



HHS Public Access

Author manuscript

J Proteome Res. Author manuscript; available in PMC 2015 October 23.

Published in final edited form as:

J Proteome Res. 2009 March ; 8(3): 1293–1303. doi:10.1021/pr800637z.

Proteomic Expression Signature Distinguishes Cancerous and Non-Malignant Tissues in Hepatocellular Carcinoma

Nikki P. Lee^{a,b,*}, Lei Chen^{a,d,*}, Marie C. Lin^c, Felice H. Tsang^a, Chun Yeung^a, Jirun Peng^d, Xisheng Leng^d, Laura Beretta^e, Stella Sun^{a,b}, Philip J. Day^f, and John M. Luk^{a,b}

^aDepartment of Surgery, The University of Hong Kong, Pokfulam, Hong Kong

^bCenter for Cancer Research, The University of Hong Kong, Pokfulam, Hong Kong

^cDepartment of Chemistry, The University of Hong Kong, Pokfulam, Hong Kong

^dDepartment of Surgery, Peking University, People's Hospital, Beijing, China

^eFred Hutchinson Cancer Research Center, Seattle, WA, USA

^fQuantitative Molecular Medicine, The Manchester Interdisciplinary Biocentre, University of Manchester, Manchester, United Kingdom

Abstract

Hepatocellular carcinoma (HCC) is an aggressive liver cancer but clinically validated biomarkers that can predict natural history of malignant progression are lacking. The present study explored the proteome-wide patterns of HCC to identify biomarker signature that could distinguish cancerous and non-malignant liver tissues. A retrospective cohort of 80 HBV-associated HCC was included and both the tumor and adjacent non-tumor tissues were subjected to proteome-wide expression profiling by 2-DE method. The subjects were randomly divided into the training (n=55) and validation (n=25) subsets, and the data analyzed by classification-and-regression tree algorithm. Protein markers were characterized by MALDI-ToF/MS and confirmed by immunohistochemistry, western blotting and qPCR assays. Proteomic expression signature composed of six biomarkers (haptoglobin, cytochrome b5, progesterone receptor membrane component 1, heat shock 27 kDa protein 1, lysosomal proteinase cathepsin B, keratin I) was developed as a classifier model for predicting HCC. We further evaluated the model using both leave-one-out procedure and independent validation, and the overall sensitivity and specificity for HCC both are 92.5% respectively. Clinical correlation analysis revealed that these biomarkers were significantly associated with serum AFP, total protein levels and the Ishak's score. The described model using biomarker signatures could accurately distinguish HCC from non-malignant tissues, which may also provide hints and guidance on how normal hepatocytes are transformed to malignant state during tumor progression.

To whom correspondence should be addressed: Dr. John Luk, Department of Surgery, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong. Fax: +852-28199636. jmluk@hku.hk.

*These authors contributed equally.

Conflict of interest statement

None declared

Keywords

hepatocellular carcinoma; tumor markers; proteomics; diagnosis and prognosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignancies, and is the third leading cause of cancer death worldwide.¹ Chronic infection with hepatitis B or C virus (HBV or HCV) is the major etiologic factor that accounts for about 80% of all HCC cases. More than 50% of the HCC incidences are diagnosed in China where HBV vaccination program has just begun and hepatitis B is still prevalent, posing a serious health threat and burdening the already burdened medical care system. Surgical treatments including hepatectomy, liver transplantation, locoregional ablation and transarterial chemoembolization therapies are the common therapies for HCC.² However, the procedures are costly and the clinical outcomes still remain dismal. The overall 5-year survival rate is less than 10%, and about one third of the HCC patients relapse within 1 year after curative surgery. The poor prognosis is mainly attributed to the late diagnosis and the high recurrence rate of the neoplasm.³

Effective management of HCC relies on accurate detection of the disease in a high-risk population (subjects with cirrhosis or HBV carriers); allowing clinicians to identify patients at the earliest curable stages when the tumor are usually resectable and to deliver effective interventions that contribute to the reduction in mortality and morbidity. Circulating α -fetoprotein (AFP) in serum has been widely used as a conventional biomarker for HCC surveillance.⁴ Elevated serum AFP level is also a useful prognostic indicator for poor survival rate and tumor recurrence in HCC patients.⁵ Nevertheless, serum AFP is not particularly efficient in detecting HCC at early stages, and its specificity is often shadowed by high false positive signals from patients with other liver-related diseases such as viral hepatitis and cirrhosis.⁶ In this regard, several groups have recently suggested several soluble tumor markers such as glypican-3,⁷ γ -glutamyltransferase II,⁸ des- γ -carboxyprothrombin,⁹ lens culinaris agglutinin-reactive AFP¹⁰ and α 1-antitrypsin,¹¹ that may improve the detection rate for early HCC. Nevertheless, their clinical utilities remain to be further determined in multicenter clinical studies.

In spite of our recent belief that a panel of biomarkers is required for early diagnosis of liver cancer, the precise set of such molecules that can be used to differentiate HCC from nonmalignant and normal liver tissues is not yet available. The technological advance in proteomics profiling promises to be an effective strategy for cancer biomarkers discovery.^{12–15} Differential proteome analysis of the matched tumor and non-tumor tissues also enable us to delineate the global changes in the expression patterns, shedding new insights into the molecular understanding of tumor progression and identifying novel therapeutic targets for early interventions.¹⁶

In this study, we analyzed the proteomic expression profiles by 2-dimensional gel electrophoresis (2-DE) of resected tumor and adjacent non-malignant tissue samples from 80 HCC patients. “Artificial intelligence” algorithm was then applied for analysis of the

profiling data in order to generate a unique biomarker signature that could distinguish HCC with high accuracy from the non-malignant and normal liver samples. Clinical correlation of each biomarker with different clinicopathological features in HCC was also evaluated.

