Imaging, Diagnosis, Prognosis

CD24 Is a Novel Predictor for Poor Prognosis of Hepatocellular Carcinoma after Surgery

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Abstract

Purpose: To investigate the role of CD24 in tumor invasion and prognostic significance in hepatocellular carcinoma (HCC).

Experimental Design: CD24 expression was measured in stepwise metastatic HCC cell lines, tumor, peritumoral tissues, and normal liver tissues by quantitative real-time PCR and Western blot. The role of CD24 in HCC was investigated by CD24 depletion using small interfering RNA. Tumor tissue microarrays of 314 HCC patients who underwent resection between 1997 and 2000 were used to detect expression of CD24, β-catenin, and proliferating cell nuclear antigen. Prognostic significance was assessed using Kaplan-Meier survival estimates and log-rank tests.

Results: CD24 was overexpressed in the highly metastatic HCC cell line and in tumor tissues of patients with recurrent HCC. Depletion of CD24 caused a notable decrease in cell proliferation, migration, and invasiveness *in vitro*. Univariate and multivariate analyses revealed that CD24 was a significant predictor for overall survival and relapse-free survival. CD24 expression was correlated with poor prognosis independent of α-fetoprotein, tumor-node-metastasis stage, and Edmondson stage. High CD24 expression was significantly associated with cytoplasmic and nuclear accumulation of β-catenin ($P = 0.023$), high tumor proliferative status ($P = 0.018$), and diffused intrahepatic recurrence and distant metastasis ($P = 0.026$). Adjuvant transcatheter arterial chemoembolization after surgery reduced the rate of early recurrence (≤1 year) in CD24$^+$ HCC patients ($P = 0.024$) but had no significant effect in CD24$^-$ patients ($P = 0.284$).

Conclusions: Overexpression of CD24 in HCC was associated with high invasiveness and metastatic potential, high tumor proliferation status, and activation of the Wnt/β-catenin pathway. CD24 may be a novel predictor for poor prognosis of HCC patients after surgery. (Clin Cancer Res 2009;15(17):5518–27)

Hepatocellular carcinoma (HCC) is one of the most prevalent tumor types, and both incidence and mortality rates of HCC have increased in recent years (1). Surgery, which includes liver resection and transplantation, remains the most effective treatment for HCC, but the high rate of recurrence or metastasis after surgery (50-70% at 5 years) hinders further improvements in HCC survival (2). Cancer classification using biomarkers can identify patients with a high risk of recurrence or metastases who may be difficult to identify using traditional clinicopathologic indexes (3). Availability of reliable biomarkers for HCC would help clinicians select therapeutic strategies for individual patients and provide personalized therapy according to the predicted risk of recurrence (4).

CD24 is a small, heavily glycosylated, mucin-like cell surface protein that is expressed in a wide variety of human malignancies, including B-cell lymphoma (5), renal cell carcinoma (6),

Translational Relevance

Cancer classification using biomarkers can identify patients with a high risk of recurrence or metastases who may be difficult to identify using traditional clinicopathologic indexes. Availability of reliable biomarkers for hepatocellular carcinoma (HCC) would help clinicians select therapeutic strategies for individual patients and provide personalized therapy. CD24 is a small, heavily glycosylated, mucin-like cell surface protein that is expressed in a wide variety of human malignancies. In this study, our data showed that CD24 expression was associated with tumor cell invasiveness and metastasis, high proliferative status, and abnormal activation of the Wnt/β-catenin pathway. High expression of CD24 was associated with a poor outcome of HCC patients after surgery, especially in α-fetoprotein–normal patients and those with early-stage and well-differentiated HCC, the outcomes of which are very difficult to predict using conventional clinical indexes. Cancer therapy targeted against CD24 may be a promising strategy for the treatment of HCC metastasis and recurrence.

small cell and non–small cell lung carcinoma (7), nasopharyngeal carcinoma (8), and breast cancer (9). It has been reported that down-regulation of CD24 inhibits proliferation and induces apoptosis in tumor cells (10, 11), whereas increased expression of CD24 increases tumor growth and metastasis (12). In recent years, the prognostic significance of CD24 has been reported in several tumor types (7, 9, 13, 14). However, only limited data on CD24 RNA expression in HCC have been published (15–17), no data on CD24 protein levels in HCC are available, and the mechanism of CD24 involvement in HCC progress has not been elucidated.

In our previous study, we found that CD24 is highly expressed in patients with recurrent HCC using gene microarray data, and suggested that CD24 may act as a biomarker for HCC recurrence, in agreement with another recent report (15). In this study, we explored the expression of CD24 in stepwise metastatic human HCC cell lines and tumor tissues by quantitative real-time PCR (qRT-PCR) and Western blot analyses. The effects of knocking down CD24 with small interfering RNA (siRNA) were also investigated. We further investigated the expression of CD24 and its clinical relevance in 314 HCC patients with long-term follow-up by using tissue microarrays. The expression of proliferating cell nuclear antigen (PCNA) and β-catenin was also investigated. We found that high expression of CD24 in HCC tissues was associated with high proliferation status and β-catenin accumulation and was an independent prognostic factor for HCC patients after surgery.

Materials and Methods

Patients and specimens. Fifty HCC tissue samples used in qRT-PCR were randomly collected from patients undergoing liver resection in our institute between 2000 and 2002. Another 10 hepatitis B virus–related HCC tissues and paired adjacent nontumor cirrhotic liver tissues for further validation were also selected from the same sample bank; 10 normal liver tissues were taken from the biopsy tissues of healthy living donors for transplantation. All specimens were collected in the operating theater immediately (≤15min) after resection of the tumors and then were snap frozen in liquid nitrogen and stored at -80°C.

Tumor specimens used in tissue microarrays analysis were consecutively chosen from 314 HCC patients who underwent liver resection in the Liver Cancer Institute, Zhong Shan Hospital, Fudan University between 1997 and 2000. The clinicopathologic characteristics of the patients were summarized in Supplementary Table S1.

The enrollment criteria for all patients in this study were (*a*) definitive HCC diagnosis by pathology based on WHO criteria (18); (*b*) no prior anticancer treatment; (*c*) surgical resection, defined as complete resection of all tumor nodules with the cut surface being free of cancer by histologic examination (19); (*d*) availability of suitable formalin-fixed, paraffin-embedded tissues and frozen tissues; and (*e*) availability of complete clinicopathologic and follow-up data. Tumor differentiation was defined according to the Edmondson grading system (20). Liver function was assessed by Child-Pugh classification. Tumor staging was defined according to the Sixth Edition of Tumor-Node-Metastasis (TNM) Classification of International Union Against Cancer. Ethical approval for human subjects was obtained from the research ethics committee of Zhong Shan Hospital, and informed consent was obtained from each patient.

