Cytokeratin 10 and Cytokeratin 19: Predictive Markers for Poor Prognosis in Hepatocellular Carcinoma Patients after Curative Resection

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Abstract

Purpose: Cytokeratin 10 (CK10) was found to be expressed differently in human hepatocellular carcinoma (HCC) cell lines with different metastatic potentials in our previous research. The aim of this study was to assess the value of CK10 alone or in combination with cytokeratin 19 (CK19) in predicting tumor recurrence after curative resection in HCC patients.

Experimental Design: CK10 expression in stepwise metastatic HCC cell lines and tumor tissues from 50 HCC patients was investigated using immunofluorescence assay, quantitative real-time reverse transcription-PCR, and Western blot analyses. Tumor tissue microarrays of 300 HCC patients who underwent curative resection between 1997 and 2000 were used to detect the expressions of CK10 and CK19. Clinicopathologic data for these patients were evaluated. The prognostic significance was assessed using Kaplan-Meier survival estimates and log-rank tests.

Results: CK10 was overexpressed in the high metastatic HCC cell line and in tumor tissues of recurrent patients. Both univariate and multivariate analyses revealed that CK10 was a significant predictor for overall survival (OS) and disease-free survival, and that CK19 was a significant predictor for OS. CK10 expression was correlated with poor prognosis regardless of α-fetoprotein, tumor-node-metastasis stage, and vascular invasion. The 7-year OS and disease-free survival rates in CK10+ and/or CK19+ patients were 30.0% and 37.6%, respectively, which were significantly lower than that of CK10−/CK19− patients (56.1% and 60.0%, respectively; \( P < 0.001 \)).

Conclusion: CK10 is associated with HCC invasiveness. CK10 alone, or in combination with CK19, can be a novel predictor for poor prognosis of HCC patients after curative resection.

Hepatocellular carcinoma (HCC) is one of the most prevalent tumor types; its incidence and mortality rates have increased in recent years (1). The high rate of recurrence or metastases after curative resection has hindered improvements in HCC survival (2, 3). Molecular signatures that define the risk of recurrence and metastatic potential of HCC have been difficult to identify. Such markers would allow appropriate therapeutic regimens to be applied earlier in the disease course. Although several prognostic biomarkers in HCC have been reported recently (4–7), there still remains a lack of ideal biomarkers available that can be widely used in clinical settings (8, 9).

Cytokeratins are typical epithelial cell markers expressed in a tissue-specific and differentiation-dependent manner. It has been reported that cytokeratins were extensively present in many malignant epithelial cells (10–13) and tended to be altered in association with increases in metastatic ability and malignancy (14). Several cytokeratins were proven to be present in HCC (15, 16) and relate with poor prognosis and metastatic potential (17–22).

In our previous research, cytokeratin 10 (CK10) expression was detected by in vitro phage display and proteomics technology in MHCC97-H but not in MHCC97-L, two human HCC cell lines created at our institute (with the same genetic background but different metastatic potentials; refs. 17, 18, 23–25). However, the expression difference in HCC cell lines still need to be confirmed, and the possible value of CK10 in the clinic is worth further exploring.
Cytokeratin 19 (CK19), as a biliary/progenitor cell marker of liver, was found to be expressed differently in MHCC97-H and MHCC97-L (18) and is correlated with HCC metastasis and recurrence, as confirmed in our previous studies (19, 21). In recent researches, CK19 has been reported as a prognostic marker in HCC after operation (20, 22). Because the number of patients who participated in those researches is not enough, and the bias of traditional immunochemistry may affect the results, the prognostic value of CK19 in HCC patients needs to be further investigated.

The tissue microarray (TMA) technology can permit high-throughput in situ analysis of specific molecular targets in hundreds or thousands of tissue specimens at once and provide a highly parallel procedure (26). In this study, we further validated CK10 expression in HCC cell lines and tumor tissues and explored the predictive values of CK10 and CK19 for the prognosis of HCC patients by TMA, which contained a consecutive series of Chinese HCC patients (300 cases) following curative resection at our institute from 1997 to 2000.

**Materials and Methods**

**Patients and specimens.** Fifty cases used in quantitative real-time reverse transcription-PCR (qRT-PCR) were randomly collected from patients undergoing curative resection between 2000 and 2002. The 50 HCC tissues were collected immediately upon resection of the tumors in the operating theater. Tumor specimens were taken from areas of the tumors; necrotic tissues were avoided. The specimens were then snapped into liquid nitrogen and were stored at -80°C. Nine frozen tissues used in Western blot analysis were randomly chosen from these 50 cases.