PATIENTS AND METHODS

Clinical Specimens

Tumor and adjacent non-malignant tissues were collected from 80 patients who were diagnosed with primary HCC and elected for hepatic surgery at Queen Mary Hospital (Pokfulam, Hong Kong) between 1998 and 2006. The demographic data and clinicopathological features of the patients were summarized in Table 1. In addition, 10 normal liver tissues obtained from residual donor grafts of healthy subjects were also included in this study as control. This study was vetted and approved by the Institutional Ethics Committee, and all the tissues were collected with informed consent from patients. Tissues were snap-frozen in liquid nitrogen after surgical resection and stored at -80°C until use. H&E staining was performed for histology examination, and samples showing tissue homogeneity (>90%) were used in this study.

2-DE Proteomics Profiling

Proteins were extracted from 10 mg tissues using the ReadyPrep Sequential Extraction Kit (Bio-Rad, Hercules, CA) and the concentration determined by PlusOne 2-D Quant Kit (GE Healthcare, Uppsala, Sweden). Proteomic expression profiling of liver tissue (15 μg soluble lysates) was done by 2-DE gel electrophoresis using the IPGphor system and the Ettan™ DALTsix Electrophoresis Unit (GE Healthcare) as previously described.^{13,17,18} The silver-stained gel was scanned using a GS-800 Calibrated Densitometer (Bio-Rad) and a gray scale format of $2,700 \times 2,200$ pixels was used. As shown in Fig. 1, the digitized images were analyzed using the PDQuest software version 7.2 (Bio-Rad) for spot detection, matching, and normalization. Protein spot abundance was reported as relative value in ppm.

MALDI-ToF/ToF MS

Protein spots of interest were excised from the gel and subjected to destaining, washing, and trypsin digestion (MS grade, Promega, Madison, WI) at 37°C overnight. The digested peptides were recovered, reconstituted, purified by ZipTip® (Millipore, Bedford, MA) and then mixed with an equal volume of matrix (10 mg/ml α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA) for mass spectrometer analysis (4800 MALDI-TOF/TOF Analyzer, Applied Biosystems Inc., Foster City, CA).

Statistics and data-mining analysis

Sample size was estimated and calculated using Statmate program for Windows (GraphPad Software, Inc., San Diego, CA). After pilot experiments, a sample size of about 60 cases was used (90% power) to detect a statistically significant difference in intensity of protein spots between tumor and non-tumor samples. One-way ANOVA followed by Tukey or Dunnett's T3 tests, Student's *t*-test and Pearson Chi-Square Test were used to analyze spot intensities among normal, adjacent non-malignant and tumor samples. The discrimination performance was evaluated by plotting the receiver operator characteristic (ROC) curve and by

calculating the area under the ROC curve (AUC). $P < 0.05$ was considered as statistically significant. SPSS for Windows (version 13, SPSS, Inc., Chicago, IL) was used for statistical and correlation analysis in this study.

For data-mining analysis of the proteome datasets by classification and regression tree (CART) algorithm, the Biomarker Pattern Software (Ciphergen Biosystems, Inc., Fremont, CA) was employed to facilitate the decision-tree model building using the Gini impurity criterion for splitting and estimation using 10-fold cross-validation. The splitting decision in this case is based on the intensity of a spot or cluster at a defined cut-off value, with the following terminal nodes (tumor, non-tumor and normal) (Fig. 2). Before the data-mining analysis, the proteome datasets were randomly divided into (i) the “training” group (n = 120 cases; 55 tumor, 55 paired non-tumor and 10 normal) for model building, and (ii) the “blinded validation” group (n = 50 cases; 25 tumors and 25 non-tumors) for evaluation of the diagnostic model accuracy on discriminating HCC cases.

Quantitative PCR

Total RNA was extracted from frozen liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer’s instructions. After DNase treatment, RNA was reverse-transcribed to cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems), and the resulting products were used as templates for qPCR using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 200 nM gene-specific primers (*Hp2*: 5’- CAGAAAATGCAACAGCGAAA-3’; 5’- TAACCCACACGCCCTACTTC-3’; *CYB5A*: 5’- CGCCTTGATGTATCGCCTAT-3’, 5’- AAATTTGAGCGCAGAAAGGA-3’; *PGRMC1*: 5’- CCTCTGCATCTTCTGCTCT-3’, 5’- CGTTGATGGCCATGAGTATG-3’; *HSPB1*: 5’- ACGAGCATGGCTACATCTCC-3’, 5’- CTTTACTTGGCGGCAGTCTC-3’; *KRT1*: 5’- AGGAGGTGGACGTGGTAGTG-3’, 5’- GAGGGCAGACAGGACCATAA-3’; *CTSB*: 5’- AGAATGGCACACCCTACTGG-3’, 5’- TGCATTTCTACCCCGATCTC -3’) in a final volume of 20 μ l. The cycling parameters were set as followings: 50°C for 2 min, 1 cycle; 95°C for 2 min, 1 cycle; 95°C for 15s and 60°C for 30s, 50 cycles. Calculation and confirmation of the qPCR were performed according to previous procedures.^{18,19}

Immunohistochemistry

Tissue sections at 4- μ m thick were used for immunohistochemical staining. After blocking with 2% BSA in PBS, the sections were incubated at 4°C overnight with the following primary antibodies (1:100 dilution) against haptoglobin (Rockland Immunochemicals, Gilbertsville, PA), cytochrome b5, heat shock protein 27, cathepsin B (Santa Cruz Biotechnology), and keratin I (Invitrogen). For negative controls, primary antibodies were substituted with the corresponding purified isotype-matched immunoglobulins (Invitrogen or Sigma). Horseradish peroxidaseconjugated secondary antibodies (1:700 in blocking solution) were used, and signals were developed using diaminobenzidine solution (Invitrogen) and counterstained with hematoxylin (Vector Laboratories, Burlingame, CA).^{20,21}

Western blot analysis

Protein extracts (25 μ g) were separated on 10% polyacrylamide SDS gels and electroblotted on nitrocellulose membranes for western blotting according to previous procedures.^{22–24} After blocking with 5% non-fat dry milk in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6), the membrane was incubated with the primary antibodies as described above at 4 °C overnight, followed by peroxidase-conjugated secondary antibody (Invitrogen). Mouse anti-human β -actin antibody (Santa Cruz Biotechnology, CA) at 1:1000 dilution was included in parallel as a loading reference control. After substrate development (ECL detection reagents, GE Healthcare), densitometry data were analyzed by Quantity One (Bio-Rad) for protein quantification of each marker normalized with β -actin.

