Association of Mortalin (HSPA9) with Liver Cancer Metastasis and Prediction for Early Tumor Recurrence*

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Hepatocellular carcinoma (HCC) is well known for poor prognosis and short survival because of high recurrence rate even after curative surgery. Today there is no available biomarker or biochemical test to indicate HCC recurrence, and this study aims to identify protein markers that can discriminate postoperative patients with early recurrence (ER), i.e. disease relapsed within the first year. In this study, 103 hepatitis B-related HCC patients were recruited, and 68 of them were used for ER-related biomarker discovery study. Proteomic expression patterns of matched tumor and adjacent non-tumor tissues from these patients plus 16 normal liver tissues were delineated by the two-dimensional gel electrophoresis differential profiling method. Significant protein spots were evaluated by hierarchical clustering analysis. SSP4612 that yielded the highest receiver operating characteristic (ROC) curve value for the ER subgroup of HCC was subsequently identified by tandem mass spectrometry, and the corresponding expression patterns were further confirmed by quantitative PCR, Western blot, and immunohistochemistry. Correlation analysis with clinicopathological data was also examined. Proteomic profiling analysis revealed overexpression of mortalin (gene HSPA9) in HCC when compared with the non-tumor and normal liver tissues (area under the curve (AUC) = 0.821). Furthermore, elevated mortalin level was also detected in the ER subgroup of HCC versus the recurrence-free state (where no cancer recurs for >1 year) (AUC = 0.833, sensitivity = 90.9%, specificity = 71.4%). Metastatic HCC cell lines also exhibited higher levels of mortalin and HSPA9 mRNA. Clinically, mortalin overexpression in HCC was closely associated with advanced tumor stages and venous infiltration, having implications for increased malignancy and aggressive behavior. Mortalin (HSPA9) is associated with HCC metastasis and thus suggested as a tumor marker for predicting early recurrence, which may have immediate clinical applications for cancer surveillance after curative surgery. Molecular & Cellular Proteomics 7: 315–325, 2008.

Patients diagnosed with advanced tumor stages and neoplastic metastasis are usually untreatable and very often survive briefly. Liver cancer is one of the prevalent and lethal malignancies and represents a tumor type of highly invasive and aggressive behavior with ability to adapt to new environment. Because of its asymptomatic features during malignant progression, liver cancer is often diagnosed at very late stage when conventional and effective treatment options become unavailable (1).

Hepatocellular carcinoma (HCC) is the most common liver malignancy, accounting for the third most common cause of cancer-related deaths worldwide, especially in parts of Asia and Africa with estimated >680,000 new cases and half million of deaths annually (2, 3). Recent epidemiological studies have also projected an alarming increase of HCC in both Japan and the United States. The only curative treatments for HCC are surgical resection or liver transplantation (4, 5). To date, conventional chemotherapeutic regimen is ineffective against HCC with a response rate between 5–10%, and no single drug or “cocktail” could prolong the patient’s survival. Even though after curative surgery, the long-term prognosis of HCC still remains poor, that is largely attributable to the high tumor recurrence rate (about 56% within the first year after surgery) (6).

The current paradigm of HCC development is invariably associated with liver damage caused by chronic hepatitis, heavy alcoholic intake, or toxin poisoning, sequentially leading to liver cirrhosis, dysplastic lesions, and eventually invasive carcinoma. Our current belief is that these agents can target the liver progenitor cells, leading to their proliferation and malignant transformation (7). One of the key targets in liver carcinogenesis is p53, which can be functionally inactivated by the X protein of HBV (8). Other established lesions in HCC include activation of the cyclin D1 (CCND1), c-met or