Follow-up and tumor recurrences. Patients were followed up every 2 months during the first postoperative year and at least every 3 to 4 months thereafter until March 15, 2008. In tissue microarray group, the median follow-up was 67 months (range, 2-133 months). After surgery, patients with a high risk of recurrence, evidenced by clinical features such as vascular invasion and microsatellite lesions, were given one to three courses of prophylactic transcatheter arterial chemoembolization (TACE; doxorubicin, cisplatin, 5-fluorouracil, and iodized oil). All patients were prospectively monitored by serum α-fetoprotein (AFP) assay, abdomen ultrasonography, and chest X-ray every 1 to 6 months depending on the postoperative time. A computed tomography scan of the abdomen was done every 6 months. Bone scan or magnetic resonance imaging was done if localized bone pain was reported. If recurrence was suspected, a computed tomography scan or magnetic resonance imaging was done immediately. The most common causes of death were recurrence, metastasis, or complicated liver cirrhosis. Patients with confirmed recurrence received further treatment, which followed a set protocol based on the size, site, and number of tumor nodules, and liver function. Briefly, if the recurrent tumor was localized, a second liver resection, radiofrequency ablation, or percutaneous ethanol injection was suggested. If the recurrent tumor was multiple or diffused, TACE was administered. External radiotherapy was given if lymph node or bone metastasis was found; otherwise, symptomatic treatment was provided. Overall survival (OS) was defined as the interval between surgery and death or the last observation taken. For surviving patients, the data were censored at the last follow-up. Relapse-free survival (RFS) was defined as from the date of resection until the detection of tumor recurrence, death, or the last follow-up assessment. For RFS analysis, the data were censored for patients without signs of recurrence. Using 12 months as the cutoff value, all recurrences were divided into early recurrence (*n* = 66) and late recurrence (*n* = 103; ref. 21).

Cell lines. Four human HCC cell lines were used in this study: HepG2 (purchased from the America Type Culture Collection) and MHCC97L, MHCC97H, and HCCLM3, human HCC cell lines with low and high lung metastatic potential, respectively, which were established at our institute (22, 23). All cell lines were routinely maintained in high0glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified incubator under 5% CO_2.

RNA isolation and qRT-PCR. Total RNA was extracted from cell lines and frozen tumor specimens using Trizol reagent (Invitrogen) according to the manufacturer's instructions. CD24 mRNA expression

in HCC cell lines and tumor tissues from 50 HCC patients was measured by qRT-PCR using an ABI7300 instrument (Applied Biosystems). qRT-PCR was done using a SYBR PrimeScript RT-PCR Kit (Takara) according to the manufacturer's instructions. β-Actin was used as an internal control. The primers were as follows: CD24 (Genbank NM_013230) forward primer 5′-TGCTCCTACCCACGCAGATT-3′ and reverse primer 5′-GGCCAACCCAGAGTTGGAA-3′ and β-actin (Genbank NM_001101.3) forward primer 5′-TTGTTACAG-GAAGTCCCTTGCC-3′ and reverse primer 5′-ATGCTAT-CACCTCCCCTGTGTG-3′. Relative mRNA levels were calculated based on the Ct values, corrected for β-actin expression, according to the equation: $2^{-\Delta Ct}$ [ΔCt = Ct (CD24) - Ct (β-actin)]. All experiments were done in triplicate.

Western blot analysis. CD24 protein expression in cell lines and tissue samples was detected by Western blotting. Briefly, cells were washed twice with ice-cold PBS and frozen tumor specimens were ground under liquid nitrogen. Total protein was extracted in lysis buffer for 45 min on ice. Equal amounts of protein were separated by 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore) using a mini trans-blot apparatus (Bio-Rad Laboratories). Membranes were blocked with PBS-0.05% Tween 20 containing 5% nonfat dry milk for 1 h and incubated with monoclonal mouse anti-human CD24 (1:500; Santa Cruz Biotechnology) or glyceraldehyde-3-phosphate dehydrogenase antibody (1:5,000; Chemicon) for 2 h at room temperature. Membranes were then washed three times with PBS-0.05% Tween 20 and incubated with horseradish peroxidase–conjugated goat anti-mouse IgG (Chemicon) at a 1:10,000 dilution for 1 h. Blots were developed using an enhanced chemiluminescence kit (Pierce). Each experiment was repeated at least three times.

siRNA-mediated CD24 silencing. The target siRNA sequences used were as follows: CD24-149 5′-CAACUAAUGCCACCACCAATT-3′, CD24-1934 5′-CAGUAGUCUUGAUGACCAATT-3′, and control 5′-UU-CUCCGAACGUGUCACGU-3′. RNA duplexes were synthesized by Genepharma. MHCC97H and HCCLM3 were used in siRNA analysis and transfection of siRNA was done using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

MTT assay, cell migration, and Matrigel invasion assays. Cells were aliquoted into a 96-well plate (5,000 per 100 µL/well), incubated for 24 h, and treated with CD24 siRNA for 6 h. After replacing the medium with 100 µL DMEM containing 10% fetal bovine serum, 10 µL CCK-8 solution (Dojindo) was added at the indicated time points and plates were incubated for a further 2 h. The absorbance at 450 nm was measured to determine the number of viable cells in each well. All experiments were done three times.

Cell migration was evaluated using the scratch wound assay. Cells were cultured for 2 days to form a tight cell monolayer and then serum starved for 16 h. Following the serum starvation, the cell monolayer was wounded with a 200 µL plastic pipette tip. The remaining cells were washed twice with culture medium to remove cell debris and incubated at 37°C with normal serum–containing culture medium. At the indicated times, migrating cells at the wound front were photographed and the percentage of the cleaned area at each time point compared with time 0 was measured using Image-Pro Plus version 6.2 software.