RESULTS

Differential proteomic analysis of HCC tissues: tumor vs non-tumor

The 2-DE-based proteomic profiling approach was employed to analyze 80 pairs of tumor and the matched non-tumor liver tissues resected from HCC patients who received curative hepatic surgery. The samples were randomly divided into 2 groups, (i) for model construction in the training set and (ii) for blind validation in the testing set. There was no significant difference ($P < 0.05$) between the 2 sample sets with respect to demographic and clinicopathological data such as gender, age, hepatitis, tumor pathology, and AFP level, etc (Table 1). In addition, a panel of 10 normal liver tissues from donor residual grafts was also included as healthy subject controls for comparison.

Fig. 1A showed a representative HCC proteome by 2-DE and the coordinates of the six discriminative biomarkers (as described below: SSP8103, SSP0026, SSP0023, SSP6108, SSP3102, SSP7711). Differential expression patterns of the six selected biomarkers that were composed of the proteomic signature discriminating among the HCC, non-tumor and normal liver tissues were depicted in Fig. 1B. Overall, there were 849 proteins commonly shared. Distribution of all the protein spots differentially expressed among different tissue types was summarized in the Venn diagram (Fig.1C). There were 21 and 56 exclusively expressed in the tumor and non-tumor livers, respectively, whereas 148 (HCC vs normal) and 282 (HCC vs non-tumor) were significantly different in their protein levels by One-way ANOVA ($P < 0.05$).

Proteomic classification model for distinguishing HCC from non-malignant tissues

To facilitate building a proteomic classification model, the CART algorithm data-mining method was employed using the Biomarker Pattern Software to analyze all the normalized, matched protein spot intensities from each proteome profile (Total $n = 120$; 55 HCC, 55 non-malignant, 10 healthy controls) in the training set. Figure 2A showed the list of candidate decision-tree outputs that were generated, and the optimal tree model with minimal cross-validation cost at 0.223 was taken and illustrated in Figure 2B. The choice binary decision tree consisted of six distinct biomarker classifiers: SSP8103, SSP0026, SSP0023, SSP6108, SSP7711, and SSP3102 (Figure 3A), and all of these biomarkers showed significant discriminative performance when analyzed by the ROC curves to distinguish at least one phenotype among the normal, non-malignant and tumor tissues (Fig.

3B). The sensitivity and specificity of the six-biomarker proteomic signature model for the training and the validation sets were described in Table 2.

Initially, the model was able to discriminate different sample types (tumor, non-tumor, normal liver) at high accuracy with 94.5% sensitivity and 98.1% specificity for HCC in the training set. We then further evaluated the model using both leave-one-out procedure and independent validation. In the blind validation test (samples $n = 50$), the biomarker signature remained at good performance with 88% sensitivity and 81.5% specificity respectively. Overall, based on the 170 samples that were tested, the performance of the 6-biomarker proteomic signature was satisfactorily with 92.5% at both the sensitivity and specificity to discriminate HCC from the non-malignant and normal liver tissues.

The Six-Biomarker Proteomic Signature

The six biomarker composition in the CART diagnostic model were subsequently identified by MALDI-ToF/ToF and unambiguously confirmed by tandem MS/MS, with at least 2 mass matched trypsin digested peptides in each protein spot, and each experiment was repeated at least twice. As presented in Table 3, the protein biomarker **SSP8103** was identified as haptoglobin (*Hp2*), which possesses protease and hemoglobin binding activities; **SSP0026** as cytochrome b5 (*CYB5A*), a major phase I xenobiotic metabolic enzyme subtype in liver parenchyma; **SSP0023** as progesterone receptor membrane component I (or progesterone receptor) (*PGRMC1*; other name *Hpr6.6*), a novel class of steroid receptor expressed on the cytoplasmic membrane, which is linked to cellular survival and possibly cancer progression as well; **SSP6108** as heat shock 27 kDa protein 1 (or HSP27) (*HSPB1*), one of the chaperones that is involved in protein folding and protection against cellular stress, which have been implicated in tumor development and progression in HCC and other malignancies; **SSP7711** as keratin I (*KRT1*), a cytoskeleton component in normal cells, and **SSP3102** as lysosomal proteinase cathepsin B (or cathepsin B) (*CTSB*) of the double-chain form, an active form of cysteine proteinase that was suggested to mediate cell death in tumors. Their relative mRNA expression values in the normal, adjacent non-tumor, and tumor tissues were evaluated by quantitative PCR (qPCR) (Fig. 4A). Except for the progesterone receptor with no commercial antibody available at present, western blot and immunohistochemistry were also performed for the other five protein biomarkers to examine the expression level and localization in the tumor and non-tumor tissues (Fig. 4B and 4C). In general, the qPCR data were comparable to the 2-DE differential expression profile pattern, except for the *PGRMC1* gene that yielded higher mRNA level in the tumors (Fig. 4A). The results derived from western blot and immunohistochemistry were largely comparable with those derived from 2-DE experiments. Higher expressions of haptoglobin, cytochrome b5, cathepsin B, keratin I were found in adjacent non-tumor tissues, whereas high expression of heat shock protein 27 was associated with tumor tissues (Fig. 4B). Immunohistochemistry analysis showed strong expression of heat shock protein 27 in HCC while immunostaining of haptoglobin, cytochrome b5, and cathepsin B were stronger in the non-tumor tissues. By contrast, the immunoreactivities of keratin I appeared weak in both cases (Fig. 4C).