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1 The abbreviations used are: HCC, hepatocellular carcinoma; AFP, α-fetoprotein; ER, early recurrence (4–12 months postsurgery); RF, recurrence free (no recurrence within the first year after surgery); HBV, hepatitis B virus; ROC, receiver operating characteristic; HSP, heat shock protein; NT, non-tumor; AUC, area under the curve.
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TABLE I
Demographic features and clinicopathological data of 68 HBV-associated HCC patients

| Clinicopathological parameters | ER (n = 33) | RF (n = 35) | p       |
|-------------------------------|------------|------------|---------|
| Recurrence-free length (days) | 246.6 ± 53.9 | 855.2 ± 622.6 | 0.000094a |
| Sex                           |            |            |         |
| Male                          | 28 (84.8%) | 29 (82.9%) | 0.9529b |
| Female                        | 5 (15.2%)  | 6 (17.1%)  |         |
| Age                           | 52.9 ± 12.4 | 52.7 ± 10.8 | 0.5116a |
| AFP (ng/ml)                   | 15,686 ± 57,716 | 2,211 ± 8,321 | 0.167a  |
| Tumor size (cm)               | 10.25 ± 5.60 | 6.72 ± 4.15  | 0.1215a |
| Venous Infiltration           |            |            |         |
| Yes                           | 19 (57.6%) | 12 (34.3%) | 0.088b  |
| No                            | 14 (42.4%) | 23 (65.7%) |         |
| New TNM stage                 |            |            |         |
| I                             | 6 (18.2%)  | 22 (61.8%) | 0.004b  |
| II                            | 16 (48.5%) | 8 (23.5%)  |         |
| IIIB                          | 5 (15.2%)  | 1 (2.9%)   |         |
| IV                            | 4 (12.1%)  | 4 (11.8%)  |         |
| New Edmonson grade            |            |            |         |
| 0–2 (well differentiated)     | 9 (27.3%)  | 11 (31.4%) | 0.884b  |
| 3 (moderately differentiated) | 17 (51.5%) | 18 (51.4%) |         |
| 4 (poorly differentiated)     | 7 (21.2%)  | 6 (17.1%)  |         |

a Analyzed by independent t test.
b Analyzed by χ² test and Fisher’s exact test.

c-myc oncogenes, as well as mutations or dysregulation in the ERK/MAPK, Ras/PI3 kinase, and Wnt/β-catenin signaling pathways (9, 10). Still, how specific lesions interact to produce its aggressive phenotypes remains poorly understood.

MS-based proteomic profiling of human clinical tissues is a powerful tool to investigate cancer biomarkers and therapeutic targets (11, 12). In this study, we used the differential proteomic approach to profile tumorous and matched noncancerous tissues from 103 HBV-associated HCC subjects, with attempts to identifying new tumor markers and characterize the spontaneous lesions arising in the aggressive (or metastatic) phenotype of HCC. The protein marker SSP4612 with high discriminative capacity (receiver operating characteristic (ROC) curve >0.8 for the ER subgroup of HCC versus RF state) was analyzed by tandem MS. The findings were further confirmed by quantitative PCR, Western blot, and immunohistochemistry. Mortalin (HSPA9) was found as a tumor marker with the ability to distinguish a subgroup of HCC patients who relapsed within the first year after curative surgery. In clinical correlation analysis, elevated protein level of mortalin in HCC was significantly associated with advanced tumor stages (III-IV) and positive venous infiltration. HCC cell lines with metastatic phenotypes also exhibited high level of mortalin and HSPA9 mRNA. These findings suggest that mortalin may be used as a predictive marker for early tumor recurrence so as to facilitate cancer surveillance after tumor resection in HCC patients.

MATERIALS AND METHODS

Patients and Clinical Specimens—The present study included 103 Chinese patients with chronic hepatitis B who had undergone hepatic resection for HCC at the Department of Surgery, Queen Mary Hospital, Pokfulam, Hong Kong between 1998 and 2005 (supplemental Table S1). Curative resection was defined as complete excision of the tumor with clear microscopic margin, and no residual tumors detected by CT scan or angiography at 1 month after surgery. All postoperative patients were closely followed up as outpatients and monitored prospectively for tumor recurrence by a standard protocol including measurement of serum α-fetoprotein (AFP) level and ultrasonography or contrast CT scan every 2–4 months (13). Suspected intrahepatic recurrence was confirmed by hepatic angiography, postlipiodol CT scan, and if necessary, percutaneous fine-needle aspiration cytology.

