From Proteomic Analysis to Clinical Significance

OVEREXPRESSION OF CYTOKERATIN 19 CORRELATES WITH HEPATOCELLULAR CARCINOMA METASTASIS*

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To better understand the mechanism underlying the hepatocellular carcinoma (HCC) metastasis and to search potential markers for HCC prognosis, differential proteomic analysis on two well-established HCC cell strains with high and low metastatic potentials, MHCC97-H and MHCC97-L, was conducted using two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/time-of-flight mass spectrometry. Cytokeratin 19 (CK19) was identified and found to be overexpressed in MHCC97-H as compared with MHCC97-L. This result was further confirmed by two-dimensional Western blot analysis and immunofluorescence assay. Furthermore, one-dimensional Western blot analysis showed consistently increased CK19 expression in progressively more metastatic cells. Immunohistochemical study on 102 human HCC specimens revealed that more patients in the CK19-positive group had overt intrahepatic metastases (satellite nodules, \( p < 0.05 \); vascular tumor emboli, \( p < 0.001 \); tumor node metastasis staging, \( p < 0.001 \)). CK19 fragment CYFRA 21-1 levels measured in sera from nude mice model of human HCC metastasis with radioimmunoassay increased in parallel with tumor progression and rose remarkably when pulmonary metastases occurred. The results demonstrated that overexpression of CK19 in HCC cells is related to metastatic behavior. Serum CK19 level might reflect the pathological progression in some HCC and may be a useful marker for predicting tumor metastasis and a therapeutic target for the treatment of HCC patients with metastases. Molecular & Cellular Proteomics 3:73–81, 2004.

Metastasis is the most life-threatening event in cancer patients. For hepatocellular carcinoma (HCC),¹ the fourth leading cause of cancer death worldwide and the second in China, surgery remains the treatment of choice, but the 5-year recurrence rate after section is as high as 40–70% (1). Metastatic recurrence is the main obstacle to the improvement of treatment efficacy. Pathologic factors indicative of tumor invasiveness such as portal vein invasion, intrahepatic metastasis, presence of satellite nodules, large tumor size, and advanced tumor node metastasis (TNM) stage, are the best-established risk factors for recurrence (2). However, the molecular events promoting invasiveness of HCC cells are still hardly known and routine biomarkers for HCC prognosis are not yet available.

For a better insight into the mechanisms of HCC metastasis, a high metastatic human HCC cell line, MHCC97, its clonal cells, MHCC97-H and MHCC97-L, with high and low metastatic potentials, and progressively more metastatic cells from lung metastatic lesions were established via repeated in vivo selection (3–5). These cells provide appropriate model systems with similar genetic background for the comparative study on the molecular events involved in HCC metastasis.

Among the currently available techniques, proteomics permits the analysis of thousands of modified or unmodified proteins simultaneously and becomes increasingly popular for identifying biomarkers for early detection, classification, and prognosis of tumors, as well as pinpointing targets for improved treatment outcomes (6). We had used this approach to identify differentially expressed proteins between human HCC and normal liver cell line and investigated malignant growth-associated proteins in human hepatoma cells transfected with antisense-epidermal growth factor receptor (7, 8). In this study, this technique was combined with immunology methods to study MHCC97-H and MHCC97-L for screening metastasis-associated proteins.

Cytokeratins represent important structural components of the epithelial cytoskeleton, and their expression is remarkably tissue specific, suggesting that the type of cytokeratins present in the cells is related to their biological function (9). Recent references point to the importance of cytokeratin in tumor progression, invasion, and metastasis. Recent references have shown that cytokeratin expression is associated with poor survival of breast cancer patients and that cytokeratins are involved in the invasion and metastasis of tumors (10, 11). In a study of breast cancer cell lines, cytokeratin 19 was found to be overexpressed in a metastatic cell line as compared to a nonmetastatic cell line (12). In addition, a high expression of cytokeratin 19 has been reported in ovarian cancer cell lines (13). These findings suggest that cytokeratin 19 may play a role in the metastatic process. In a recent study, cytokeratin 19 was found to be overexpressed in prostate cancer cell lines and to be associated with the presence of lymph node metastases (14). These results provide evidence that cytokeratin 19 expression may be a useful marker for predicting metastasis in prostate cancer. In a study of cervical cancer cell lines, cytokeratin 19 was found to be overexpressed in a metastatic cell line as compared to a nonmetastatic cell line (15). These findings suggest that cytokeratin 19 may play a role in the metastatic process. In a recent study, cytokeratin 19 was found to be overexpressed in prostate cancer cell lines and to be associated with the presence of lymph node metastases (16). These results provide evidence that cytokeratin 19 expression may be a useful marker for predicting metastasis in prostate cancer.