Diagnosis performance and clinical relevance of each individual biomarker

The diagnosis test performance of individual protein biomarkers in the proteomic signature model were calculated and summarized in Table 4. While HSP27 (SSP6108) appeared to be the key biomarker for distinguishing the tumor type, both the cytochrome b5 (SSP0026) and haptoglobin (SSP8103) were indicators of healthy state in the normal liver. To discriminate the non-malignant liver tissues, the indicative biomarkers were progesterone receptor (SSP0023) and cathepsin B (SSP3102) as well as cytochrome b5 and haptoglobin at lower cut-off thresholds. By contrast, keratin I (SSP7711) was insufficient *per se* as a diagnostic biomarker based on its expression level alone.

Next we evaluated their potential clinical values by Pearson correlation analysis. Expression level of each protein biomarker in the panel was examined for their association with various clinicopathological features in HCC (Table 5). The results showed that cytochrome b5, progesterone receptor and keratin I were shown to have significant relevance with serum AFP (>400 ng/ml), total proteins levels and the Ishak's score, respectively. Ishak's score is a scoring system that takes architectural disturbance, fibrosis, and cirrhosis into account in assessing the liver status.²⁵

DISCUSSION

To identify potential diagnostic biomarkers for HCC, we performed proteomics profiling analysis on matched tumor and non-tumor tissues resected from 80 HCC patients mostly associated with HBV together with 10 normal liver controls. First, the CART diagnostic model was developed by supervised algorithm analysis of 120 proteome profiles in the training set. Second, it was subjected to validation test with separate blind samples (25 pairs of T/NT tissues) and gave similar efficiency. As a whole, based on all the 170 samples (the largest cohort studied in this kind), the described CART model performed satisfactorily (overall sensitivity and specificity were 92.5%).

The decision-tree analysis also led to the identification of six highly distinctive protein biomarkers (as reflected by ROC in Figure 3 and AUC in Table 4) that correspond to tumor, non-malignant and normal liver tissues. By using the MALDI-ToF/ToF and tandem MS/MS analytical methods, the biomarkers were identified as (i) haptoglobin – a fragment isoform of 42 kDa, (ii) cytochrome b5, (iii) progesterone receptor– a fragment isoform of 21 kDa, (iv) HSP27, (v) cathepsin B, and (vi) keratin I, respectively. Dysregulated expression of these protein biomarkers was also reported to have implications in tumor development and progression in various malignancies (Table 3). Our present findings indicated that cytochrome b5 (*CYB5A*), progesterone receptor (*PGRMC1*) and keratin I (*KRT1*) respectively correlated significantly with the following clinical parameters in HCC: - serum AFP, total protein levels and the Ishak's score, whilst the other three biomarkers did not reveal any significant association. Noteworthy from other studies, decreased expression of cytochrome b5 in HCC patients was associated with increased level of serum AFP,^{26,27} whereas progesterone receptor level was elevated in cirrhotic livers as well as in other cancerous tissues.²⁸ Keratin I level was also found higher in HCC tissues, which seemed to link with the histopathological condition of liver tissue damages. This study supports further

investigations into the molecular understanding and regulatory mechanisms of these biomarkers in hepatocarcinogenesis.

There is a trend of down regulation of metabolic biomarkers from healthy to diseased liver tissues to HCC. Haptoglobin (*Hp2*) functions as a heme-binding protein, together with transferrin. Previous studies have identified the *Hp2* β and $\alpha 2$ chains as potential serum biomarkers of HBV-associated liver inflammation.²⁹ In most chronic liver disease or HCC tissues, the haptoglobin level was found down-modulated as revealed by a similar proteomic approach.³⁰ The present study also revealed lower mRNA level of *Hp2* in tumor and non-tumor tissues as compared to the normal liver. Lower hemoglobin-binding capacity and increase in free iron-mediated oxidative stress may become a predisposing factor for hepatocarcinogenesis. Another example is cytochrome b5, which is one of the phase I catabolic enzymes of xenobiotics in normal liver. Down-regulation of cytochrome b5 was shown in HCV-related HCC in other studies.^{26,27} The inhibition of the detoxification enzymes in tumor may be related to the disruption of the membrane-bounded polysome attachment that is required for their synthesis in the endoplasmic reticulum. Thus, loss of haptoglobin and cytochrome b5 seems to be of general phenomenon in HCC that may indicate potential risk for tumor initiation or predisposition.

Tumors are complex biological systems, and tumorigenesis results from a progressive sequence of genetic alterations and post-translational protein modifications that promote malignant transformation of normal cells and neoplasm invasiveness. Progesterone receptor (*PGRMC1*), a novel class of membranous progesterone receptor, has been underscored in the cytochrome p450-dependent oxidative damage response pathway as demonstrated in breast cancer cells. Over-expression of progesterone receptor has been indicated in breast, cervix, colon and thyroid cancers.²⁸ In this study, the observed molecular weight of progesterone receptor, 17 kDa, is smaller than the native progesterone receptor (Hpr6.6) of 22 kDa, probably due to post-translational modifications or enzymatic cleavage process in the tumor microenvironment. The *PGRMC1* 17 kDa-form was first described in HCC here and would require more detailed structure-function characterization once specific antibodies are available.