Invasive potential was measured using an *in vitro* Boyden chamber assay with some modifications. Briefly, 5×10^4 siRNA-transfected cells in 0.1 mL serum-free DMEM were added to the well of 8 µm pore membrane Boyden chamber (Costar) coated with Matrigel. The bottom chamber contained 10% fetal bovine serum in DMEM as a chemoattractant. Cells were allowed to invade for 48 h and any cells that had not penetrated the filters were then removed by scrubbing with cotton swabs. Chambers were fixed for 20 min at room temperature with 4% formaldehyde in PBS, stained in 0.1% crystal violet for 30 min, and rinsed in water. Cells that migrated to the bottom surface of the filter were considered to have invaded through the matrix and were counted
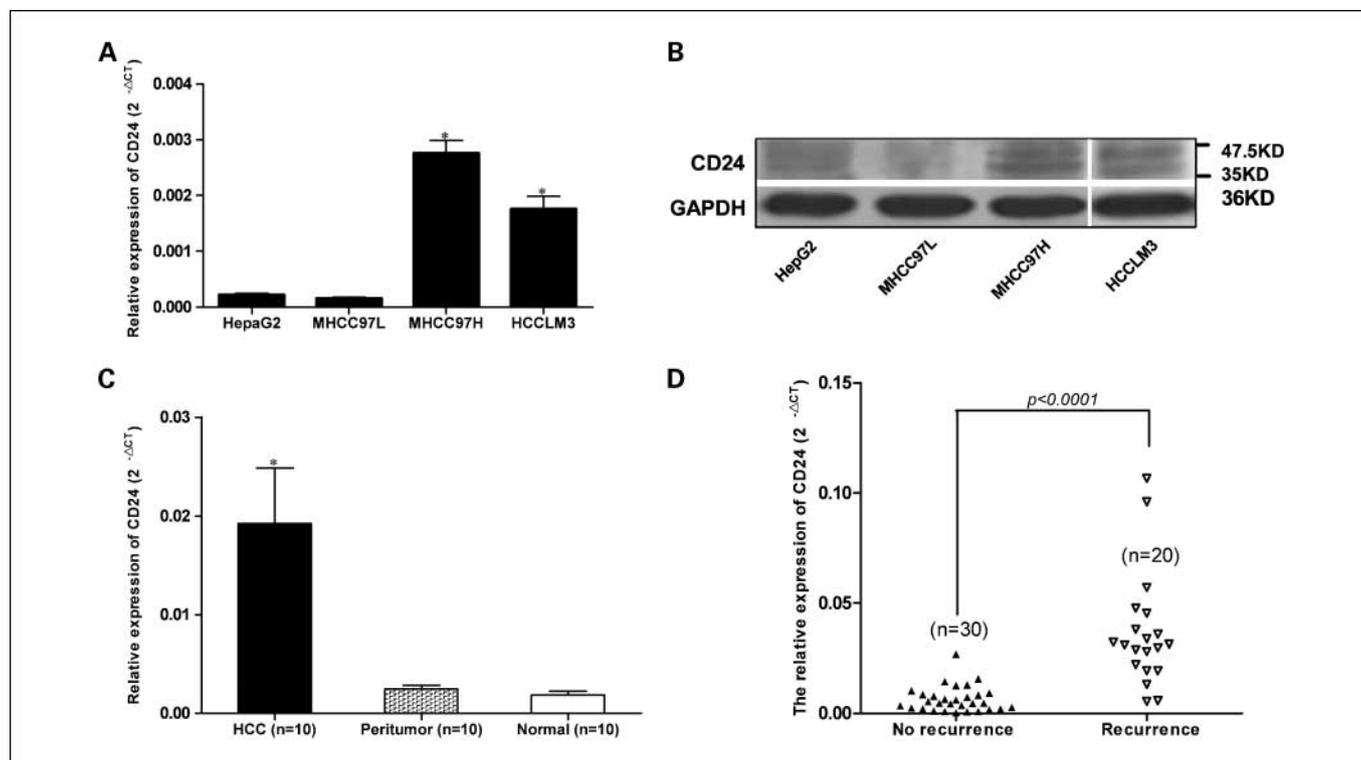
Fig. 1. Expression of CD24 in HCC cell lines and tissue samples. *A,* relative CD24 mRNA levels in different cell lines by qRT-PCR analysis. *, $P < 0.05$, compared with HepaG2 and MHCC97L. *B,* Western blot analysis of CD24 protein level in HepG2, MHCC97L, MHCC97H, and HCCLM3. *C,* relative CD24 mRNA levels among normal liver, peritumoral tissues with cirrhosis, and HCC tissues. CD24 was higher expressed in HCC tissues compared with peritumoral tissues and normal liver tissues. *D,* scatter plot of CD24 mRNA expression in 50 HCC tissue samples. Patients suffering HCC recurrence (20 of 50) had higher CD24 mRNA expression than those without recurrence (30 of 50).

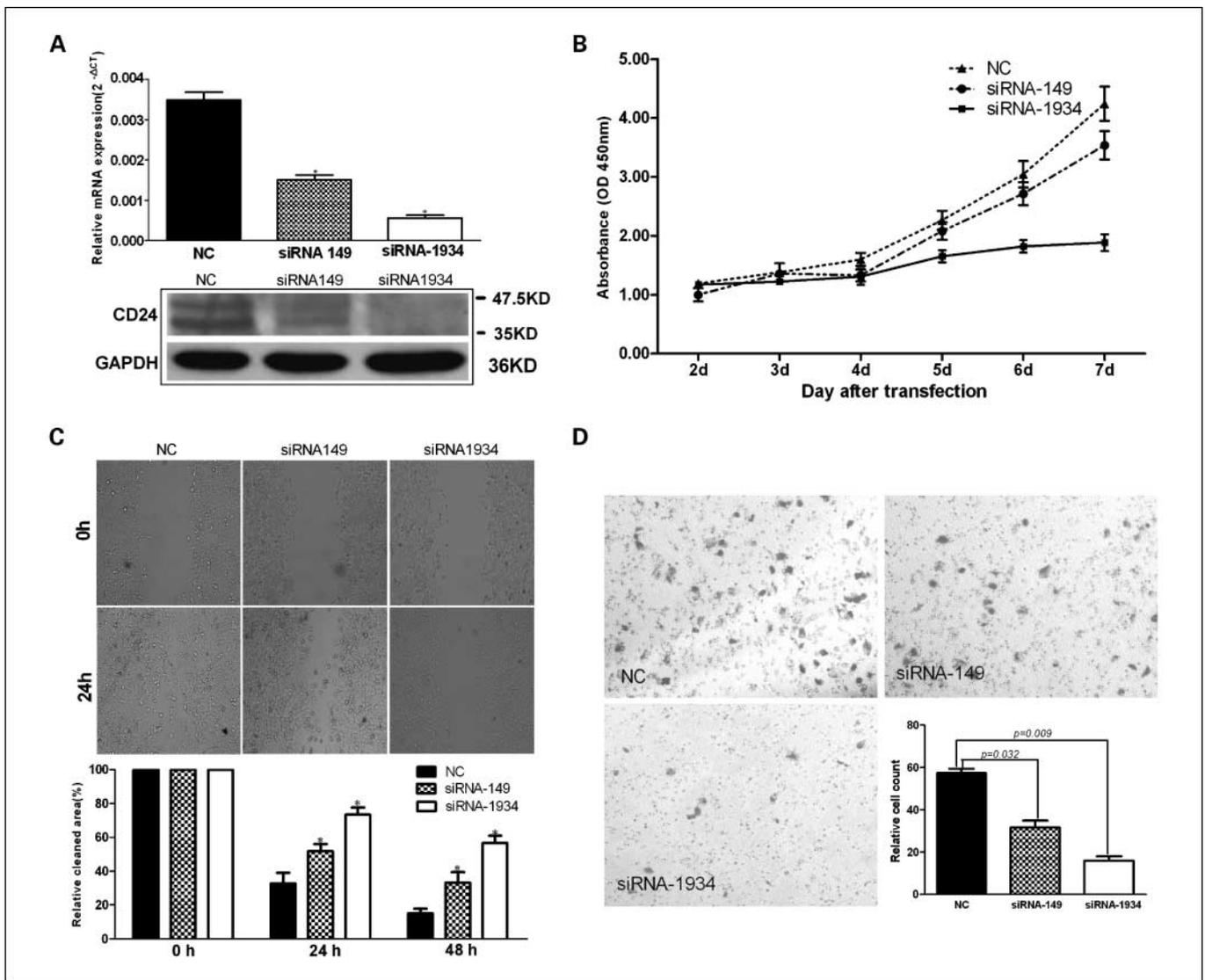**Fig. 2.** Function analysis after CD24 siRNA in MHCC97H. CD24 expression in MHCC97H was decreased significantly after treated by siRNA for 72 h according to qRT-PCR and Western blot analysis. *A,* cell proliferation was detected by MTT assay. Mean ± SD from individual experiments with three replicate assays. *B,* monolayer of negative siRNA or CD24 siRNA MHCC97H cells was abraded and then monitored at 0, 24, and 48 h for wound channel closure. The cleaned area was measured and plotted as the percentage of the original time point (0 h). Mean ± SD from three independent experiments (*C*). Invasive behavior tested by transwell Matrigel invasion assays (*D*). Magnification, ×100 (*C*) and ×200 (*D*).