Disease-free survival time was defined as the interval between operation and the confirmed diagnosis of tumor recurrence. Early recurrence (ER) was defined as tumor recurred within the period between the 4th and 12th months after curative surgery, whereas recurrence free (RF) was defined for those patients surviving disease free for more than 12 months postoperation. Cases in whom cancer recurred less than or equal to 3 months were also excluded from the present study because they might be caused by undetectable remnant tumor(s) from the operation or other unknown causes. Those cases that did not have long enough follow-up time were also not included.

Among these patients, 68 cases were suitable for this study, and the clinicopathological data were summarized in Table I. Whereas there were 35 subjects belonging to the RF subgroup, an ER subgroup of 33 HCCs was diagnosed with intrahepatic recurrence but without distant metastasis after surgical resection. There were no differences between the two groups except for the disease-free duration and tumor stages. In addition, 16 normal liver tissues from residual grafts were included in this study as reference control. HCC from the non-HBV group consisted of approximately 10% incidence in this locality and 12 such cases were also used for comparison. The Institutional Review Board for Human Ethics approved this study and informed consent was obtained from all patients for collecting the specimens.
**Cell Lines**—Primary and metastatic HCC cell lines: MIHA, Hep3B, Huh7, HepG2, PLC, 97L, 97H, H2M, and H2P were described in previous studies (14, 15). They were maintained in DMEM with high glucose supplemented with 10% heat-inactivated fetal bovine serum, 100 mg/ml penicillin G, and 50 μg/ml streptomycin. Hep(AD38) was maintained in the above medium supplemented with 100 μg/ml kanamycin and 400 μg/ml G418. All cell lines were cultured in a humidified atmosphere with 5% carbon dioxide.

**Protein Extractions and Two-dimensional Gel Electrophoresis**—Tissues were immediately snap-frozen in liquid nitrogen after surgical resection and stored at −80 °C until use. For each tissue sample, 6-μm section was prepared and stained with hematoxylin and eosin to examine the pathological histology. Only tissues of >90% homogenous properties were included for two-dimensional gel electrophoresis analysis as reported (16). Briefly, protein lysates were extracted from 10- to 20-mg tissues using the ReadyPrep Sequential Extraction Kit (Bio-Rad, Hercules, CA). After ultracentrifugation at 20,800 × g for 60 min at 15 °C, pellets were dissolved in 0.1 ml extraction solution maintained in the above medium supplemented with 100 mg/ml kanamycin and 400 μg/ml G418. All cell lines were cultured in a humidified atmosphere with 5% carbon dioxide.

**In-gel Enzymatic Digestion and Mass Spectrometry**—Spots of interest were excised and digested with trypsin (MS grade, Promega, Madison, WI). Briefly, protein spots were washed in 50 mM ammonium bicarbonate buffer and then destained in potassium ferricyanide. The protein spots were then dehydrated in acetonitrile and dried in a SpeedVac before being rehydrated on ice for 40 min in trypsinization buffer (20 ng/ml trypsin, 20 mM NH₄HCO₃, pH 8.0). Proteins were digested overnight at 37 °C. Peptides were extracted with 25 mM NH₄HCO₃, 50% acetonitrile, and 0.1% trifluoroacetic acid (v/v) sequentially. All extracts were saved, pooled, then lyophilized, and redissolved in 0.1% trifluoroacetic acid. After desalting with a Millipore ZIP plate (Millipore), samples were finally dissolved in 5 mg/ml α-cyano-4-hydroxy-cinnamic acid matrix in 50% acetonitrile, 0.1% trifluoroacetic acid. MS and MS/MS spectra were obtained using the ABI 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems) operating in a result-dependent acquisition mode. Peptide mass maps were acquired in positive ion reflector mode (2 kV accelerating voltage) with 2500 laser shots per spectrum. Monoisotopic peak masses were automatically determined within the mass range 1000–4000 Da with a signal to noise ratio minimum set to 5 and a local noise window width of m/z 200. Up to five of the most intense ion signals were selected as precursors for MS/MS acquisition, excluding common trypsin autolysis peaks and matrix ion signals. In MS/MS-positive ion mode, 4000 spectra were averaged, collision energy was 2 kV, and default calibration was set. Monoisotopic peak masses were automatically determined with a signal to noise ratio minimum set to 5 and a local noise window width of m/z 200. The MS together with MS/MS spectra were searched against the NCBI nr database using the software GPS Explorer, version 3.6 (Applied Biosystems) and MASCOT version 2.1 (Matrix Science) with the following parameter settings: 65 ppm mass accuracy, trypsin cleavage, one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionines allowed as variable modification, peptide mass tolerance set to 50 ppm, fragment tolerance set to ± 0.2 Da, and minimum ion score confidence interval for MS/MS data set to 99%.