Specific members of stress response chaperones or heat shock proteins are frequently upregulated in tumor tissues of various malignancies.³¹ In this study, the HSP27 level was drastically elevated in tumor tissues. It has been suggested to inhibit apoptosis and promote cellular survival in tumor and regulate endothelial cell migration, the key mechanisms that are responsible for tumor proliferation and malignant progression.³² In liver, up-regulated expression of HSP27 and other HSP members such as HSP70, GRP75 and GRP78, may be closely related to the pro-survival mechanisms in HCC in response to unfavorable conditions (e.g. hypoxia and nutrient depletion) that were induced during cancer development.^{15,30}

Upregulation of matrix proteinase enzymes and dysregulation of cytokeratins are often detected in the pre-malignant and lesion sites. Cathepsin B is a cysteine proteinase that is often over-expressed in colon, gastric, and lung cancers.³³ Its function was suggested to help degrade fibronectin, and laminin and some types of collagen during extracellular matrix

remodeling,³⁴ a process essential for tumor migration and invasion. There was a positive correlation of cathepsin B with increased angiogenesis in colorectal cancer and patient ages. Inhibition of the proteinase was reported to decrease cell invasion, angiogenesis and metastasis in various cancers.³⁵ In our study, its expression was higher in the adjacent non-tumor tissues. Keratin I is one of the cytoskeletal and microfibrillar keratins in normal cells, and is first found as one of the cytokeratins in amyloid deposits in amyloidosis. Our present findings also indicated its clinical correlation with the degree of liver damage as reflected by the Ishak's score. Cytokeratin profiling in several cancers also indicated its diagnostic utility in squamous cell carcinoma and HCC,³⁶ while other cytokeratin family members, such as cytokeratin 8, 10, and 18, were shown to be down-regulated in the context of HCC.²⁶

Indeed, query might exist justifying the use of this panel of protein markers to diagnose liver cancer because we only show their expressions in clinical tissues, but not in serum. Although we have not demonstrated the circulation of these protein markers in patients' sera, there are reports in the literature showing that some of these markers, such as cathepsin B and HSP27, are actually found in the bloodstream under certain clinical, or even HCC, conditions. The serum HSP27 level is elevated in HCC patients when compared to those with hepatitis B virus infection.³⁷ A higher serum level of cathepsin B is observed in patients with HCC and cirrhosis than normal subjects.³⁸ An elevated serum haptoglobin level can distinguish HCC patients from those patients with chronic liver diseases and hepatitis.^{37,39} However, no study reporting on the presence of the fragment isoform of haptoglobin that we identified in this study in blood circulation is yet performed. Besides, no report demonstrates the presence of cytochrome b5 in human serum. Based on these previous studies, it is believed that some of them might be secreted into the blood circulation in the normal liver condition or during liver tumorigenesis. It is of noted that some known serological markers of HCC nowadays, such as glypican-3, are first identified in liver tissues⁴⁰ and later confirmed to be present in patients' sera.^{41,42} For this purpose, large-scale and retrospective studies should be followed to illustrate their presence in blood circulation of normal subjects and patients. Results found in this paper form the foundation for further investigating the applicability of using this panel of markers for HCC diagnosis in future studies.

In conclusion, we have developed a proteomic signature model composed of six discriminative biomarkers that allow us to discriminate HCC from non-malignant and normal liver tissues at high accuracy. Some of these biomarkers are functionally related to pre-conditioning the stromal microenvironment or directly involved in the pro-survival and invasive mechanisms of HCC during tumor progression. HSP27 appears to be the key identifier for HCC whereas haptoglobin and cytochrome b5 are indicators for normal and healthy state of the liver.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the clinical supports and technical assistance provided by Wan-Ching Yu, Pauline Leung, Brian Lam and Stanley Lam of the Department of Surgery of The University of Hong Kong.

Financial Support: The work was supported by the Research Grants Council of Hong Kong (N_HKU 718/03), Innovation and Technology Fund (ITS/120/07) and Sun Chieh Yeh Research Foundation for Hepatobiliary and Pancreatic Surgery.

Abbreviations

2-DE	2-dimensional gel electrophoresis
AFP	alpha fetoprotein
CART	classification-and-regression tree
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
MALDI-ToF MS	matrix assisted laser desorption ionization-time of flight mass spectrometry
ROC	receiver operating characteristics
T/NT	tumor/non-tumor

REFERENCES

- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics 2002. *CA Cancer J Clin.* 2002; 55(2): 74–108. [PubMed: 15761078]
- Poon RT, Fan ST. Is primary resection and salvage transplantation for hepatocellular carcinoma a reasonable strategy? *Ann Surg.* 2004; 240(5):925–928. [PubMed: 15492581]
- Poon RT, Ng IO, Fan ST, Lai EC, Lo CM, Liu CL, Wong J. Clinicopathologic features of long-term survivors and disease-free survivors after resection of hepatocellular carcinoma: a study of a prospective cohort. *J Clin Oncol.* 2001; 19(12):3037–3044. [PubMed: 11408499]
- Lok AS, Lai CL. alpha-Fetoprotein monitoring in Chinese patients with chronic hepatitis B virus infection: role in the early detection of hepatocellular carcinoma. *Hepatology.* 1989; 9(1):110–115. [PubMed: 2461890]
- Farinati F, Marino D, De Giorgio M, Baldan A, Cantarini M, Cursaro C, Rapaccini G, Del Poggio P, Di Nolfo MA, Benvegna L, Zoli M, Borzio F, Bernardi M, Trevisani F. Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? *Am J Gastroenterol.* 2006; 101(3):524–532. [PubMed: 16542289]
- Daniele B, Bencivenga A, Megna AS, Tinessa V. Alpha-fetoprotein and ultrasonography screening for hepatocellular carcinoma. *Gastroenterology.* 2004; 127 Suppl 1(5):S108–S112. [PubMed: 15508073]
- Moriguchi H, Sato C. The values and limitations of glypican-3 as a novel tumor marker for hepatocellular carcinoma from clinical and economic viewpoints. *Gastroenterology.* 2004; 127(2): 679–680. [PubMed: 15300602]
- Pompili M, Addolorato G, Pignataro G, Rossi C, Zuppi C, Covino M, Grieco A, Gasbarrini G, Rapaccini GL. Evaluation of the albumin-gamma-glutamyltransferase isoenzyme as a diagnostic marker of hepatocellular carcinoma-complicating liver cirrhosis. *J Gastroenterol Hepatol.* 2003; 18(3):288–295. [PubMed: 12603529]
- Marrero JA, Su GL, Wei W, Emick D, Conjeevaram HS, Fontana RJ, Lok AS. Des-gamma carboxyprothrombin can differentiate hepatocellular carcinoma from nonmalignant chronic liver disease in American patients. *Hepatology.* 2003; 37(5):1114–1121. [PubMed: 12717392]
- Khien VV, Mao HV, Chinh TT, Ha PT, Bang MH, Lac BV, Hop TV, Tuan NA, Don LV, Taketa K, Satomura S. Clinical evaluation of lentil lectin-reactive alpha-fetoprotein-L3 in histology-proven hepatocellular carcinoma. *Int J Biol Markers.* 2001; 16(2):105–111. [PubMed: 11471892]
- Lee HB, Yoo OJ, Ham JS, Lee MH. Serum alpha 1-antitrypsin in patients with hepatocellular carcinoma. *Clin Chim Acta.* 1992; 206(3):225–230. [PubMed: 1376649]
- Sun S, Lee NP, Poon RT, Fan ST, He QY, Lau GK, Luk JM. Oncoproteomics of hepatocellular carcinoma: from cancer markers' discovery to functional pathways. *Liver Int.* 2007; 27(8):1021–1038. [PubMed: 17845530]