under a light microscope. Assays were done three times using triplicate wells.

Tissue microarrays and immunohistochemistry. Tissue microarray blocks were constructed as described previously (24). Briefly, all HCC tissues were reviewed by two histopathologists and representative tumor areas free from necrotic and hemorrhagic materials were premarked in the paraffin blocks. Two core biopsies of 1 mm in diameter were taken from the donor blocks and transferred to the recipient paraffin block at defined array positions. Four different tissue microarray blocks were constructed. In addition, we build a tissue microarray chip containing 100 cases of peritumoral livers, which were randomly chosen from 314 cases. Consecutive sections of 4 μm thickness were taken on 3-aminopropyltriethoxysilane–coated slides (Shanghai Biochip).

The primary antibodies used in immunohistochemistry were CD24 (monoclonal mouse, clone 24C02/SN3b, diluted 1:100; Neomarkers), β-catenin (monoclonal mouse, clone 14, diluted 1:1,000; Transduction Lab), and PCNA (monoclonal mouse, clone PC-10, diluted 1:100;

DAKO). Immunohistochemistry was carried out using a two-step protocol (Novolink Polymer Detection System; Novocastra) as described previously (24, 25). Briefly, after microwave antigen retrieval, tissues were incubated with primary antibodies for 60 min at room temperature or overnight at 4°C. Following 30 min incubation with secondary antibody (Novolink Polymer RE7112), sections were developed in 3,3′-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. Negative control slides in which the primary antibodies were omitted were included in all assays.

To validate the concordance between tissue microarrays and whole tumor sections, we further detected the expression of all the biomarkers in 50 corresponding whole tumor sections randomly chosen from the 314 cases by immunohistochemistry.

Evaluation of immunohistochemical variables. Immunohistochemical staining was assessed by two independent pathologists without knowledge of patient characteristics. For CD24 staining, we determined the percentage of cells with a positive score for staining in the cytosol or membrane in the whole biopsy cylinder. In detail,

the scoring procedure was as follows: the staining intensity was first scored (0, negative; 1, weak; 2, moderate; 3, high) and then the percentage of positive cells was scored (0, 0% positive cells; 1, 1-10% positive cells; 2, 11-50% positive cells; 3, >50% positive cells). The final score of each sample was obtained by multiplying the scores for staining intensity and percentage of cells. Samples were classified as negative when the final scores were 0 to 3 and positive when 4 to 9. For β-catenin, we regarded >10% cells with cytoplasmic or nuclear staining as a positive result (26). For PCNA, a positive result was re-corded when >50% of the cells exhibited strong staining (27). All cases that were given a different score by the two pathologists were discussed at a multiheaded microscope until consensus was reached. Duplicate spots for each tumor showed a good level of homogeneity for the percentage of cells stained and intensity of staining. The higher score was taken as the final score in cases of a difference between duplicate tissue cores.