**Western Blot Analysis**—About 25 μg protein lysates was loaded for 12% SDS-PAGE. After electrophoretic transfer, membrane was washed with TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6). The membrane was incubated in blocking solution (5% nonfat dry milk and 1% bovine serum albumin in TBS-T) with gentle shaking at room temperature for 1 h. Incubation with mouse anti-mortalin (sp5-825; Stressgen, Victoria, Alberta, Canada) at 1:5000 dilution in blocking solution was performed at 4 °C overnight. On the next day, membrane was washed with TBS-T (3 × 10 min) and incubated with horseradish peroxidase conjugated goat antimouse antibody at 1:5000 (in blocking solution) for 1 h at room temperature. After 3 more washes, immunoreactive proteins were visualized with ECL detection reagents (Amersham Biosciences). Goat anti-human actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:10000 in blocking solution) was included as the protein loading control. Quantification of Western blot result was calculated based on the mortalnin/actin ratio of each sample.

**Immunohistochemistry**—Clinical tissue specimens were embedded in paraffin and sectioned at 4-μm thick for immunohistochemical analysis as described (14, 17). Endogenous peroxidase activities were quenched with hydrogen peroxide and sections were blocked with 3% normal goat serum (w/v in phosphate-buffered saline) and 1% bovine serum albumin (w/v in phosphate-buffered saline) (blocking solution). Mouse anti-mortalin antibody (10 μg/ml in blocking solution) was added to the sections and incubated at 4 °C overnight. Thereafter, sections were incubated with the horseradish peroxidase-conjugated goat antimouse immunoglobulin for 30 min at room temperature. Signal was detected using a ready-to-use DAKO EnVision system, horseradish peroxidase-diaminobenzidine (Dako, Glostrup, Denmark). Sections were counterstained with hematoxylin (Vector Laboratories, Burlingame, CA) and viewed at five different fields per section under light microscope. For the negative isotope controls, the primary antibody was replaced with purified mouse immunoglobulin (1:500 dilution, Zymed Laboratories, Invitrogen, Carlsbad, CA). Images were viewed with Nikon epifluorescent upright microscope E600 (Nikon, Tokyo, Japan), captured with 3-CCD color camera DC-330 (DAGE-MTI, Inc, Michigan City, IN), and analyzed by the Metamorph v3.0 (Universal Imaging Corporation, West Chester, PA).

**TaqMan Q-PCR for HSPA9 mRNA Expression**—HSPA9 mRNA levels were quantified by Q-PCR using TaqMan probes and oligonucleotide primers for HSPA9 and ribosomal 18s designed by the ABI PRISM Primer Express Software (Applied Biosystems). Fluorescent dyes, FAM and VIC, were used as reporter signals and ribosomal 18s was used as an internal control. 

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followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. All the reactions were performed at least in duplicates and analyzed using the ABI Prism 7700 detection system (Applied Biosystems). Statistical Analyses—SPSS for Windows (version 13.0, Chicago, IL) was used to perform statistical analysis using the spot intensity quantified on two-dimensional gel electrophoresis proteomic expression patterns. One-way analysis of variance (ANOVA) was used to analyze spot intensities in tumor, non-tumor, and normal liver samples. Hierarchical clustering analysis of the expression profile of the significant protein spots between the tumor, non-tumor, and normal liver samples. The correlation between protein expression intensity (ppm) and the clinicopathological data was performed by non-parametric correlation analysis. The ROC curve and area under the curve (AUC) were used to select candidate biomarkers of HCC and early tumor recurrence (20). ROC plots were constructed by using the vector of 1 – SPE, where SPE is the test specificity at a cutoff value on the x-axis and the vector of corresponding sensitivity values on the y-axis. The best possible cut-off points were defined at the highest Youden index. The predictive performance of mortalin, serum AFP, advanced new TNM stage, and venous infiltration were also calculated using tabular methods to evaluate the usefulness of these prognostic parameters as individual or combined tests for ER of HCC according to the diagnosis test as previously described (21, 22).