13. Yi X, Luk JM, Lee NP, Peng J, Leng X, Guan XY, Lau GK, Beretta L, Fan ST. Association of mortalin (HSPA9) with liver cancer metastasis and prediction for early tumor recurrence. *Mol Cell Proteomics*. 2008; 7(2):315–325. [PubMed: 17934217]
14. Luk JM, Lam BY, Lee NP, Ho DW, Sham PC, Chen L, Peng J, Leng X, Day PJ, Fan ST. Artificial neural networks and decision tree model analysis of liver cancer proteomes. *Biochem Biophys Res Commun*. 2007; 361(1):68–73. [PubMed: 17644064]
15. Luk JM, Lam CT, Siu AF, Lam BY, Ng IO, Hu MY, Che CM, Fan ST. Proteomic profiling of hepatocellular carcinoma in Chinese cohort reveals heat-shock proteins (Hsp27, Hsp70, GRP78) up-regulation and their associated prognostic values. *Proteomics*. 2006; 6(3):1049–1057. [PubMed: 16400691]
16. Feng JT, Shang S, Beretta L. Proteomics for the early detection and treatment of hepatocellular carcinoma. *Oncogene*. 2006; 25(27):3810–3817. [PubMed: 16799622]
17. Luk JM, Su YC, Lam SC, Lee CK, Hu MY, He QY, Lau GK, Wong FW, Fan ST. Proteomic identification of Ku70/Ku80 autoantigen recognized by monoclonal antibody against hepatocellular carcinoma. *Proteomics*. 2005; 5(7):1980–1986. [PubMed: 15832367]
18. Lee NP, Leung KW, Cheung N, Lam BY, Xu MZ, Sham PC, Lau GK, Poon RT, Fan ST, Luk JM. Comparative proteomic analysis of mouse livers from embryo to adult reveals an association with progression of hepatocellular carcinoma. *Proteomics*. 2008; 8(10):2136–2149. [PubMed: 18425728]
19. Wang XQ, Luk JM, Leung PP, Wong BW, Stanbridge EJ, Fan ST. Alternative mRNA splicing of liver intestine-cadherin in hepatocellular carcinoma. *Clin Cancer Res*. 2005; 11(2 Pt 1):483–489. [PubMed: 15701831]
20. Luk JM, Wang PP, Lee CK, Wang JH, Fan ST. Hepatic potential of bone marrow stromal cells: development of *in vitro* co-culture and intra-portal transplantation models. *J Immunol Methods*. 2005; 305:39–47. [PubMed: 16150456]
21. Lee NP, Tong MK, Leung PP, Chan VW, Leung S, Tam PC, Chan KW, Lee KF, Yeung WS, Luk JM. Kidney claudin-19: localization in distal tubules and collecting ducts and dysregulation in polycystic renal disease. *FEBS Lett*. 2006; 580:923–931. [PubMed: 16427635]
22. Wong WS, Luk JM. Signaling mechanisms of pertussis toxin-induced myelomonocytic cell adhesion: role of tyrosine phosphorylation. *Biochem Biophys Res Commun*. 1997; 236(2):479–482. [PubMed: 9240464]
23. Lee NP, Leung KW, Wo JY, Tam PC, Yeung WS, Luk JM. Blockage of testicular connexins induced apoptosis in rat seminiferous epithelium. *Apoptosis*. 2006; 11(7):1215–1229. [PubMed: 16699959]
24. Lee NP, Tsang S, Cheng RH, Luk JM. Increased solubility of integrin betaA domain using maltose-binding protein as a fusion tag. *Protein Pept Lett*. 2006; 13(5):431–435. [PubMed: 16800794]
25. Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN, et al. Histological grading and staging of chronic hepatitis. *J Hepatol*. 1995; 22(6):696–699. [PubMed: 7560864]
26. Blanc JF, Lalanne C, Plomion C, Schmitter JM, Bathany K, Gion JM, Bioulac-Sage P, Balabaud C, Bonneu M, Rosenbaum J. Proteomic analysis of differentially expressed proteins in hepatocellular carcinoma developed in patients with chronic viral hepatitis C. *Proteomics*. 2005; 5(14):3778–3789. [PubMed: 16097030]
27. Kim W, Oe Lim S, Kim JS, Ryu YH, Byeon JY, Kim HJ, Kim YI, Heo JS, Park YM, Jung G. Comparison of proteome between hepatitis B virus- and hepatitis C virus-associated hepatocellular carcinoma. *Clin Cancer Res*. 2003; 9(15):5493–5500. [PubMed: 14654528]
28. Crudden G, Loesel R, Craven RJ. Overexpression of the cytochrome p450 activator hpr6 (heme-1 domain protein/human progesterone receptor) in tumors. *Tumour Biol*. 2005; 26(3):142–146. [PubMed: 15970648]
29. He QY, Lau GK, Zhou Y, Yuen ST, Lin MC, Kung HF, Chiu JF. Serum biomarkers of hepatitis B virus infected liver inflammation: a proteomic study. *Proteomics*. 2003; 3(5):666–674. [PubMed: 12748946]