Tumor specimens used in TMA studies were obtained from 300 consecutive patients with HCC who underwent curative resection at Liver Cancer Institute, Zhongshan Hospital, Fudan University between 1997 and 2000.

The inclusion and exclusion criteria of the patient cohorts include (a) having a distinctive pathologic diagnosis of HCC, (b) having no anticancer treatment before liver resection, (c) having curative liver resection, (d) having suitable formalin-fixed, paraffin-embedded tissues or frozen tissues, and (e) having a complete clinicopathologic and follow-up data.

Curative resection was defined as complete resection of all tumor nodules and the cut surface being free of cancer by histologic examination; having no cancerous thrombus in the portal vein (main trunk or two major branches), hepatic veins, or bile duct; and having no extrahepatic metastasis (27). HCC diagnosis was based on the WHO criteria (28, 29). Tumor differentiation was defined according to the Edmondson grading system (29). Tumor staging was defined according to the 6th edition of tumor-node-metastasis (TNM) classification of Unio Internationale Contra Cancrum. Most patients (81.3%) had
hepatitis B virus background, and only five patients had hepatitis C
virus. Almost all the patients (299 of 300) were in the Child-Pugh A
classification. The clinicopathologic characteristics of 300 patients were
summarized in Supplementary Table S1. Ethical approval for human
subjects was obtained from the research ethics committee of Zhongshan
Hospital; informed consent was obtained from each patient.

Follow-up and treatment for tumor recurrences. Patients were
followed up every 2 mo during the first postoperative year and at least
every 3 to 4 mo afterward. Follow-up was finished on March 15, 2007.
The median follow-up was 75 mo (range, 4-121 mo). Most patients
died from intrahepatic recurrence, distal metastasis, or complicated
liver cirrhosis. Seven patients died of unrelated diseases, such as
diabetes, myocardial infarction, and cerebral accident without recur-
rence. These seven patients were looked upon as censored values in
analysis. All patients were monitored prospectively by serum
α-fetoprotein (AFP), abdomen ultrasonography, and chest X-ray every
1 to 6 mo, according to the postoperative time. For patients with test
results suggestive of recurrence, computed tomography and/or mag-
netic resonance imaging were used to verify whether intrahepatic
recurrence and/or distal metastasis had occurred. A diagnosis of
recurrence was based on typical imaging appearance in computed
tomography and/or magnetic resonance imaging scan and an elevated
AFP level. Patients with confirmed recurrence received further
treatment, which followed the same protocol based on tumor size,
site, 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, transcatheter arterial chemo-
embolization was the choice. External radiotherapy was given if lymph
node or bone metastasis was found. Otherwise, symptomatic treatment
was provided.

Cell lines. MHCC97-H and MHCC97-L, human HCC cell lines with
high or low metastatic potential (19, 23–25) established at our
institute, were used in these studies. l-02, a human normal hepatocyte
cell line, was purchased from the Institute of Biochemistry and Cell
Biology, Chinese Academy of Sciences (30). All the cell lines were
routinely maintained and cultured as described previously (19).

RNA isolation and qRT-PCR. qRT-PCR was used to measure CK10
mRNA expression in cell lines and tumor tissues from 50 HCC patients.
Total RNA was extracted from cell lines and frozen tumor specimens
using the Trizol reagent (Invitrogen). Two micrograms of total RNA
were reverse transcribed using RevertAid first-strand cDNA synthesis kit
(Fermentas). RT-PCR was done before qRT-PCR. CK10 mRNA
expression was quantified using the TaqMan PCR Master Mix kit
(Appplied Biosystems) and ABI PRISM 7300 Sequence Detection System.
The reaction system was done following the manual. The primers and
probes used were as follows: CK10 (Genbank NM_000421) forward primer: 5'-AGGGGGCAGTTTCGGAGGTG-3', reverse primer: 5'-AAGTAGGAAGCCAGGCGGTCATT-3', probe: 5'-FAM-TGGAGGCGGCTTTGGAGGAGGCT-TAMRA-3'. 18s (Genbank: NR_003286) forward primer: 5'-GCCCGAAGCGTTTACTTTGA-3', reverse primer: 5'-TCCATTATTCCTAGCTGCGGTATC-3', probe: 5'-FAM-AAAGCAGGCCCGAGCCGCC-TAMRA-3'. Relative mRNA levels were calculated based on the Ct values, corrected by the 18s rRNA expression, according to the following equation: $2^{-\Delta \Delta Ct} = 2^{(Ct \text{CK10} - Ct \text{18s})} - 2^{(Ct \text{18s})}$.