Statistical analysis. Statistical analyses were done using SPSS 15.0 for windows (SPSS). Cumulative survival time was calculated by the
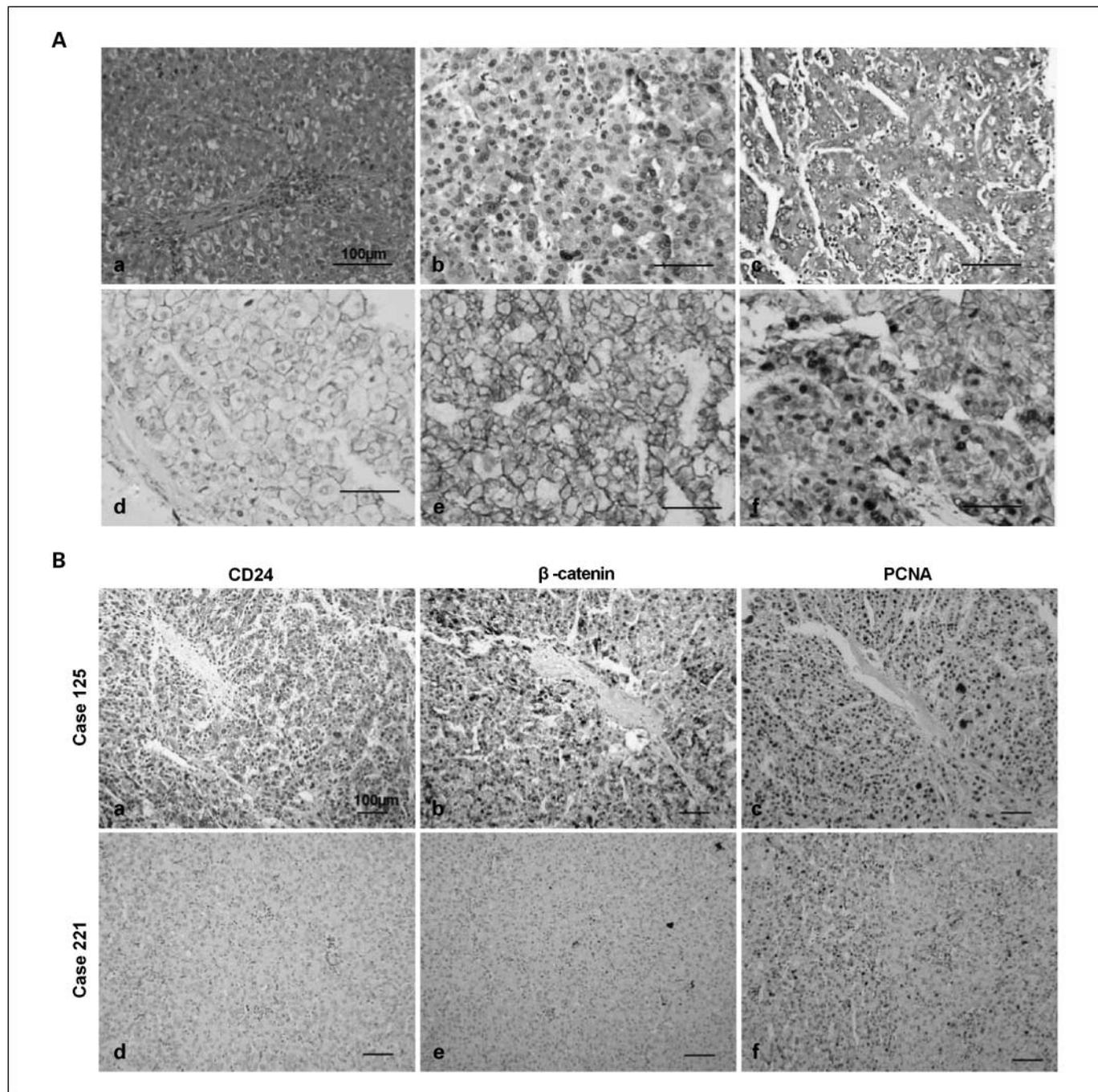
Fig. 3. Expression of CD24, PCNA, and β-catenin in tissue microarrays by immunochemistry analysis. *A,* H&E staining (*a*), membrane staining for CD24 (*b*), cytoplasm expression (*c*), β-catenin expression in membrane (*d*), cytoplasm (*e*), and nuclear (*f*). Magnification, ×200. Bar, 100 μm. *B,* CD24^{+} HCC tissue (*a*) with β-catenin accumulation in cytoplasm and nuclear (*b*) with high PCNA expression (*c*), whereas CD24^{-} HCC tissue (*d*) with β-catenin–negative expression (*e*) low PCNA expression (*f*). Magnification, ×100. Bar, 100 μm.

Table 1. Univariate analyses of factors associated with survival and recurrence (*n* = 314)

Variables	OS		RFS	
	Hazard ratio (95% confidence interval)	*P*	Hazard ratio (95% confidence interval)	*P*
Sex (male vs female)	0.94 (0.64-1.40)	0.772	1.23 (0.79-1.93)	0.361
Age, y (>52 vs ≤52)	0.90 (0.68-1.19)	0.461	0.87 (0.64-1.18)	0.374
Hepatitis B surface antigen (positive vs negative)	1.20 (0.81-1.77)	0.355	1.23 (0.81-1.88)	0.323
Hepatitis C virus (positive vs negative)	1.45 (0.46-4.53)	0.527	1.89 (0.70-5.09)	0.211
Child-Pugh score (B vs A)	2.73 (0.68-11.02)	0.158	2.68 (0.66-10.83)	0.166
Liver cirrhosis (yes vs no)	1.73 (0.92-3.27)	0.092	1.87 (0.95-3.65)	0.069
γ-Glutamyl transferase, units/L (>54 vs ≤54)	1.95 (1.45-2.61)	<0.0001	1.54 (1.13-2.09)	0.006
Alanine aminotransferase, units/L (>75 vs ≤75)	1.18 (0.77-1.79)	0.446	1.30 (0.84-2.00)	0.235
AFP, ng/mL (>20 vs ≤20)	1.13 (0.85-1.50)	0.406	1.04 (0.77-1.40)	0.81
Tumor differentiation (III-IV vs I-II)	1.53 (1.13-2.08)	0.006	1.38 (0.99-1.92)	0.055
Tumor encapsulation (none vs complete)	1.38 (1.03-1.83)	0.029	1.01 (0.74-1.37)	0.948
Tumor size, cm (>5 vs ≤5)	1.58 (1.19-2.10)	0.002	1.30 (0.96-1.76)	0.091
Tumor number (multiple vs single)	1.78 (1.24-2.55)	0.001	1.61 (1.08-2.39)	0.018
Vascular invasion (yes vs no)	1.95 (1.35-2.80)	0.0003	1.76 (1.17-2.65)	0.007
TNM stage (II + III vs I)	1.97 (1.46-2.66)	<0.0001	1.88 (1.36-2.60)	0.0001
Satellite (yes vs no)	2.93 (1.91-4.48)	<0.0001	1.59 (0.93-2.72)	0.089
Cancer of Liver Italian Program stage (2 + 3 + 4 vs 0 + 1)	1.64 (1.18-2.28)	0.003	1.43 (0.99-2.05)	0.054
Adjuvant TACE (yes vs no)	0.87 (0.65-1.17)	0.358	0.99 (0.73-1.34)	0.939
CD24 (positive vs negative)	1.80 (1.35-2.41)	<0.0001	2.57 (1.89-3.49)	<0.0001

NOTE: Cox proportional hazards regression model.

Kaplan-Meier method and analyzed by the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. The χ^2 test, Fisher's exact probability, and Student's *t* test were used for comparison between groups. *P* < 0.05 was considered statistically significant.

Results

CD24 expression in HCC cell lines and HCC tissues. CD24 expression increased in parallel with the metastatic potential of HCC cell lines: the CD24 mRNA and protein level in MHCC97H and HCCLM3 cells was significantly higher than that in MHCC97L and HepG2 cells (*P* < 0.05; Fig. 1A and B).