30. Lee IN, Chen CH, Sheu JC, Lee HS, Huang GT, Yu CY, Lu FJ, Chow LP. Identification of human hepatocellular carcinoma-related biomarkers by two-dimensional difference gel electrophoresis and mass spectrometry. *J Proteome Res.* 2005; 4(6):2062–2069. [PubMed: 16335951]
31. Beere HM. Stressed to death: regulation of apoptotic signaling pathways by the heat shock proteins. *Sci STKE.* 2001; 2001(93):RE1. [PubMed: 11752668]
32. Garrido C, Schmitt E, Cande C, Vahsen N, Parcellier A, Kroemer G. HSP27 and HSP70: potentially oncogenic apoptosis inhibitors. *Cell Cycle.* 2003; 2(6):579–584. [PubMed: 14512773]
33. Kruszewski WJ, Rzepko R, Wojtacki J, Skokowski J, Kopacz A, Jaskiewicz K, Drucis K. Overexpression of cathepsin B correlates with angiogenesis in colon adenocarcinoma. *Neoplasma.* 2004; 51(1):38–43. [PubMed: 15004658]
34. Creemers LB, Jansen ID, Docherty AJ, Reynolds JJ, Beertsen W, Everts V. Gelatinase A (MMP-2) and cysteine proteinases are essential for the degradation of collagen in soft connective tissue. *Matrix Biol.* 1998; 17(1):35–46. [PubMed: 9628251]
35. Lakka SS, Gondi CS, Yanamandra N, Olivero WC, Dinh DH, Gujrati M, Rao JS. Inhibition of cathepsin B and MMP-9 gene expression in glioblastoma cell line via RNA interference reduces tumor cell invasion, tumor growth and angiogenesis. *Oncogene.* 2004; 23(27):4681–4689. [PubMed: 15122332]
36. Tanaka S, Hirohashi K, Uenishi T, Yamamoto T, Hamba H, Kubo S, Tanaka H, Shuto T, Ogawa M, Kinoshita H. A mixed hepatocellular carcinoma and cholangiocarcinoma: dual expression of biliary-type cytokeratin and hepatocyte specific marker. *Hepatogastroenterology.* 2004; 51(57):839–841. [PubMed: 15143930]
37. Feng JT, Liu YK, Song HY, Dai Z, Qin LX, Almofti MR, Fang CY, Lu HJ, Yang PY, Tang ZY. Heat-shock protein 27: a potential biomarker for hepatocellular carcinoma identified by serum proteome analysis. *Proteomics.* 2005; 5(17):4581–4588. [PubMed: 16240287]
38. Leto G, Tumminello FM, Pizzolanti G, Montalto G, Soresi M, Gebbia N. Lysosomal cathepsins B and L and Stefin A blood levels in patients with hepatocellular carcinoma and/or liver cirrhosis: potential clinical implications. *Oncology.* 1997; 54(1):79–83. [PubMed: 8978598]
39. Ang IL, Poon TC, Lai PB, Chan AT, Ngai SM, Hui AY, Johnson PJ, Sung JJ. Study of serum haptoglobin and its glycoforms in the diagnosis of hepatocellular carcinoma: a glycoproteomic approach. *J Proteome Res.* 2006; 5(10):2691–2700. [PubMed: 17022640]
40. Zhu ZW, Friess H, Wang L, Abou-Shady M, Zimmermann A, Lander AD, Korc M, Kleeff J, Buchler MW. Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. *Gut.* 2001; 48(4):558–564. [PubMed: 11247902]
41. Nakatsura T, Kageshita T, Ito S, Wakamatsu K, Monji M, Ikuta Y, Senju S, Ono T, Nishimura Y. Identification of glypican-3 as a novel tumor marker for melanoma. *Clin Cancer Res.* 2004; 10(19):6612–6621. [PubMed: 15475451]
42. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, Filmus J. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. *Gastroenterology.* 2003; 125(1):89–97. [PubMed: 12851874]
43. Lim SK, Ferraro B, Moore K, Halliwell B. Role of haptoglobin in free hemoglobin metabolism. *Redox Rep.* 2001; 6(4):219–227. [PubMed: 11642712]
44. Gordon S. Homeostasis: a scavenger receptor for haemoglobin. *Curr Biol.* 2001; 11(10):R399–R401. [PubMed: 11378405]
45. Madsen M, Graversen JH, Moestrup SK. Haptoglobin and CD163: captor and receptor gating hemoglobin to macrophage lysosomes. *Redox Rep.* 2001; 6(6):386–388. [PubMed: 11865982]
46. Porter TD. The roles of cytochrome b5 in cytochrome P450 reactions. *J Biochem Mol Toxicol.* 2002; 16(6):311–316. [PubMed: 12481306]
47. Schenkman JB, Jansson I. The many roles of cytochrome b5. *Pharmacol Ther.* 2003; 97(2):139–152. [PubMed: 12559387]
48. Lewis DF, Ito Y. Human cytochromes P450 in the metabolism of drugs: new molecular models of enzyme-substrate interactions. *Expert Opin Drug Metab Toxicol.* 2008; 4(9):1181–1186. [PubMed: 18721112]
49. Mifsud W, Bateman A. Membrane-bound progesterone receptors contain a cytochrome b5-like ligand-binding domain. *Genome Biol.* 2002; 3(12) RESEARCH0068.