RESULTS

Differential Proteomic Analysis of HCC—Expression profiling analysis by two-dimensional gel electrophoresis yielded a total of 1433 protein spots in HCC proteomes (Fig. 1A). After gel-to-gel matching and normalization, 365 protein spots were found to display differential expression patterns among the tumor, non-tumor, and normal liver by one-way ANOVA. The HCCs, there were 52 spots significantly different between the ER and RF subgroups by Student’s t test. Furthermore, hierarchical clustering analysis was performed to
segregate the protein spots according to individual expression similarity among these groups. As exemplified in Fig. 2, we observed 2 major clusters in the HCC specimens, i.e. the ER and RF subgroups separating from each other. In addition, the 4 normal liver subjects were also clustered together accordingly. Thus, the proteomic expression profiles of HCC exhibited distinguishing traits, which can be exploited to identify potential biomarkers for liver carcinoma.

To facilitate the discovery of biomarkers that could distinguish HCC tumors from the non-tumor and normal liver tissues, ROC curve analysis was conducted for the protein spots identified from the 103 matched tumor and adjacent non-tumor tissues and the 16 normal liver tissues. A total of 36 protein species in the HCC proteomes was found to have an AUC value >0.7. Among these, SSP4612 protein spot was selected for further characterization for its ability to distinguish HCC from the normal liver and non-tumor samples (AUC, 0.821; 82.4% sensitivity and 68.1% specificity) (Fig. 3A). The protein expression levels of SSP4612 in HCC, non-tumor, and normal livers were 5240 ± 2865 (n = 68), 2906 ± 1627 (n = 68), and 2583 ± 1735 (n = 16), respectively (Fig. 1B). There was no significant difference between the non-tumor and normal liver groups, whereas the protein level of SSP4612 was significantly higher in the HCC tissues (p < 0.001). The on-gel physical properties of SSP4612 were MW of ~74 kDa and pl at 5.0 (Fig. 1A).

SSP4612 Marker Was Overexpressed in the ER Subgroup of HCC—Protein marker SSP4612 was also found to be able to distinguish the ER subgroup among the 68 HCC samples by ROC analysis (AUC = 0.833, sensitivity = 90.90%, specificity = 71.4%) (Fig. 3B). The expression level of SSP4612 was significantly higher (by 1.82-fold) in the ER subgroup (6766 ± 3224, n = 33) versus in the RF (disease-free >1 year) (3713 ± 2099, n = 35) (p = 0.0142; by t test) (Fig. 1C). When we categorized

Fig. 2. Tree view diagram of hierarchical clustering of the pattern of variations in the expression of 52 protein spots with significantly altered levels between tumor, non-tumor, and normal liver (one-way ANOVA). Representative samples displayed are 7 HCCs of the ER subgroup, 7 HCCs of RF subgroup, and 4 normal controls. The protein marker SSP4612 is indicated by an arrow. Red grid, up-regulation; green grid, down-regulation; black grid, no expression difference. ER, early recurrence; RF, recurrence free; N, healthy donor.
the HCC samples into different tumor stages and vascular invasions, mortalin levels were also higher in the advanced TNM stages III-IV and positive venous infiltration (data not shown).

**MS/MS Identification of SSP4612 Marker as Mortalin**—Because of its potential association with the aggressive characteristics of HCC, SSP4612 was subsequently subjected to detailed characterization by tandem MS/MS and UPLC Q-TOF Premier MS analysis. The SSP4612 marker was identified to be mortalin (other names: GRP75, mthsp75, or heat shock 70 kDa protein 9), gene product encoded by HSPA9. Details of the peptides matched list were shown in Table II. The digested peptides sequence coverage was 34.2%. The predicted MW and PI are 74 kDa and 6.0, respectively, and the subcellular localization of this protein is found in mitochondria, microsome, and cytoplasm.