¹ The abbreviations used are: HCC, hepatocellular carcinoma; CK, cytokeratin; 2-DE, two-dimensional electrophoresis; IEF, isoelectric focusing; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; IF, intermediate filament; TNM, tumor node metastasis; AFP, α-fetoprotein.
studies have indicated that cytokeratins play an important role in the regulation of cell migration and invasion. The accumulation of cytokeratin 16 (CK16) correlated with a re-organization of keratin filaments to a perinuclear position away from the direction of migration as a prerequisite for cell migration into the wound site (10). Cell transected with human cytokeratin 8 (CK8) and cytokeratin 18 (CK18) had higher migratory and invasive abilities (11). Direct evidence has been provided to link overexpression of cytokeratins in human melanomas with increased migratory and invasive activity in vitro (12). The mechanism responsible for the differential expression of metastatic properties rests in the unique interaction, either direct or indirect, of cytokeratin intermediate filaments (IFs) with specific integrins interacting with the extracellular matrix (12, 13).

Here cytokeratin 19 (CK19) was identified and found to be overexpressed in MHCC97-H as compared with MHCC97-L by proteomic approach. This result was further confirmed by two-dimensional Western blot analysis and immunofluorescence assay. Furthermore, one-dimensional Western blot analysis showed consistently increased CK19 expression in progressively more metastatic cells. Immunohistochemical study on human HCC specimens revealed that more patients in the CK19-positive group had overt intrahepatic metastases. Serum CK19 fragment CYFRA 21-1 levels in nude mice model demonstrated that CK19 might be a useful marker for predicting the possibility of tumor metastasis and a therapeutic target for the treatment of HCC patients with metastases.

EXPERIMENTAL PROCEDURES

Cell Culture and Sample Preparation—MHCC97-H and MHCC97-L cells were established from the same parent cell line, MHCC97. After orthotopic inoculation of MHCC97-H and MHCC97-L to recipient nude mice, respectively, spontaneous pulmonary metastasis occurred in 100 and 40% (4). A series of progressively more metastatic HCC cell strains designated as HCCLM1–5 were also established via repeated in vivo selection from MHCC97-H cells (5). These cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, cultured in Dulbecco

dition (MALDI) Time-of-Flight (TOF) Mass Spectrometry (MS)—Protein identification was performed as described previously (16) with some modifications. Briefly, spots were cut out from the gels, destained for 20 min in 30 mM KCN/100 mM Na2S2O3 1:1 (v/v), and washed with Milli-Q water until the gels became clear. Background gel was cut out and used as a negative control. Then they were kept in 0.2 M NH4HCO3 for 20 min, lyophilized, and digested overnight in 12.5 ng trypsin/μL in 0.1 M NH4HCO3. The peptides were extracted three times by 50% acetonitrile with 0.1% trifluoroacetic acid and lyophilized. Then 0.5 μL of peptides was mixed with the α-cyano-4-hydroxycinnamic acid matrix (Sigma, St. Louis, MO), applied onto the target, air dried, and analyzed by Bruker REFLEX III MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Protein identification using peptide mass fingerprint was performed by Mascot search (www.matrixscience.com; MatrixScience Ltd, London, UK) against the NCBI nonredundant protein database. The species is Homo sapiens. The errors in peptide masses were in the range of 0.01–0.1%. One missed tryptic cleavage site per peptide was allowed during the search. Cysteines were carboxamidomethylated, and methionines were considered as oxidation. Proteins matching more than four peptides and Mascot scores higher than 63 were considered significant (p < 0.05).