We found that the expression of CD24 was higher in HCC tissues than in peritumoral tissues and normal liver tissues (*P* < 0.05; Fig. 1C). There were no significant differences between peritumoral tissues and normal liver tissues. The patients suffering HCC recurrence (20 of 50) had higher expressive levels of CD24 mRNA than those without recurrence (30 of 50; *P* < 0.0001; Fig. 1D).

Inhibition of CD24 by siRNA attenuated proliferation and reduced migration and invasiveness of HCC cells. To further examine the functional role of CD24 in HCC cells, MHCC97H and HCCLM3 were transfected with siRNA duplexes against CD24. In MHCC97H, successful knockdown of CD24 expression was confirmed by qRT-PCR and Western blot analysis (Fig. 2A). Down-regulation of CD24 by siRNA caused significant suppression of cell proliferation in the 72 h after transfection (*P* < 0.001; Fig. 2B). In wound-healing migration assay, microscopic examination at 24 and 48 h revealed a significant delay in the wound closure rate of CD24-siRNA MHCC97H compared with the control cell line, in which the wound was closed by 48 h (Fig. 2C). Decreased CD24

expression was also accompanied by a decrease in the invasiveness of HCC cells as measured by transwell Matrigel invasion assays (*P* < 0.001; Fig. 2D). The similar effects of inhibition of CD24 by siRNA were also observed in HCCLM3 (Supplementary Fig. S1A-D).

Immunohistochemical characteristics. When observed by H&E staining and excluding necrotic, hemorrhagic, and fibrotic components, the cancer cells within a tumor were relatively homogeneous. CD24 stained the membrane or cytoplasm of tumor cells and exhibited a variety of staining patterns with respect to staining intensity and percentage of positive cells (Fig. 3A). Most cells showed diffuse or focal staining patterns with intermediate or strong staining intensity. We observed 33.12% (104 of 314) with positive score for CD24 in 314 HCC patients. Most of peritumoral tissues showed negative or low CD24 expression; only 13 of 100 cases showed positive expression (Supplementary Fig. S2A and B). There was significant difference in CD24 expression between tumor and peritumoral tissues (*P* < 0.0001).

There were three patterns of β-catenin expression: membranous, cytoplasmic, and nuclear staining (Fig. 3A). Cytoplasmic and nuclear staining of β-catenin was observed in 40.13% (126 of 314) of patients. PCNA was localized in the nucleus in 28.03% of cases that showed positive staining. Representative images and statistics are shown in Fig. 3 and Supplementary Table S1. The expression levels of these three biomarkers in the tissue microarray analysis were consistent with expression observed in the corresponding whole tumor sections (data not shown).

Prognostic factors. For the whole study population, the OS and RFS rates were 68.10% and 59.55% at 3 years, 54.30% and 48.81% at 5 years, 43.51% and 43.36% at 7 years, and 39.10% and 39.35% at 9 years, respectively. On univariate analysis,
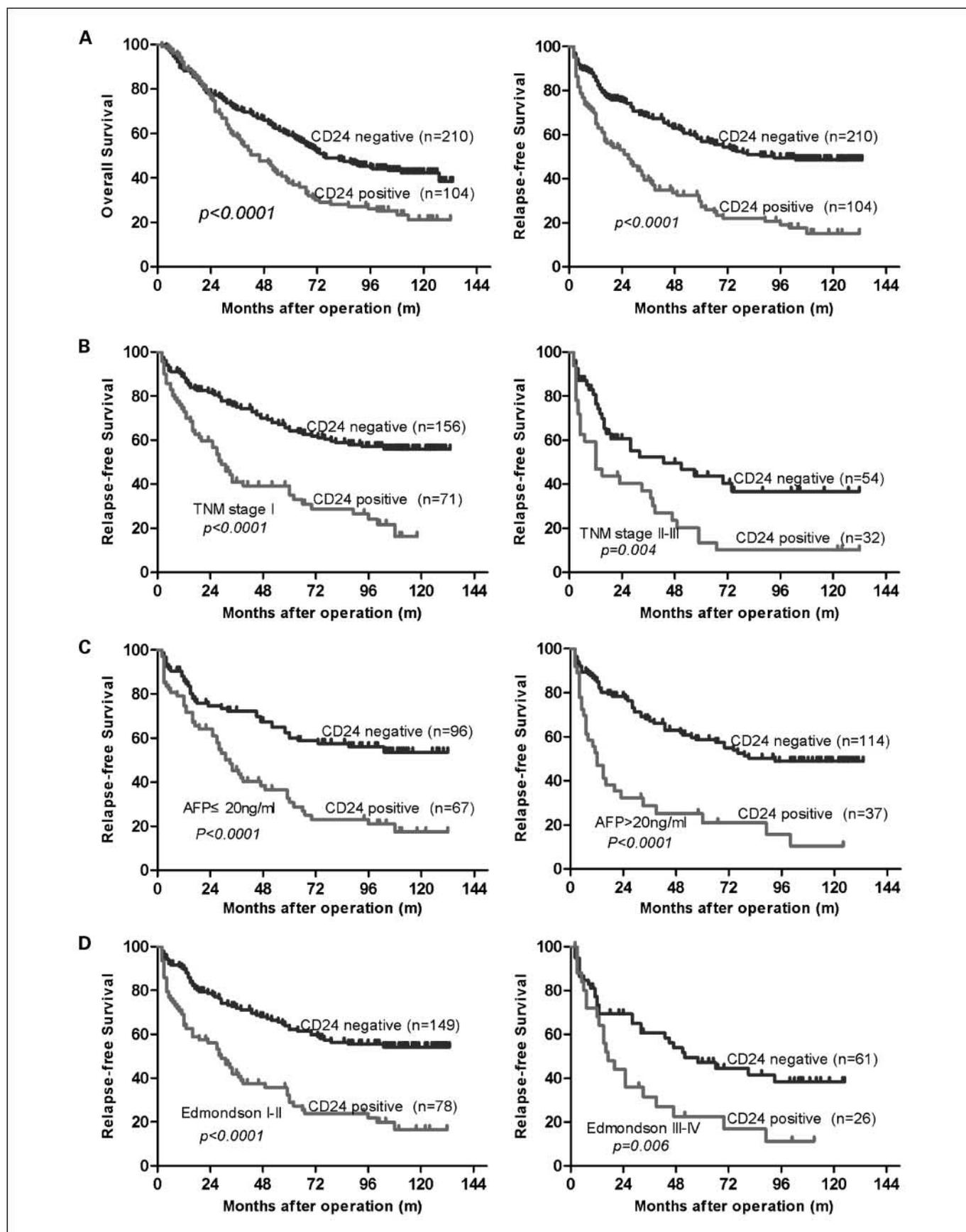
Imaging, Diagnosis, Prognosis

Fig. 4. Kaplan-Meier analysis of RFS and OS for CD24 expression. Kaplan-Meier analysis of RFS and OS for CD24 expression in 314 cases (*A*). Prognostic role of CD24 in TNM stage I to II and II to III (*B*), AFP ≤20 ng/mL and >20 ng/mL (*C*), and Edmondson I-II and III-IV (*D*).