**Western blot analysis.** CK10 expression in cell lines and tumor specimens from nine HCC patients was detected by Western blotting. Cells were briefly washed with ice-cold PBS twice, and frozen tumor

![Fig. 3](image)

**Fig. 3.** The prognostic significance assessed by using Kaplan-Meier survival estimates and log-rank tests. Comparisons of OS and DFS by CK10 (A and B), CK19 (C and D), CK10/CK19 (E and F), CK10 and TNM stage (G and H), CK10/CK19 and TNM stage (I and J), and CK10 and AFP (K and L).

### Table 1. Univariate analyses of factors associated with survival and recurrence

<table>
<thead>
<tr>
<th>Variables</th>
<th>OS Hazard ratio (95% CI)</th>
<th>P</th>
<th>DFS Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female vs male)</td>
<td>1.02 (0.67-1.55)</td>
<td>0.925</td>
<td>1.14 (0.71-1.83)</td>
<td>0.587</td>
</tr>
<tr>
<td>Age, y (&lt;52 vs &gt;52)</td>
<td>0.88 (0.65-1.19)</td>
<td>0.401</td>
<td>0.91 (0.65-1.27)</td>
<td>0.573</td>
</tr>
<tr>
<td>HBsAg (positive vs negative)</td>
<td>1.11 (0.74-1.65)</td>
<td>0.624</td>
<td>1.37 (0.86-2.21)</td>
<td>0.189</td>
</tr>
<tr>
<td>HCV (positive vs negative)</td>
<td>1.42 (0.45-4.47)</td>
<td>0.545</td>
<td>1.15 (0.28-4.64)</td>
<td>0.849</td>
</tr>
<tr>
<td>Child-Pugh score (A vs B)</td>
<td>1.63 (0.23-11.65)</td>
<td>0.627</td>
<td>1.68 (0.23-12.01)</td>
<td>0.61</td>
</tr>
<tr>
<td>Liver cirrhosis (no vs yes)</td>
<td>1.83 (1.06-3.17)</td>
<td>0.030</td>
<td>1.62 (0.91-2.86)</td>
<td>0.101</td>
</tr>
<tr>
<td>ALT, units/L (&lt;75 vs &gt;75)</td>
<td>1.35 (0.99-1.83)</td>
<td>0.087</td>
<td>1.26 (0.89-1.77)</td>
<td>0.193</td>
</tr>
<tr>
<td>AFP, ng/mL (&lt;20 vs &gt;20)</td>
<td>1.34 (0.99-1.82)</td>
<td>0.06</td>
<td>1.17 (0.84-1.64)</td>
<td>0.353</td>
</tr>
<tr>
<td>Tumor differentiation (I-II vs III-IV)</td>
<td>1.47 (1.07-2.03)</td>
<td>0.019</td>
<td>1.40 (0.98-2.01)</td>
<td>0.066</td>
</tr>
<tr>
<td>Tumor encapsulation (complete vs none)</td>
<td>1.69 (1.25-2.33)</td>
<td>0.001</td>
<td>1.43 (1.00-2.03)</td>
<td>0.048</td>
</tr>
<tr>
<td>Tumor size, cm (&lt;5 vs &gt;5)</td>
<td>1.64 (1.21-2.22)</td>
<td>0.001</td>
<td>1.42 (1.02-2.02)</td>
<td>0.041</td>
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<tr>
<td>TNM stage (I vs II-III)</td>
<td>2.30 (1.64-3.23)</td>
<td>&lt;0.001</td>
<td>1.97 (1.33-2.91)</td>
<td>0.001</td>
</tr>
<tr>
<td>Tumor number (single vs multiple)</td>
<td>1.85 (1.25-2.73)</td>
<td>0.002</td>
<td>1.55 (0.98-2.45)</td>
<td>0.061</td>
</tr>
<tr>
<td>Vascular invasion (no vs yes)</td>
<td>2.63 (1.59-4.37)</td>
<td>&lt;0.001</td>
<td>2.23 (1.23-4.06)</td>
<td>0.008</td>
</tr>
<tr>
<td>CK10 (negative vs positive)</td>
<td>1.72 (1.19-2.48)</td>
<td>0.004</td>
<td>2.37 (1.61-3.48)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CK19 (negative vs positive)</td>
<td>1.82 (1.28-2.59)</td>
<td>0.001</td>
<td>1.37 (0.90-2.09)</td>
<td>0.145</td>
</tr>
<tr>
<td>CK10+/CK19- vs CK10+ and/or CK19+</td>
<td>1.92 (1.41-2.62)</td>
<td>&lt;0.001</td>
<td>2.02 (1.43-2.84)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**NOTE:** Cox proportional hazards regression model was used in univariate analysis. Abbreviations: 95% CI, 95% confidence interval; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus.
specimens were ground with liquid nitrogen. The overall protein of cell lines and tumor specimens 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 fluoride membranes (Millipore) using a mini trans-blot (Bio-Rad Laboratories). Membranes were then blocked with PBS with 0.05% Tween 20 containing 5% nonfat dry milk for 1 h and incubated with a monoclonal mouse anti-human CK10 (1:200, Chemicon) or glyceraldehyde-3-phosphate dehydrogenase (1:5,000, Chemicon) antibody for 2 h at room temperature. Membranes were then washed thrice with PBST and incubated with horseradish peroxidase–conjugated goat anti-mouse IgG (Chemicon) at 1:10,000 dilution for 1 h. The blots were developed using an enhanced chemiluminescence kit (Pierce). Each experiment was repeated at least thrice.