Western Blot—Protein was separated by 10% SDS-PAGE or by 2-DE as described above and electroblotted to Immobilon-P membrane (Millipore, Bedford, MA). After treating with 5% fat-free dried milk in 1× TBST (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20, and 0.001% thimerosal) for 1 h at room temperature, the membranes were incubated with mouse anti-human CK19 (1:1000 dilution; Dako, Glostrup, Denmark) for 1 h at room temperature, followed by horse-radish peroxidase-conjugated secondary antibody for 1 h at room temperature. Actin was used as an internal positive control. Target proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence Detection of CKs—Trilicate cultures of MHCC97-H and MHCC97-L cell clones were washed in phosphate-buffered saline (pH 7.4) and fixed in methanol at −20 °C for 5 min. The cells were first incubated with mouse anti-human keratin 8, 18, and 19 primary antibodies (Dako) (1:100 dilution for CK6 and CK18, 1:50 dilution for CK19) for 30 min at 37 °C, then incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody (1:300 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. The negative control was performed without adding primary antibody. The cells were washed in phosphate-buffered saline three times each for 3 min. The slides were sealed in glycerin, and CK expression and layout pattern was observed by fluorescent microscopy (Zeiss, Oberkochen, Germany) at 450-nm wavelength.

Immunohistochemical Detection of CK19—One hundred two HCC specimens from 88 male and 14 female patients with an average age of 51 (range 26–72) were obtained from the Eastern Hepatobiliary Surgery Hospital in the period of May 2002 to July 2003. Ninety-two (90.2%) patients had serological evidence of hepatitis B virus infection. The α-fetoprotein (AFP) level was above 25 μg/liter in 77 cases (75.5%). Tumor size was smaller than 5 cm in 30 patients and larger than 5 cm in 72 patients. Eighty-two patients (80.4%) had complication of cirrhosis. There were 27 cases (26.5%) in TNM stage I (T1N0M0) and II (T2N0M0 and T1N1M0) and 75 (73.5%) in stage III (T2N1M0, T2N1M1 and T3N0M0 and IV (any T, any N and M) (17). Capsule invasion was found in 64 cases, macroscopic portal vein tumor emboli in 9 patients, and microscopic surrounding liver vascular cancer emboli in 19 patients. Metastatic satellite nodules were found in 12 cases. After routine pathological study, these specimens were processed for immunohistochemical detection of CK19 expression with anti-CK19 primary antibody (1:500 dilution; Dako). The slides were lightly counterstained with Harris hematoxylin and viewed under a light microscope. Uninfected liver tissues from surgically resected hepatic cav-
ernous hemangiomas were used as a negative control. Preoperative liver function tests were normal in all these patients and were negative for hepatitis B and C. An irrelevant primary antibody (pre-immune rabbit serum) was used to replace CK19 primary antibody on a consecutive section from CK19-positive staining followed by hematoxylin and eosin staining. CK19-positive staining was categorized as HCC-CK19\(^+\) if over 25% of the tumor cells were positively stained and as HCC-CK19\(^−\) if stained otherwise.

Serum CK19 Fragment CYFRA 21-1 Detection in Metastatic Nude Mice Model of Human HCC—Highly spontaneous metastatic nude mice model of human HCC was used to study the relationship between serum CK19 fragment CYFRA 21-1 levels and tumor progression. A total of 5 × 10\(^6\) cells/0.2 ml of HCCLM3 cell strain (5) were injected subcutaneously into each of 30 4-wk-old male BALB/c-\(nu/nu\) nude mice obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science, and kept in specific pathogen-free