**Mortalin (HSPA9) Up-regulation in HCC ER Subgroup and Metastatic Cell Lines**—Overexpression of mortalin protein and HSPA9 mRNA levels in the ER subgroup of HCC was further confirmed by other established methods (Fig. 4), using a separate blind set of HBV-associated HCC samples (ER, n = 20; RF, n = 20). In accordance with the proteomic expression data, mortalin level was generally higher in ER compared with RF group as shown by both the Q-PCR and immunoblot (Fig. 4A). Strong expression or staining intensity of mortalin was detected in the ER subgroup, whereas the RF tumors had lower levels of mortalin. Transcriptional analysis by TaqMan quantitative PCR also revealed up-regulation (by 1.78-fold) of HSPA9 gene level in the ER tumor (fold ratio, 15.03 ± 2.43 versus normal liver) compared with that in the RF group (8.43 ± 2.84), and significant difference was determined between the 2 groups (p = 0.0159, by Mann-Whitney test). By Western blot, the relative ratio of mortalin/actin in the ER subgroup was about 2.2-fold higher than the RF subgroup (0.993 ± 0.176 versus 0.452 ± 0.141, p < 0.01) (Fig. 4A, right panel) and selected images were shown in Fig 4B. As revealed by immunohistochemical staining (Fig. 4C), strong immunoreactivity of mortalin was observed in the ER subgroup of HCC, and the staining was essentially localized in the cytoplasm of tumor cells. No signal was observed in the interstitial tissues or parenchyma. In addition, mortalin was nondetectable in the non-tumor tissues or the isotype-matched control section.

**Tumor recurrence is in close association with the metastatic potential or aggressive behavior of neoplasm.** To examine whether mortalin is linked to the aggressive phenotype, we assessed the gene expression levels of HSPA9 and the protein level of mortalin in a panel of primary HCC cell lines with different metastatic potential. Our findings derived from the Q-PCR analysis evidenced that HSPA9 mRNA abundance was increased in the metastatic HCC cell line (H2M) when compared with its parental clone (H2P) of lower metastatic potential (23). In general, the level of HSPA9 was higher in the primary HCC cell lines (Hep3B and Huh7) compared with MIHA (immortalized normal hepatocytes) and non-tumorigenic HepG2 (Fig. 5). Similar findings were also observed by Western blot analysis (supplemental Fig. S1).

**Mortalin Predicts the Incidence of HCC Recurrence after Hepatic Surgery**—Next, we analyzed the predictive performance of mortalin as a prognostic biomarker for postoperative...
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Analysis software: GPS Explorer Workstation (version 3.6); database: MASCOT, NCBInr.

### TABLE II
Summary of protein spot SSP4612 identified by MALDI-TOF/TOF tandem mass spectrometry

| Protein name:         | Accession no: | Protein score: | Total ion score: | Protein MW: | Protein Peptide/sequence coverage (%) | Peptide species count: |
|-----------------------|---------------|----------------|------------------|-------------|-------------------------------------|-----------------------|
| HSPA9 protein (mortalin) [Homo sapiens] | gi|21040386 | 283 | 182 | 74093.1 | 16 | 34.2 |

Calculation of precision mass and observed mass with ppm error

| Observation | Start sequence | End sequence | Ion Score | CI (%) |
|-------------|----------------|--------------|-----------|--------|
| 1045.5387  | 1045.5472      | ± 0.0085     | 8         | 647    |
| 1242.6801  | 1242.6726      | ± 0.0075     | 6         | 207    |
| 1290.6801  | 1290.6686      | ± 0.0121     | 9         | 395    |
| 1430.6945  | 1430.6899      | ± 0.0076     | 5         | 601    |
| 1446.7621  | 1446.756       | ± 0.0061     | 4         | 378    |
| 1450.7173  | 1450.7067      | ± 0.0106     | 7         | 86     |
| 1493.6471  | 1493.7147      | ± 0.0076     | 45        | 486    |
| 1569.8345  | 1569.7917      | ± 0.0028     | 27        | 160    |
| 1592.9523  | 1592.9259      | ± 0.0024     | 17        | 499    |
| 1690.8429  | 1690.8369      | ± 0.006      | 4         | 293    |
| 1694.8496  | 1694.8408      | ± 0.0088     | 5         | 188    |
| 1724.8715  | 1724.8575      | ± 0.014      | 8         | 108    |
| 1792.8568  | 1792.8235      | ± 0.033      | 19        | 362    |
| 1808.9025  | 1808.8655      | ± 0.036      | 20        | 469    |
| 2055.9617  | 2055.9482      | ± 0.0135     | 7         | 266    |
| 2422.1951  | 2422.2156      | ± 0.0205     | 8         | 369    |