**Immunofluorescence assay.** CK10 expression in cell lines was also detected by immunofluorescence assay. Cells cultured on glass slides were fixed by acetone for 15 min. After treating with 0.2% Triton X-100 for 2 min, the fixed cells were blocked with bovine serum albumin and stained with mouse anti-human CK10 FITC-conjugated monoclonal antibody (1:100, Chemicon) for 1 h at 37°C. A negative control (primary antibody omitted) was included on every slide. After rinsing in PBS, the slides were counterstained with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc.) and examined under a fluorescent microscope (Olympus BX 40). Each experiment was repeated at least thrice.

**TMA and immunohistochemistry.** TMA was constructed as previously described (6). Briefly, all the HCC tissues were reviewed by two histopathologists, and representative 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. Three different TMA blocks were constructed. In addition, we built TMA-containing 100 cases of peritumoral livers that were randomly chosen from 300 cases. Each TMA contained 200 cylinders. Consecutive sections of 4 μm in thickness were taken on 3-aminopropyltriethoxysilane–coated slides (Shanghai Biochip Co., Ltd.).

Monoclonal antibodies against human CK10 (1:100) and CK19 (1:50) were purchased from DakoCytomation. Immunohistochemistry was carried out using a two-step protocol (Novolink Polymer Detection System) as previously described (6, 31). After microwave antigen retrieval, tissues were incubated with primary antibodies for 60 min at room temperature. Following a 30-min incubation with secondary antibody (Novolink Polymer Re7112), the sections were developed in 3,3′-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. Negative control slides with the primary antibodies omitted were included in all assays.

**Evaluation of immunohistochemical variables.** Immunohistochemical staining was assessed by three independent pathologists without knowledge of patient characteristics. Scores were assigned as intensity and percentage of positive staining with cytoplasm in the whole cylinder. Discrepancies were resolved by consensus among three pathologists with a multilab microscope. There existed at least 12 independent and intact computerized microscopic fields for the duplication of each case in TMA sections. Five independent microscopic fields (>400) were selected for each sample to ensure representativeness and homogeneity. All the tumor cells within each microscopic field were counted, and then the positive rates of CK10 or CK19 cells were calculated. The criterion for achieving a positive score of CK10 was based on the published evidence when moderate or intense immunoreactivity presented in >1% of the cells (32). CK19 was considered positive if moderate or intense staining presented in ≥5% of the tumor cells (20). The higher score was considered as a final score in case of a difference between duplicate tissue scores. The concordance between scores from different scores of the same tumor was greater than 90%.