![Fig. 1. CK19 was identified as differentially expressed protein between MHCC97-H and MHCC97-L using 2-DE and MALDI-TOF-MS. A, proteomic profiling of representative gel selected from MHCC97-L. 2-DE was performed with silver staining. The image was analyzed by PDQuest software, MHCC97-L was selected as the master gel, to which MHCC97-H and MHCC97-L gels were compared. The major proteins were identified by MALDI-TOF-MS. Anx, annexin; CK, cytokeratins; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HSP, heat shock proteins; PDI, Protein disulfide isomerase precursor; Mn-SOD, manganese superoxide dismutase; Tim, triosephosphate isomerase; E-FABP, fatty acid binding protein S; CRP55, calreticulin precursor. Framed area, area of the gel used as the basis for Fig. 1B. B, enlargement of differentially expressed spot SSP7615 by MHCC97-H and MHCC97-L. C, MALDI-TOF mass spectrum obtained from spot SSP 7615 after trypsin digestion.](image-url)
facilities. It is more efficient to choose HCCLM3 cells for the establishment of the mouse model because it could produce prominent lung metastasis after subcutaneous injection (5). After tumor cell inoculation, the mice were randomly divided into six groups of five mice in each group. At the end of the second, third, fourth, fifth, sixth, and seventh week, one group of mice was sacrificed respectively under deep anesthesia by peritoneal injection of 3% phenobarbital chloride. The blood was drawn and centrifuged at 10,000 \( \times g \) at 4°C for 10 min to separate the serum and then kept at \(-80^\circ C\). The tumor weight of each mouse was recorded. The lungs were fixed in 10% neutral formalin solution and processed for conventional pathological study. Pulmonary metastasis was recorded according to the procedures used before (5). Serum CYFRA 21-1 levels in nude mice model of human HCC were determined by radioimmunoassay using a commercial detection kit (ELISA-CYFRA 21-1; CIS Bio International, Gif-Sur-Yvette, France) in accordance with the protocols.

**Statistical Analysis**—Student’s t test and Fisher’s exact test were used to compare the clinicopathologic parameters between HCC-CK19+ and HCC-CK19- groups for age, hepatitis B antigen, tumor size, serum AFP level, TNM stage, capsule invasion, metastatic satellite nodules, and blood vessel emboli. Values of \( p < 0.05 \) were considered statistically significant. For the comparison of serum CK19 levels in different animal groups, analysis of variance and q test was used.

**RESULTS**

**Proteomic Analysis of MHCC97-H and MHCC97-L Cells and CK19 Differential Expression**—From cell culture to 2-DE, the experiments were repeated three times and silver-stained gels were analyzed by PDQuest software. The average protein spots detected were 1030 ± 113 in MHCC97-H and 1007 ± 107 in MHCC97-L. The scatter plots showed good correlation between these two kinds of cells with a correlation coefficient of 0.81, implying similarity in expression of most proteins and differences of only a small number of proteins between them (18). A representative gel image was selected from MHCC97-L (Fig. 1A).

In total, 69 spots (including both housekeeping and differentially expressed proteins) were identified on the preparative silver-stained gels using MALDI-TOF-MS analysis (18). Some of the identified proteins are shown in Fig. 1A. Among them, spot SSP 7615 was only present in MHCC97-H (Fig. 1B), and the MALDI-TOF mass spectrum of peptides derived from 7615 is shown in Fig. 1C. Mascot search using the peptide mass fingerprint data indicated that 18 peptides were matched with peptides from CK19, giving sequence coverage of 50% and a summary score of 240. Such results suggested that CK19 was the identity of spot 7615 with high confidence. CK8 and CK18 were also identified in MHCC97-H and HCCLM1-5.

**Fig. 2. Two-dimensional Western blot analysis for CK19 expression in MHCC97-H and MHCC97-L.** Eighty micrograms of whole-cell lysates were separated by 2-DE as describe above, and proteins were electrotheretically transferred onto the membrane. Immunoblotting was performed by anti-CK19 monoclonal antibody, horseradish peroxidase-conjugated secondary antibody, and detected by enhanced chemiluminescence.

**Fig. 3. Immunofluorescence detection of CK IFs in MHCC97-H and MHCC97-L cells.** A total of 1 × 10⁶ cells were cultured on slide chamber for 24 h, washed, fixed, and treated with monoclonal antibodies against CK8, CK18, and CK19, followed by fluorescein isothiocyanate-conjugated secondary antibody, and viewed by immunofluorescent microscopy (1000×). The negative control was performed without adding primary antibody. Both CK8 and CK18, which are positive in HCC, were well developed in MHCC97-H and MHCC97-L, forming fine network pattern. In contrast, CK19 was aberrantly expressed in MHCC97-H but invisible in MHCC97-L cells.