Table 2. Multivariate analyses of factors associated with OS and RFS (*n* = 314)

Variables	OS		RFS	
	Hazard ratio (95% confidence interval)	*P*	Hazard ratio (95% confidence interval)	*P*
γ-Glutamyl transferase, units/L (>54 vs ≤54)	1.70 (1.26-2.29)	<0.001	1.49 (1.10-2.03)	0.011
Tumor differentiation (III-IV vs I-II)	1.31 (0.96-1.80)	0.088	NA	
Tumor encapsulation (none vs complete)	1.27 (0.94-1.71)	0.117	NA	
Tumor size, cm (>5 vs ≤5)	1.14 (0.84-1.56)	0.390	NA	
Tumor number (multiple vs single)	1.42 (0.98-2.07)	0.063	1.41 (0.95-2.11)	0.090
Vascular invasion (yes vs no)	1.80 (1.24-2.63)	0.002	1.78 (1.18-2.68)	0.006
Satellite (yes vs no)	2.30 (1.45-3.67)	<0.001	NA	
CD24 (positive vs negative)	1.82 (1.35-2.45)	0.0001	2.55 (1.88-3.47)	<0.0001

NOTE: Cox proportional hazards regression model. The clinicopathologic variables were adopted for their prognostic significance by univariate analyses.
Abbreviation: NA, not applicable.

tumor number, tumor size, vascular invasion, encapsulation, differentiation, satellite lesions, TNM stage, serum γ-glutamyl transferase level, and Cancer of Liver Italian Program score were prognostic factors for OS and/or RFS (Table 1).

CD24 was prognostic for OS (*P* < 0.0001) and RFS (*P* < 0.0001; Table 1). The 5-year OS and RFS rates of the CD24⁻ group were significantly higher than those of the CD24⁺ group (62.2% and 59.5% versus 38.5% and 27.3%; *P* < 0.0001 for OS and RFS; Fig. 4A). The median RFS was 27 months for CD24⁺ patients and was not reached for CD24⁻ patients, and the median OS was 46 months for CD24⁺ patients compared with 88 months for CD24⁻ patients.

As TNM stage and Cancer of Liver Italian Program score were associated with several clinical indexes such as tumor size, tumor number, and vascular invasion, we did not enter them into multiple analyses with these indexes to avoid potential bias. On multivariate analysis, tumor CD24 expression status was defined as an independent prognostic factor for both RFS (*P* < 0.0001) and OS (*P* = 0.0001). CD24⁺ patients were more than two times more likely to suffer from relapse than CD24⁻ patients (hazard ratio, 2.55; 95% confidence interval, 1.88-3.47; Table 2).

We further investigated the predictive value of CD24 within subgroups (TNM stage I versus II-III, AFP ≤20 versus >20 ng/mL, Edmondson I-II versus III-IV, and early versus late recurrence) and found that the prognostic significance of CD24 was retained (Fig. 4B-D). In the TNM stage I group, the 5-year RFS rates of CD24⁻ patients was 64.2% compared with 34.6% for CD24⁺ patients (*P* < 0.0001; Fig. 4B). In the AFP ≤20 ng/mL group, the 5-year RFS rate was 60.7% for CD24⁻ patients compared with 30.8% for CD24⁺ patients (*P* < 0.0001; Fig. 5C). For the Edmondson stage I to II group, the 5-year RFS rate was 63.4% for CD24⁻ patients compared with 29.0% for CD24⁺ patients (*P* < 0.0001; Fig. 4D). With respect to time of recurrence, the prognostic significance of CD24 existed in both early recurrence and late recurrence groups (*P* < 0.0001 for both; Supplementary Fig. S3A and B).

Correlation of CD24 expression with PCNA, β-catenin, and clinicopathologic features. A high level of CD24 expression was associated with cytoplasmic or nuclear staining of β-catenin (51 of 75 for high CD24 expression versus 53 of 135 for low CD24 expression; *P* = 0.023). The proportion of patients with high PCNA expression was significantly higher among patients with CD24⁺ HCC than among those with CD24⁻ HCC (38 of 104 versus 50 of 210; *P* = 0.018; Fig. 3B). Patients with high CD24 expression were more likely to have low AFP levels (*P* = 0.002) and had a greater propensity for multiple tumors (*P* = 0.068) and liver cirrhosis (*P* = 0.051). Sites of HCC recurrence were classified into three groups: type I was local recurrence in remnant liver with a single or double lesion (*n* = 119), type II was a multinodular (≥3) or diffuse pattern consisting of many nodules scattered throughout the remnant liver (*n* = 23), and type III was extrahepatic metastasis (*n* = 27). High CD24 expression was associated with type II and III recurrence (30 of 79 for high CD24 versus 21 of 90 for low CD24; *P* = 0.039). Detailed analyses are shown in Supplementary Table S2.

Relationship between CD24 expression and adjuvant TACE. In this study, we found that adjuvant TACE after surgery did not improve the OS and RFS rates of the whole study population (Supplementary Fig. S4A). Further analysis of early and late recurrence groups showed that adjuvant TACE after surgery reduced the early recurrence rate compared with no adjuvant TACE treatment (*P* = 0.033; Supplementary Fig. S4B). To further assess whether CD24 acted as a marker of sensitivity to adjuvant TACE, we repeated the above analyses for subgroups of patients who received adjuvant TACE or did not receive adjuvant TACE. We found that adjuvant TACE reduced the early recurrence rate in CD24⁺ HCC patients (*P* = 0.024) but had no significant effect in CD24⁻ patients (*P* = 0.284; Supplementary Fig. S4C). Adjuvant TACE had no effect on late recurrence rates among CD24⁺ and CD24⁻ patients (*P* > 0.05 for both; Supplementary Fig. S4D).

When compared CD24 expression with the clinicopathologic factors, we found there had no significant difference in peritumoral tissues (Supplementary Table S3).