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**Fig. 3** Continued.
Statistical analyses. Statistical analyses were done by SPSS 12.0 for Windows (SPSS). The χ² test, Fisher's exact probability, and Student's t test were used for comparison between groups. If variances in groups were not homogeneous, the nonparametric Mann-Whitney U test was used. Cumulative survival time was calculated by the Kaplan-Meier method and analyzed by the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazard regression model. Multivariate analysis was done using a Cox regression model with forward stepwise manner. P < 0.05 was considered statistically significant.

Results

The expression of CK10 in cell lines. CK10 mRNA expression detected by qRT-PCR in MHCC97-L was significantly higher than that in MHCC97-L (P = 0.012) and L-02 (P = 0.0003; Fig. 1A). According to Western blot analysis, CK10 was only detected in MHCC97-L, whereas it was not found in MHCC97-L and L-02 (Fig. 1B). Anti-CK10 immunofluorescence assay was detected moderately in the cytoplasm of MHCC97-L cells. A weak signal was present in MHCC97-L and L-02 cells (Fig. 1D).

The expression of CK10 in tissue samples. In qRT-PCR array, those patients suffering HCC recurrence had higher CK10 mRNA expression (18 of 50) than those without recurrence (32 of 50; P = 0.001; Supplementary Table S2). According to Western blot analysis, CK10 was expressed in two of four patients in recurrent group, but not in the five patients without recurrence (Fig. 1C).

Immunohistochemical characteristics in TMA. Cancer cells were relatively homogenous within a tumor, excluding necrotic, hemorrhagic, and fibrotic components when observed by H&E staining (Fig. 2A and B). HCC cells showed a variety of staining patterns with cytokeratin markers. The common expression profiles of CK10 were both focal and scattered, whereas few displayed a diffuse pattern of staining in cytoplasm. Occasionally, one or two single cells were observed with low intensity at high magnification fields (Supplementary Fig. S1D). There was no positive staining of CK10 in tumor mesenchyme portions and peritumoral liver tissues. Most cases in the peritumoral liver TMA were either negative or with only several scattered positive cells with faint staining in the whole cylinder (Supplementary Fig. S1F).

CK10 was considered positive when there were >1% of tumor cells showing moderate or high staining (mean, ~10%). Of the 250 negative patients, there were 162 patients without any CK10 expression and 88 patients with no more than 1% positive tumor cells. Fifty-eight of the 88 (>60%) patients with ≤1% positive cells did not recur. Thirty-one CK10+ patients presented more than 10% positive tumor cells, of which 9 patients presented positive cells more than 1% in corresponding whole tumor sections (Supplementary Tables S3 and S4).

Validation of CK10 expression in whole tumor sections. To validate the concordance between TMAs and whole tumor sections, we further detected CK10 expression of all CK10+ group (50 cases) and 50 cases randomly chosen from CK10- group in the corresponding whole tumor sections. We found that CK10 expression in whole tumor sections were in accordance with the results in TMAs; all the CK10+ cases in TMAs showed positive staining (>1%), and none of the negative cases presented positive cells more than 1% in corresponding whole tumor sections (Supplementary Tables S3 and S4).

Prognostic factors. The 7-year overall survival (OS) and disease free-survival (DFS) rates were 58.6% and 51.2%, respectively, in the whole study population. Univariate analysis showed that TNM stage, vascular invasion, and tumor size and encapsulation were unfavorable predictors for OS and DFS. Liver cirrhosis, tumor differentiation, and tumor number were associated with OS. Age, sex, hepatitis B and C virus infection background, liver cirrhosis, and Child-Pugh score had no prognostic significance for OS and DFS (Table 1).

CK10 was found to be prognostic for OS (P = 0.004) and DFS (P < 0.001; Table 1). The 7-year OS and DFS rates in CK10+ group were significantly higher than those of the CK10+ group (51.5% and 56.2% versus 28.0% and 28.6%; Fig. 3A and B).

Almost all of the CK10+ patients with normal preoperation AFP (AFP ≤20 ng/mL; 14 of 15) died from recurrence within 5 years (Fig. 3K and L). Most of these patients were in TNM stage I (12 of 15) and without vascular invasion (13 of 15). The clinicopathologic characteristics are shown in Supplementary Table S5.