**Fig. 4. Western blot analysis for CK19 expression in HCC cell series.** Fifteen micrograms of whole-cell lysates were separated by 10% SDS-PAGE, and proteins were electrotheretically transferred onto the membrane. Immunoblotting was performed by anti-CK19 monoclonal antibody, horseradish peroxidase-conjugated secondary antibody, and detected by enhanced chemiluminescence. Equal protein loading was evidenced by detection of β-actin level using a monoclonal anti-β-actin antibody. CK19 was expressed in all cells obtained from five rounds of lung metastasis selection, HCCLM1-5. Increasing CK19 expression level was observed along with the progressively more metastatic potentials of these cells.
MHCC97-L cells but without significant changes.

**Verification of CK19 Differential Expression by Western Blot and Immunofluorescence Assay**—To confirm the above proteomic result, we employed two-dimensional Western blot, which has the advantage of enhanced sensitivity and the ability to detect protein isoforms assigned from post-translational modifications. A trace amount of CK19 isoforms in MHCC97-L was detected, but its expression was much higher in MHCC97-H (Fig. 2). Furthermore, the immunofluorescence assay demonstrated that both MHCC97-H and MHCC97-L expressed CK8 and CK18 normally, with the same distribution and layout pattern, forming a well-extended filament network. However, the expression and distribution pattern of CK19 was quite different between these two kinds of cells, being well developed in MHCC97-H but almost invisible in MHCC97-L cells (Fig. 3).

**Expression of CK19 in Serial Metastatic Potential HCC Cell Strains**—HCCLM1-5 cells, with progressively higher metastatic potential obtained from successive lung metastasis selections of MHCC97-H (5), were examined for CK19 expression. As indicated in Fig. 4, CK19 showed consistently increased expression from HCCLM1 to HCCLM5, suggesting a positive correlation of CK19 expression with their metastatic potentials.

**CK19 Expression in HCC Specimens**—We further studied the CK19 expression in clinical HCC tissues using immunohistochemistry analysis. Among 102 HCC specimens, CK19 was positive in 12.7% (13/102) of the cases. When the CK19-positive group was compared with the CK19-negative group, more patients had poor tumor differentiation (grade III/IV tumors 13/13 (100.0%) versus 62/89 (69.7%), \( p < 0.001 \)) and intrahepatic metastases (satellite nodules 5/13 (38.5%) versus 7/89 (7.9%), \( p < 0.05 \); tumor emboli in blood vessels 11/13 (84.6%) versus 17/89 (19.1%), \( p < 0.001 \)). There was no significant correlation between CK19 expression with age, tumor size, and hepatitis B antigen seropositivity (Fig. 5, Table I).

**Tumor Development in Nude Mice Model of Human HCC and Serum CK19 Fragment CYFRA 21-1 Level**—When the human HCC cell line HCCLM3 was injected into nude mice, tumors developed after 1 wk, showing aggressive growth. The tumor growth became accelerated from the third week on, and on the fifth week the mice showed signs of distress. Histopathological study of the lungs showed conspicuous metastases (Fig. 6). Tumor growth, lung metastasis, and serum CYFRA 21-1 levels are shown in Table II. The animal serum CYFRA 21-1 level increased in parallel with the pro-
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**TABLE I**

|                    | CK19 positive (n = 13) | CK19 negative (n = 89) | p value |
|--------------------|------------------------|------------------------|---------|
| Age (years, median) | 54.7                   | 49.9                   | >0.05   |
| HBsAg+ positive    | 92.3% (12/13)          | 89.9% (80/89)          | >0.05   |
| Tumor size         |                        |                        |         |
| >5 cm              | 69.2% (9/13)           | 70.8% (63/89)          | >0.05   |
| ≤5 cm              | 30.8% (4/13)           | 29.2% (26/89)          | >0.05   |
| AFP (>25 μg/liter) | 69.2% (9/13)           | 76.4% (68/89)          | >0.05   |
| TNM staging        |                        |                        |         |
| III/IV             | 100.0% (13/13)         | 69.7% (62/89)          |         |
| I/II               | 0.0% (0/13)            | 30.3% (27/89)          | <0.001  |
| Capsule invasion   | 61.5% (8/13)           | 62.9% (56/89)          | >0.05   |
| Satellite nodules  | 38.5% (5/13)           | 7.9% (7/89)            | <0.05   |
| Vascular tumor emboli | 84.6% (11/13) | 19.1% (17/89)          | <0.001  |