Discussion

Despite improvements in surveillance and clinical treatment strategies, the prognosis of HCC remains dismal because of its

high recurrence and metastasis rates (2). Molecular classification can help create clinical indices to define the risk of recurrence, and further investigation of these molecular biomarkers would also benefit development of novel therapeutic targets for the treatment of patients with HCC (28).

CD24 was identified as a cluster of differentiation marker of hematopoietic lineages and has subsequently been found to be overexpressed in a large variety of malignant tumors and to be associated with the metastatic phenotype (29). CD24 participates in the regulation of cell-cell and cell-matrix interactions and plays a role in cell proliferation, invasion, and metastasis, which may be based on the CD24-P-selectin interaction (10). Recent research based on transcript profiling indicated that CD24 is highly expressed in HCC and might be a good biomarker for the prediction of HCC (15); this conclusion was also reached by transcript profile analysis in our institute. Until now, systematic investigation of the prognostic significance of CD24 in HCC has not been reported, especially with long-term follow-up and a large number of patients. Moreover, the role of CD24 in HCC progression has not been clearly defined.

In this study, we showed that CD24 was overexpressed in high metastatic HCC cell lines and in patients with HCC recurrence. Depletion of CD24 by siRNA inhibited HCC cell migration and invasion significantly. A positive correlation was found between CD24 expression and type II and III recurrences in HCC patients, which are mainly caused by dissociation of tumor cells from the primary tumor and dissemination into other sites during tumor progression (30). CD24$^+$ tumor cells may be more prone to invade into the stroma and vasculature and then metastasize to remote organs, which can be testified by the results of CD24 siRNA. We also found an association between CD24 expression and high levels of PCNA on clinical specimens. PCNA is a nuclear protein that is synthesized in the G_1-S phase of the cell cycle, and high proliferative activity of tumor cells is known to be associated with tumor progression (27). These data support a dual-functional model for CD24 in cancer: both in proliferation and survival at the tumor origin and metastases as well as adhesive function during hematogenous dissemination of cancer cells, which were accordance with previous studies in bladder, colorectal, and pancreatic cancer (10, 11). In addition, our tissue microarray results showed that high CD24 expression was associated with cytoplasmic and nuclear accumulation of β-catenin, indicating activation of the Wnt/β-catenin pathway (31, 32), which plays a pivotal role during hepatocarcinogenic progression and tumor chemoresistance (31–33). There were 33.12% (104 of 314) patients with positive CD24 expression; these patients had higher recurrence rates and more dismal outcome after operation. All these findings indicate that the expression of CD24 is significantly related with aggressive phenotype of HCC; CD24 can be a new prognostic marker for HCC after operation.

HCC is a heterogeneous disease and patients with the same TNM stage of disease, histopathologic features of tumor, and treatment strategy (such as curative resection) can have different clinical outcomes (34). In particular, it is hard to predict which individuals will have tumor relapse after surgical treatment for early-stage HCC (TNM stage I). When we stratified the patient cohort by TNM stage, we found that the prognostic significance of CD24 still existed in TNM stage I HCC patients: the 5-year RFS for CD24$^+$ and CD24$^-$ patients was 34.6% versus 64.2% ($P < 0.0001$) in the TNM stage I group. The same association

existed in the well-differentiated tumor group. AFP is the most widely used tumor marker in the diagnosis and management of HCC and remains the best marker to monitor recurrence and metastasis in AFP$^+$ HCC patients after surgery (35). Until now, there was no ideal tumor marker with prognostic value in the 30% to 40% of HCC patients with normal serum AFP, in whom it is very difficult to monitor recurrence and metastasis after surgery (4, 36). When we further explored the predicted prognostic potential of CD24, we found that patients in the AFP$^-$ group can be stratified according to CD24 status into two groups with substantially different 5-year RFS (30.8% and 60.7% for CD24$^+$ and CD24$^-$ patients, respectively; $P < 0.0001$). The predictive significance of CD24 in this subgroup would help clinicians identify patients at high risk of recurrence and enable them to administer rational adjuvant therapy after surgery. Taken together, our data indicate that CD24 may be a suitable prognostic marker for HCC, especially in AFP-normal patients and those with early-stage and well-differentiated HCC, the outcomes of which are very difficult to predict using conventional clinical indexes (4, 37).

Recurrence can be classified into two groups according to the time of relapse (21, 38). Early recurrence (≤1 year) typically results from HCC dissemination before resection, whereas in late recurrence (>1 year) a tumor develops *de novo* in the liver remnant as the result of stepwise accumulation of multiple genetic alterations caused mainly by continuous virus infection and inflammation (21, 30, 39, 40). The predictive potential of CD24 was evident in both these groups, implying that CD24 not only plays an important role in tumor metastasis but also is involved in hepatocarcinogenesis of a new tumor.

In our previous study, we found that adjuvant TACE can prolong survival of patients with risk factors for residual tumor (41). Here, we show that adjuvant TACE can reduce early recurrence after surgery in CD24$^+$ patients only. This implies that adjuvant TACE may be beneficial for CD24$^+$ HCC patients, but its use in CD24$^-$ patients should be seriously considered as it has no benefit but can aggravate liver dysfunction and cause other complications (42).

The potential for targeting CD24 in cancer therapy seems promising, as CD24 is overexpressed in many human cancers but is barely detectable in normal tissues (43, 44). In this study, we also found that CD24 was negative in most of the peritumoral and normal liver tissues. In a recent report, down-regulation of CD24 in colorectal and pancreatic cancer using RNA interference or anti-CD24 monoclonal antibodies achieved significant inhibition of tumor development *in vitro* and *in vivo* (10). Our *in vitro* study using RNA interference showed that CD24 may also be a promising target molecular for treatment of HCC.

To our knowledge, this is the first report showing the potential of CD24 as a predictive biomarker in a large number of HCC patients with long-term follow-up. However, it should be noted that most HCC patients in China have a hepatitis B virus–positive background; 84.4% of our study population were hepatitis B virus–positive, which differs greatly from studies in the United States, Europe, and Japan (45). Therefore, the prognostic significance of CD24 needs to be validated in HCC patients from these areas. Prospective studies with larger patient populations are needed to further investigate the value of CD24 as a prognostic marker. *In vivo* experiments and further studies on the role of CD24 are very interesting and are an area of active research at our institute.

In conclusion, CD24 expression was associated with tumor cell invasiveness and metastasis, high proliferative status, and abnormal activation of the Wnt/β-catenin pathway. High expression of CD24 was associated with a poor outcome of HCC patients after surgery, and adjuvant TACE after surgery may benefit CD24$^+$ patients. Cancer therapy targeted against CD24 may be a promising strategy for the treatment of HCC metastasis and recurrence.

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