When we further stratified patients by TNM stage, we found that most of the CK10+ patients (41 of 50) were in TNM stage I, which may have been caused by the patients’ heterogeneous TNM stage distribution (I, n = 240; II, n = 19; III, n = 41). The 7-year OS and DFS rates of these patients were 34.2% and 34.8%, respectively, which were significantly reduced compared with CK10- patients in TNM stage I (57.5% and 59.6%; P = 0.0147 and P = 0.0006, respectively; Fig. 3G and H). There were only nine CK10+ patients in TNM stage II-III, but they all recurred within 2 years and died within 5 years. CK10+ patients were associated with high AFP level (P = 0.005) and the absence of tumor capsule (P = 0.022). CK10 expression was not related with tumor differentiation (P = 0.496; Table 2).

When the patients were stratified by other clinicopathologic characteristics, we found that CK10-positive reaction in the tumor region correlated with poor prognosis, regardless of the tumor size, number, differentiation, encapsulation, and vascular invasion (Supplementary Fig. S3).

CK19 was associated with OS (P < 0.001) and had no influence on DFS (P = 0.145; Fig. 3C and D; Table 1). Forty-five of 56 CK19+ patients were in TNM stage I, and the 7-year OS rate was 37.8% versus 57.2% for CK19- patients in TNM stage I (P = 0.006). There were 11 CK19+ patients in TNM stage II-III.
and they all died within 6 years, including 10 with HCC recurrence.

When we evaluated the combined effect of CK10 and CK19 on the prognosis of HCC patients, we found that the 7-year OS and DFS rates in CK10+ and/or CK19+ patients were 30.0% and 37.6%, respectively, which were significantly lower than those of CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)) and CK10+/CK19+ patients (70.1% and 71.8%, respectively; \( P < 0.0001 \)). In this group, we found that CK10+/CK19+ patients had a significantly shorter OS and DFS (70.1% and 71.8%, respectively; \( P < 0.0001 \)) than CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)). In addition, we found that CK10+ and/or CK19+ patients had a significantly worse OS and DFS than CK10+/CK19+ patients (70.1% and 71.8%, respectively; \( P < 0.0001 \)). In this study, we found that CK10+ and/or CK19+ patients had a significantly worse OS and DFS (70.1% and 71.8%, respectively; \( P < 0.0001 \)) than CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)). In this group, we found that CK10+/CK19+ patients had a significantly shorter OS and DFS than CK10+/CK19+ patients (70.1% and 71.8%, respectively; \( P < 0.0001 \)). In the multivariate analysis, we found that the co-index of CK10/CK19 was an independent prognosticator for OS (\( P < 0.0001 \)) and DFS (\( P < 0.0001 \)). In addition, we found that CK10+ and/or CK19+ patients had a significantly worse OS and DFS (70.1% and 71.8%, respectively; \( P < 0.0001 \)) than CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)). In this study, we found that CK10+ and/or CK19+ patients had a significantly worse OS and DFS (70.1% and 71.8%, respectively; \( P < 0.0001 \)) than CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)). In this group, we found that CK10+ and/or CK19+ patients had a significantly shorter OS and DFS than CK10+/CK19+ patients (70.1% and 71.8%, respectively; \( P < 0.0001 \)). In the multivariate analysis, we found that the co-index of CK10/CK19 was an independent prognosticator for OS (\( P < 0.0001 \)) and DFS (\( P < 0.0001 \)). In addition, we found that CK10+ and/or CK19+ patients had a significantly worse OS and DFS (70.1% and 71.8%, respectively; \( P < 0.0001 \)) than CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)). In this study, we found that CK10+ and/or CK19+ patients had a significantly worse OS and DFS (70.1% and 71.8%, respectively; \( P < 0.0001 \)) than CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)). In the multivariate analysis, we found that the co-index of CK10/CK19 was an independent prognosticator for OS (\( P < 0.0001 \)) and DFS (\( P < 0.0001 \)). In addition, we found that CK10+ and/or CK19+ patients had a significantly worse OS and DFS (70.1% and 71.8%, respectively; \( P < 0.0001 \)) than CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)). In this study, we found that CK10+ and/or CK19+ patients had a significantly worse OS and DFS (70.1% and 71.8%, respectively; \( P < 0.0001 \)) than CK10+/CK19+ patients (56.1% and 60.0%, respectively; \( P < 0.0001 \)).
MHCC97-H and MHCC97-L was confirmed by immunofluorescence assay, qRT-PCR, and Western blot analyses. Furthermore, we found that the CK10 mRNA expression in the patients with HCC recurrence increased by 10 times than that of the patients with no recurrence. This tendency was also validated by Western blot analysis in tumor specimens. Although not all the recurrent patients expressed CK10, we found that the expression of CK10 predicted very poor prognosis regardless of AFP level, tumor size, number, vascular invasion, and TNM stage—the putative prognostic factors in HCC (7, 9, 43, 44). All these findings showed that CK10 expression was associated with HCC invasiveness and metastasis and could be a new predictor for the prognosis of HCC patients.