*a* Student’s t test.
*b* Hepatitis B surface antigen.
*c* Fisher’s exact test.
*d* Including tumor emboli in the portal vein and small blood vessels.

progression of the engrafted tumor. Moreover, serum CYFRA 21-1 levels rose remarkably when pulmonary metastasis occurred (q test, *p* < 0.01).

**DISCUSSION**

A great deal of effort has been made to predict HCC behavior, but specific prognostic indicators are still lacking. Prognostic factors in HCC conventionally consist of staging with the TNM system and grading by tumor cellular differentiation (19). There are also other factors useful in prognosis but most of them are clinical. A large number of molecular biological factors have been shown to be associated with the invasiveness of HCC and have potential prognostic significance including proliferating cell nuclear antigen, Ki-67, E-cadherin, catenins, urokinase-type plasminogen activator (uPA), uPA receptor, vascular endothelial growth factor, and AFP, etc (19–25). However, routine biomarkers for HCC prognosis are not yet available. Advances in the fields of genomics and proteomics give rise to the promise for the discovery of novel markers to improve diagnosis and identify therapeutic targets for the treatment of HCC patients with metastases.

In this study, CK19 screened from comparative proteomic analysis of two HCC cell strains with different metastatic potentials has been shown to be overexpressed in high metastatic MHCC97-H cells as compared with MHCC97-L cells. Moreover, CK19 expression is gradually increased in line with the metastatic potency of HCC cell as shown in the Western blot detection of CK19 expression in a series of HCC cell lines with progressively increasing spontaneous metastatic potentials, implying the possible role of CK19 in metastasis.

CK19 is the smallest member of cytokeratin family, a group of heterogeneous IF proteins. Cytokeratins are generally expressed in the epithelia in a tissue- and differentiation-specific manner (9). In liver, for example, the fetal liver usually shows high CK8, CK18, and CK19 expression. Upon differentiation, hepatocytes lose the expression of CK19, whereas its expression is retained in bile ducts (26). Because cancer cells usually retain the IF type of their cell origin, CK19 is clinically used as a discrimination marker of HCC and intrahepatic cholangiocarcinoma (27, 28). However, Wu et al. reported that three HCC cell lines, Hep G2, Huh-7, and PLC/PRF/5, also expressed CK19 (29). In addition, hepatocytes with a bile duct cytokeratin phenotype (CK19 and CK7) appear during the early stages of aflatoxin B1-induced carcinogenesis in rat liver (30). Therefore, upon malignant transformation, the cytokeratin expression profile of hepatocytes may change. CK19 expression in MHCC97-L, MHCC97-H, and HCCLM1-5 cells confirmed the above results. Our interesting finding is that the expression level of CK19 in these HCC cells is significantly correlated with their spontaneous metastatic potentials.

The immunohistochemical study in 102 human HCC specimens demonstrated that more patients in the CK19-positive group had clinical metastasis, such as metastatic satellite nodules or tumor emboli in blood vessels. Such observations suggest that CK19-positive tumor cells might invade normal tissues more easily and gain access to the circulation, thus harboring the risk of tumor recurrence. CK19 expression was also found to be related to poor differentiation in agreement with previous reports in HCC (31, 32).