Incidence of ER (<1 year) of HCC after curative surgery. By using the diagnosis test methods, all patients were divided into either positive (ER) or negative (RF) group at a range of cutoff values based on the proteomic expression pattern and clinical diagnosis of tumor recurrence was used as the standard. As summarized in Table III, mortalin could satisfactorily discriminate the ER subgroup from other HCCs (sensitivity = 90.9%, specificity = 71.4%, Youden index = 0.623).

Likewise, the predictive performance of high serum AFP level (cutoff at 400 ng/ml), advanced tumor stages (III-IV) and presence of venous infiltration for HCC recurrence were also conducted. Overall, the studied test features of mortalin levels in HCC appeared to be superior to the clinicopathological features, such as positive venous infiltration, serum AFP level (>400 ng/ml), and advanced tumor stages (III-IV). When combined with serum AFP level (>400 ng/ml) in parallel diagnosis test, the predictive performance of mortalin overexpression for identification of the ER subgroup of HCC could be further enhanced (sensitivity = 94.0%; specificity = 94.3%).

Clinical Significance of Mortalin Overexpression in HCC—In a total of 103 HBV-associated HCC cases tested in this study, clinicopathological correlation analysis showed that mortalin overexpression in HCC was significantly associated with positive venous infiltration (r = 0.243, p < 0.05) and advanced tumor TNM stages (r = 0.358, p < 0.001). No apparent correlation with other clinical features, including serum AFP, tumor size, and new Edmonson grade, was observed (Table IV). Furthermore, there was no significant association of the mortalin expression levels among the HBV-positive (n = 103) and HBV-negative (n = 12) groups, neither in the tumor or adjacent non-tumor tissues of HCC (supplemental Table S2).

**DISCUSSION**

Tumor recurrence is closely related to the invasive behaviors and metastatic features of neoplasm, which unfortunately, and very often, is associated with poor clinical outcomes and shorter survival of cancer patients. HCC is a heterogeneous and aggressive neoplasm and even within one year after tumor resection, about half of the patients may suffer from disease relapsed (24). The present staging systems for HCC that are based on clinical and pathological findings (including number of nodules, tumor sizes, venous infiltration, and nodal metastasis) may have reached their limits of usefulness for predicting patient outcome, especially about disease-free survival. In this context, biomarker-based laboratory assays and molecular methods will offer added values. Today, the most widely used diagnostic biomarker for HCC is AFP. However, the diagnostic performance of measuring serum AFP for primary liver cancer is unsatisfactory by its low detection sensitivity (about 40% for early stages of HCC) and high false-positive rate because of benign nodules and nonmalignant liver disease (25–28). Although serum AFP at high level (>600 ng/ml) is associated with tumor recurrence and poor survival (29), the exact prognostic roles and clinical significance remain unclear.

ER after curative resection of HCC, defined as intrahepatic, regional, or systemic recurrence within the first year, is one of the most important factors affecting the patient outcomes (19,
More than 60% of patients with multiple HCCs result from intrahepatic recurrence. In the present study, we have identified mortalin (HSPA9) as a tumor biomarker able to discriminate a subgroup of HCC patients who were diagnosed with intrahepatic recurrence between 4 and 12 months after curative surgery. The identification of predictive markers for early tumor recurrence was based on two-dimensional gel electrophoresis differential proteomic profiling, unsupervised hierarchical clustering and ROC curve analyses of 152 frozen specimens from HCC patients and healthy donors. Selected from a total of 36 potential biomarkers that were significantly up-regulated in the ER subgroup, mortalin emerged with the highest detection sensitivity of 90.9%. In ER versus recurrence-free states, mortalin protein levels could distinguish between individuals who were recurrence free (no cancer for at least 1 year) and those who had early recurrence (relapsed within 1 year, except those excluded in the first 3 months), with an AUC value of 0.833. This was further supported by Western blot and mRNA analyses. Immunohistochemical staining also revealed strong immunoreactivity of mortalin in HCC from the ER subgroup. In short, the abundance of mortalin expression level is found in the following order: HCC (ER subgroup)/HCC (RF subgroup)/non-tumor/normal liver; and the differences are statistically significant between each group except that between the non-tumor and normal. Recently, other studies have also demonstrated that mortalin is frequently up-regulated in a variety of tumor tissues, including breast, brain, and colon carcinomas (30, 31). In colorectal cancer, high level of mortalin is correlated with poor clinical outcome independent of the Dukes stage (32).