CK19 is a low molecular weight cytokeratin and has been reported to correlate with HCC metastasis in our previous research (18, 19); however, the value of CK19 required to predict the prognosis of HCC patients using TMA has not been reported before. In this study, we evaluated the prognostic value of CK10 and CK19 by TMA, which contained 300 tumor specimens from HCC patients who underwent curative resection and whose consistency of immunohistochemistry was maintained steadily. The common expression profiles of CK10 were focal or scattered pattern and a range of 0% to 30% positive cells with moderate to intense staining in tumor tissues (32, 45, 46), which was in accordance with the results of qRT-PCR and Western blot analyses. We chose the 1% positive cells as the cutoff value to meet suitable specificity and low false negative, which was also according to the previous report (32). The expression pattern of CK19 was more intense and extensive compared with that of CK10. We considered CK19 positive if ≥5% of tumor cells stained positive, which was consistent with previous researches (20, 22).

According to the TMA results and clinical data, CK10 was an independent prognosticator for OS and DFS in HCC. Interestingly, unlike usual predictive markers for poor prognosis described before, which are mainly expressed in advanced HCC, we found that the survival rates of the CK10+ patients in TNM stage I were significantly reduced compared with that of CK10− patients in the same stage, which suggested that CK10 may be a promising marker for poor prognosis in early-stage HCC patients. In the clinic, it has been noted that even

### Table 3. Multivariate analyses of factors associated with OS and DFS (CK10 and CK19, respectively)

<table>
<thead>
<tr>
<th></th>
<th>Median Survival</th>
<th>Hazard ratio (95% CI)</th>
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<tbody>
<tr>
<td><strong>OS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver cirrhosis (no vs yes)</td>
<td>NA</td>
<td>69</td>
</tr>
<tr>
<td>Tumor encapsulation (complete vs none)</td>
<td>99</td>
<td>52</td>
</tr>
<tr>
<td>Tumor size, cm (&lt;5 vs &gt;5)</td>
<td>96</td>
<td>51</td>
</tr>
<tr>
<td>TNM stage (I vs II-III)</td>
<td>97</td>
<td>42</td>
</tr>
<tr>
<td>CK10 (negative vs positive)</td>
<td>88</td>
<td>50</td>
</tr>
<tr>
<td>CK19 (negative vs positive)</td>
<td>88</td>
<td>46</td>
</tr>
<tr>
<td><strong>DFS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNM stage (I vs II-III)</td>
<td>NA</td>
<td>38</td>
</tr>
<tr>
<td>CK10 (negative vs positive)</td>
<td>NA</td>
<td>20</td>
</tr>
</tbody>
</table>

**NOTE:** Multivariate analysis and Cox proportional hazards regression model were used. Variables were adopted for their prognostic significance by univariate analysis with forward stepwise selection (forward, likelihood ratio). Variables were adopted for their prognostic significance by univariate analysis (P < 0.05).

### Table 4. Multivariate analyses of factors associated with OS and DFS (the co-index of CK10 and CK19)

<table>
<thead>
<tr>
<th></th>
<th>Median Survival</th>
<th>Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver cirrhosis (no vs yes)</td>
<td>NA</td>
<td>69</td>
</tr>
<tr>
<td>Tumor encapsulation (complete vs none)</td>
<td>99</td>
<td>52</td>
</tr>
<tr>
<td>Tumor size, cm (&lt;5 vs &gt;5)</td>
<td>96</td>
<td>51</td>
</tr>
<tr>
<td>TNM stage (I vs II-III)</td>
<td>97</td>
<td>42</td>
</tr>
<tr>
<td>CK10 and CK19 (vs CK10+ or CK19+)</td>
<td>99</td>
<td>46</td>
</tr>
<tr>
<td><strong>DFS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNM stage (I vs II-III)</td>
<td>NA</td>
<td>38</td>
</tr>
<tr>
<td>CK10+ / CK19− vs CK10− and/or CK19−</td>
<td>NA</td>
<td>33</td>
</tr>
</tbody>
</table>

**NOTE:** Multivariate analysis and Cox proportional hazards regression model were used. Variables were adopted for their prognostic significance by univariate analysis with forward stepwise selection (forward, likelihood ratio). Variables were adopted for their prognostic significance by univariate analysis (P < 0.05).
after curative resection, the recurrence rate of early-stage HCC was still very high (41.25% in our series). The predictive value of CK10 is very valuable to help clinicians to distinguish high-risk recurrence in early-stage patients.

