vascularity within the tumor. The tumor was not exposed on the surface or cut surface of the liver. The background liver showed a pre-cirrhotic state. The results of immunohistochemistry are summarized in Table 1. Both the moderately to

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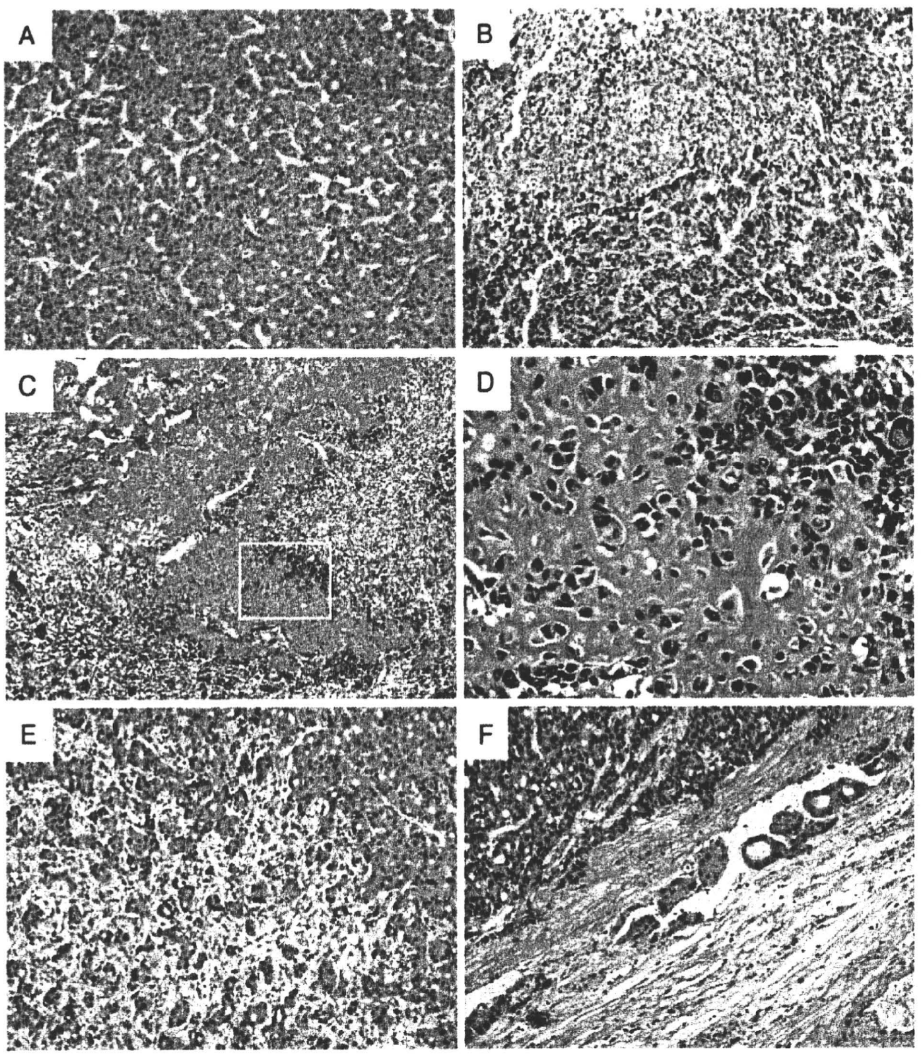

Figure 3. Histological appearance of the tumor (hematoxylin and eosin stain). (A) Moderately to well-differentiated (Edmondson II–I) hepatocellular carcinoma in the external side of the solid component. (B) The transitional component and the sarcoma component. The transitional component consists of poorly differentiated hepatocellular carcinoma and undifferentiated carcinoma components (Edmondson grades III and IV). The sarcoma component consists of fusiform and occasional bizarre cells within the solid component. (C) Neoplastic osteoid formation is observed in the sarcoma component. (D) Higher-magnification view of (C) (squared part). Sarcoma cells proliferate in the neoplastic osteoid tissue. (E) The sarcoma component invading along to the sinusoidal part of the hepatocellular carcinoma. (F) At some sites in the marginal area of the tumor, portal vein tumor thrombi composed of only the hepatocellular carcinoma component are observed.

well-differentiated HCC component and the transitional component were positive for hepatocyte antigen (Fig. 4A and B) and negative for vimentin. The sarcomatous component was positive for vimentin (Fig. 4C and D) and negative for pan-keratin and hepatocyte antigen. The Ki-67 labeling index was 5%, 58% and >90% in the moderately to well-differentiated HCC component (Fig. 4E), the transitional component (Fig. 4F) and the sarcomatous component (Fig. 4F), respectively. The immunohistochemical expression of Ki-67 increased gradually from the moderately to well-differentiated HCC component through to the transitional component, and was highest in the sarcomatous component (Fig. 4E and F). These findings suggested that the sarcomatous component in the internal area of the solid component represented a highly malignant mesenchymal tumor with ossification, showing characteristics clearly different from those of the co-existing HCC component in the external area of the solid component with a transitional component. As HCC and sarcoma components co-existed in the same tumor, the pathological diagnosis was hepatic carcinosarcoma.