Based on these findings, we further explored whether the detection of serum CK19 level could help to predict tumor metastasis. In the nude mice model of human HCC metastasis, the serum CK19 fragment CYFRA 21-1 level increased in parallel with the progression of the engrafted tumor. Interestingly, serum CYFRA 21-1 level showed a dramatic upsurge.
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**Table II**

Relationship between serum CK19 level and lung metastasis in a nude mice model

| Week    | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 | Week 7 |
|---------|--------|--------|--------|--------|--------|--------|
| Tumor weight (g) | 0.31 ± 0.13 | 0.61 ± 0.12 | 1.43 ± 0.37 | 2.38 ± 0.92 | 3.74 ± 0.96 | 4.85 ± 1.02 |
| Lung metastasis rate (%) | 0 | 0 | 20 | 100 | 100 | 100 |
| Median no. of lung metastases | 0 | 0 | 0a | 81 | 118 | 150 |
| Serum CK19 (μg/liter) | 4.79 ± 1.16 | 5.06 ± 1.21 | 8.55 ± 1.25 | 40.71 ± 9.87b | 76.29 ± 20.00b | 95.94 ± 19.68b |

a Only one mouse had lung metastases at week 4, and the total number of lung metastases was less than five, so the median number of lung metastases was 0.
b p < 0.01, compared with serum CK19 level in week 4 (analysis of variance and q test).

when pulmonary metastasis occurred. Therefore, in this tumor model system, serum CYFRA 21-1 level might be a sensitive indicator to predict metastasis. However, in previous studies, CYFRA 21-1 has been reported to be a sensitive marker in non-small-cell lung cancer (33). In addition, CYFRA 21-1 also showed diagnostic or prognostic value in various cancers, such as breast cancer, ovarian cancer, and bladder cancer (34–36). In liver cancer, Nagai et al. reported an increase of 12.3% in hepatoma diagnostic rate by adding serum CYFRA 21-1 to the routine diagnostic panel (37). Recently, Uenishi et al. reported that CYFRA 21-1 showed higher sensitivity for intrahepatic cholangiocarcinoma than three commonly used markers including AFP, carcinoembryonic antigen, and carbohydrate antigen 19-9 (38). These results indicated that the combination of CYFRA 21-1 with other pathological features and biomarkers seems to be more rational to predict HCC metastasis.

This result also showed the relationship between the elevations of serum CK19 level and general spreading of tumor cells in the animal model. As tumor metastasis is a continuous dynamic process involving releasing of tumor cells, their crossing the blood vessel barriers, and colonization at distant sites, many tumor cells are destroyed during the metastasis process. It has been demonstrated that the apoptosis of HCC cell line HuH-7 induced by tumor necrosis factor-α could result in the release of CK19 fragment CYFRA 21-1 (39). In this animal tumor model, the significant increase in serum CK19 level may be due to the destruction of large number of tumor cells released into the blood stream during the metastatic cascade. However, the possibility of CK19 fragment release from viable cancer cells suggested by Satoh et al. could not be excluded (40).

The role of CK19, or even other CKs, in tumor metastasis may be related to their function of promoting cell mobility or extracellular degradation. Chu et al. demonstrated in a murine tumor cell model that cells expressing intact CKs had higher in vitro mobility and invasiveness. He also suggested that intact CKs might anchor specific cell membrane receptors, thus inhibiting cell clustering and facilitating cell movement (11). Dobashi et al. reported that recombinant CK19 could bind strongly to laminin, a major protein in all basement membranes, provoking an immune response that damaged the basement membrane (41). Direct evidence has been provided to link overexpression of cytokeratins in human melanoma with increased migratory and invasive activity in vitro (12). Additional experimental evidence suggested that the mechanism responsible for the differential expression of metastatic properties associated with the interconverted phenotype rested in the unique interaction, either direct or indirect, of IFs with specific integrins interacting with the extracellular matrix (12, 13). Our previous study showed that MHCC97-H was more prone to trypsin digestion than MHCC97-L, implying less tight intercellular attachment in MHCC97-H. Moreover, in vitro invasive assay demonstrated that more MHCC97-H cells could penetrate matrigel and the cell mobility was faster (4). However, another study found that CK19 overexpression was correlated with decreased invasive potential of oral squamous cell carcinoma cells, suggesting that the role of CK19 in tumor metastasis may be context dependent (13).

In summary, the current study demonstrated that overexpression of CK19 in HCC cells is related to metastatic behavior. Serum CK19 level might reflect the pathological progression in some HCC and may be a useful marker for predicting tumor metastasis and a therapeutic target for the treatment of HCC patients with metastases.

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