In addition, we found that CK10+ patients were associated with high AFP level and with the absence of tumor capsule, which are two relatively putative clinicopathologic markers of HCC invasiveness and unfavorable prognosis (9, 43, 44). AFP is still a very useful marker in the clinics to supervise HCC recurrence for AFP-positive patients. Nevertheless, there were ~40% to 60% HCC patients who present normal AFP level; early detection of recurrence in these patients is difficult (3). In this study, when we stratified patients by AFP, we found that although only 15 patients in the AFP-normal group expressed CK10, and most of them were in TNM stage I and had no relation with tumor size, number, capsule, differentiation, and vascular invasion, the prognosis of these patients was very dismal and almost all of them died from HCC recurrence within 5 years. This finding indicated that CK10 may be a very good marker to predict poor prognosis in AFP-normal HCC patients.

Our research showed that CK19 was an independent predictor in OS, but had no significant difference in DFS in univariate analysis. However, when we censored the DFS at 3, 5, and 7 years, respectively, there was significant difference at 3 years (P = 0.0108; Supplementary Table S8). This finding indicated that CK19 was suitable to predict early recurrence in HCC after operation, which was in accordance with the previous report (20).

In this study, the positive rates of CK10 and CK19 revealed by TMA was not very high (i.e., ~20%) and only nine patients expressed both CK10 and CK19. When we evaluated the effect of CK10 and CK19 together, we found the predicted range was extended and sensitivity was more improved than with CK10 or CK19 alone. In multivariate analysis, the co-index of CK10/CK19 was an independent prognostic factor in both OS and DFS (P < 0.0001 and P < 0.0001).

When we explored the recurrence time of the patients successfully predicted by CK10 or CK19, we found that the predictive accuracy decreased significantly 3 years after operation (from 53.2% to 14.3%). It indicated that CK10 and CK19 were more suitable predictors for early recurrence after operation.

Heterogeneous expression of cytokeratin subtypes indicated that cytokeratins were related to tissue development, proliferation, and differentiation (38). In a previous research, changes in cytoskeletal molecules related to increase in metastatic ability and malignancy of tumor (14). As normal hepatocytes only expressed CK18 and CK8 (47), CK10 and CK19 may be molecular phenotypes associated with the process of malignant tumor cell transformation, which acquires more invasive potential. Abnormal expressions of CK10 and CK19 in tumors indicate HCC with more malignancy and invasiveness regardless of TNM stage, AFP level, and other clinicopathologic characteristics, which may explain the high recurrence rates and poor prognosis in CK10+ and/or CK19+ HCC patients. It was reported that CK10 expression was related to better tumor differentiation in esophageal cancer, as CK10 was a terminal differentiated marker during epithelial differentiation (39). In our study, CK10 expression did not parallel histologic differentiation in HCC. We believe that it may be because of the heterogeneities among different types of epithelial original tumor, which was also in accordance with other reports (48, 49).

In addition, most HCC patients in China had hepatitis B virus background (81.3% in our series) unlike with the United States, Europe, and Japan (50); hence, the prognosis significance of CK10 and CK19 should be further validated in HCC patients from those areas.

To our knowledge, this is the first report showing CK10 expression as a predictive marker of HCC invasiveness and unfavorable prognosis in HCC patients after operation, especially with early-stage and normal AFP patients. CK10 and CK19 may help physicians make informed decisions regarding adjuvant treatment following curative resection. Initiating adjuvant treatment early in CK10+ and/or CK19+ HCC patients after surgical procedures may reduce recurrence and prolong survival.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Shanghai Biochip Co., Ltd., for helping in the construction of TMAs.

References

Cytokeratin 10 and Cytokeratin 19: Predictive Markers for Poor Prognosis in Hepatocellular Carcinoma Patients after Curative Resection

Xin-Rong Yang, Yang Xu, Guo-Ming Shi, et al.


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