DISCUSSION

The World Health Organization (WHO) has defined hepatic carcinosarcoma as a malignant tumor containing an intimate mixture of carcinomatous (either hepatocellular or cholangiocellular) and sarcomatous elements, being considered

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Table 1. Summary of immunohistochemical staining in the moderately to well-differentiated hepatocellular carcinoma, transitional and sarcoma components of the tumor

	HCC component		Sarcoma component
	M–W HCC component (Edmondson II > I)	Transitional component (Edmondson III > IV)	
Hepatocyte antigen	+	+	–
Vimentin	–	–	+
Alpha-smooth muscle actin	–	–	–
Desmin	–	–	–
h-caldesmon	–	–	–
Chromogranin A	–	–	–
Synaptophysin	–	–	–
CK7	–	–	–
AE1/AE3	–	–	–
CD31	–	–	–
CD34	–	–	–
Ki-67 labeling index (%)	5	58	90

HCC, hepatocellular carcinoma; +, positive; –, negative; M–W HCC, moderately to well-differentiated hepatocellular carcinoma.

synonymous with malignant mixed tumor (1). On the other hand, Craig et al. (18) have defined hepatic carcinosarcoma as a malignant tumor with both HCC and a non-spindle cell sarcoma such as osteosarcoma, chondrosarcoma, angiosarcoma or malignant schwannoma. The WHO and Craig et al. stated that carcinosarcoma should be distinguished from carcinomas with foci of spindled epithelial cells and from the rare true 'collision' tumor consisting of carcinomatous and sarcomatous elements of distinct origin and invading each other.

Twenty-one cases of hepatic carcinosarcoma have been reported (2–17), including the present case, which met the histological definition of hepatic carcinosarcoma by the WHO criteria and had immunohistochemically demonstrable carcinoma and sarcoma components (Table 2). The patients were 15 men and 6 women with a mean age of 60 years (range, 40–84 years). The tumor diameter ranged from 4 to 21 cm with a mean of 11.4 cm. Mean patient survival time was 8.7 months, clearly indicating that hepatic carcinosarcomas have a poorer prognosis than conventional HCCs and mass-forming type CCCs. Metastasis, including intrahepatic metastasis, occurred in 15 (70%) of the 21 patients and was identified histologically in 14 of them. In 13 (93%) of these 14 patients, sarcoma components were observed in the metastatic lesions. Of the 15 patients with metastasis including intrahepatic metastasis cases, 14 (93%) died of cancer within 2 years, indicating that hepatic carcinosarcomas with metastasis have a very poor prognosis. In contrast, five (83%) of the six patients without metastasis remain free of recurrence after surgery, although the follow-up period was shorter (mean, 12.6 months), suggesting that aggressive resection is advisable for patients with hepatic carcinosarcoma without metastasis. Among the reported patients, the present one has shown the longest survival, being 30 months at the time of writing.

The proposed histogenetic mechanisms involved in the co-existence of carcinoma and sarcoma components in the same tumor include 'transformation', 'combination' and 'collision' (14). 'Transformation' means that part of a carcinoma undergoes sarcomatous transformation. 'Combination' signifies that a tumor arises from a single stem cell that differentiates into epithelial and mesenchymal tissues. 'Collision' denotes that carcinoma and sarcoma elements of distinct origin invade each other. In the present patient, the encapsulated tumor had a concentric growth pattern, composed of an HCC component in the external area and a sarcoma component in the internal area. In part of the boundary area between the carcinomatous and sarcomatous components, there was a transitional component composed of poorly differentiated HCC and undifferentiated carcinoma components (Edmondson grades III and IV). Moreover, the immunohistochemical expression of Ki-67 increased gradually from the moderately to well-differentiated HCC component through to the transitional component, and was highest in the sarcomatous component. Therefore, we speculated that the mechanism of tumor histogenesis in this case might have been 'transformation'. Further, we speculate that the sarcoma component underwent degeneration and necrotized in the central area due to the rapid cycle of proliferation, resulting in a tissue defect, while invading along the sinusoidal spaces of HCC in the external area of the solid component.

Although the previously reported patients with hepatic carcinosarcoma showed early metastasis and had a very poor

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