Mortalin (HSPA9), also known as GRP75 or mthsp75, a member of the heat shock protein (HSP) 70 family, was first identified as human mitochondrial hsp75 (33), having important roles in stress response, glucose regulation, cell proliferation, differentiation, and tumorigenesis. Despite the molecular mechanisms of mortalin in liver carcinogenesis is not fully elucidated, it is thought to exert its tumorigenic effects through various binding partners, including p53 (34), fibroblast growth factor-1 (35), interleukin-1 receptor type 1 (36), and GRP94 (37). Functional inactivation of tumor suppressor p53 is a common event in early HCC (38), and mortalin has been shown precisely in colocalization with wild-type p53, thereby resulting in cytoplasmic retention and transcriptional inactivation of p53 in tumors (39). This may provide the mech-
anistic foundation of mortalin in HCC tumorigenesis and cancer progression. Because reduced mortalin expression in immortalized cells causes senescence-like growth arrest (40), it is thus considered as a potential candidate target for cancer therapy (41).

Previous reports also demonstrated that other HSP members (Hsp27, Hsp60, Hsp70, Hsp90, GRP78, and GRP94) were overexpressed in HCC (16, 42–44), but none of them associated with tumor metastasis or recurrence. Blan et al. (44) recently reported a 2-fold (T/NT ratio) up-regulation of mortalin protein in 14 HCC patients with chronic hepatitis C virus, but its metastatic role was not shown and characterized. The present study clearly indicates that HSPA9 mRNA level is elevated in the metastatic phenotype of HCC, and its protein abundance in tumor tissues may have implications to liver cancer patients at risk of early disease relapsed. The presence of mortalin in HCC is also significantly associated with advanced tumor stages (III–IV) and venous infiltration, the two critical prognostic factors indicative of poor outcomes and cancer recurrence in HCC patients.

Along this line, we subsequently compared the predictive performance of mortalin overexpression, high serum AFP (>400 ng/ml), TNM staging III-IV, and positive venous infiltration for the identification of early tumor recurrence of HCC. Mortalin marker appeared to be superior to the other criteria that were evaluated, except the AFP and tumor stages offering better specificity (71.4% versus 80%). Nevertheless, mortalin level in HCC may be used in combination with the serum AFP level (>400 ng/ml) to improve the diagnostic performance of the test (parallel test sensitivity of 94.0% and serial test specificity of 94.3%). The identification of mortalin autoantibodies in HCC patients' serum (45) also prompts us to investigate the potential application of noninvasive blood test of mortalin level to monitor HCC progression and early recurrence prediction. Despite of these interesting findings, larger sample size in randomized studies is needed to fully assess the potential clinical value of mortalin as candidate biomarker for HCC surveillance. Clinical validation of mortalin as a predictive marker for early HCC recurrence is under way in a multicenter study.

In conclusion, our findings indicate that mortalin (HSPA9) is generally up-regulated in metastatic HCC cell lines and tumor tissues, particularly in the ER subgroup of HCC patients after curative surgery. Clinically, overexpression of mortalin in HCC is closely associated with the poor prognostic factors, such as advanced tumor stages and positive venous infiltration. Thus, mortalin (HSPA9) is suggested as a tumor marker for predicting early recurrence, which might have immediate clinical applications for cancer surveillance after curative surgery. Earlier identification of tumor recurrence would enable clinicians to offer cancer patients with individualized clinical management and more effective treatment modalities before the neoplasm spreads distantly.

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