50. Cahill MA. Progesterone receptor membrane component 1: an integrative review. *J Steroid Biochem Mol Biol.* 2007; 105(1–5):16–36. [PubMed: 17583495]
51. Losel RM, Besong D, Peluso JJ, Wehling M. Progesterone receptor membrane component 1--many tasks for a versatile protein. *Steroids.* 2008; 73(9–10):929–934. [PubMed: 18249431]
52. Ciocca DR, Oesterreich S, Chamness GC, McGuire WL, Fuqua SA. Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. *J Natl Cancer Inst.* 1993; 85(19): 1558–1570. [PubMed: 8411230]
53. Parcellier A, Schmitt E, Brunet M, Hammann A, Solary E, Garrido C. Small heat shock proteins HSP27 and alphaB-crystallin: cytoprotective and oncogenic functions. *Antioxid Redox Signal.* 2005; 7(3–4):404–413. [PubMed: 15706087]
54. Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, Garrido C. Heat shock proteins: essential proteins for apoptosis regulation. *J Cell Mol Med.* 2008; 12(3):743–761. [PubMed: 18266962]
55. Mort JS, Buttler DJ. Cathepsin B. *Int J Biochem Cell Biol.* 1997; 29(5):715–720. [PubMed: 9251238]
56. Moin K, Demchik L, Mai J, Duessing J, Peters C, Sloane BF. Observing proteases in living cells. *Adv Exp Med Biol.* 2000; 477:391–401. [PubMed: 10849765]
57. Oriolo AS, Wald FA, Ramsauer VP, Salas PJ. Intermediate filaments: a role in epithelial polarity. *Exp Cell Res.* 2007; 313(10):2255–2264. [PubMed: 17425955]
58. Magin TM, Vijayaraj P, Leube RE. Structural and regulatory functions of keratins. *Exp Cell Res.* 2007; 313(10):2021–2032. [PubMed: 17434482]

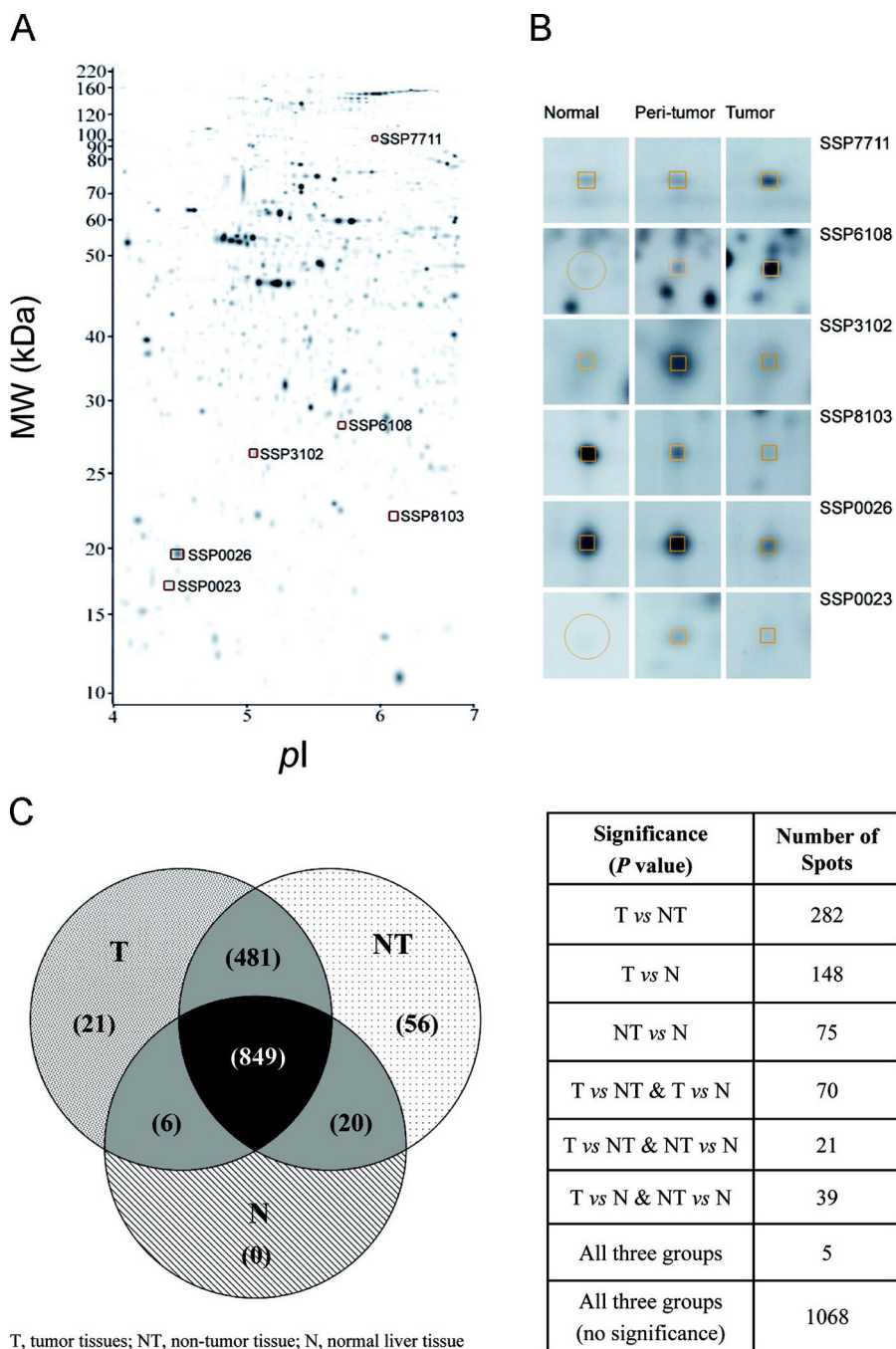


Fig. 1. Differential expression profiles of HCC proteome

(A) Liver tissue lysates were separated by IEF in the range of *pI* 4–7, followed by second dimension (MW, 10–250 kDa) of PAGE and silver staining.

(B) Representative gel pictures of the six reference points from HCC, peritumor and normal liver tissues were shown.

(C) Venn diagram showing the numerical distribution of protein spots presents in tumor (T), non-tumor (NT) and normal (N) livers. The numbers of protein spots with significant changes ($P < 0.05$) among the three different comparison groups (T vs NT, T vs N, and NT vs

N) were tabulated. Statistical analysis was performed by one-way ANOVA and student-T-test ($p < 0.05$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

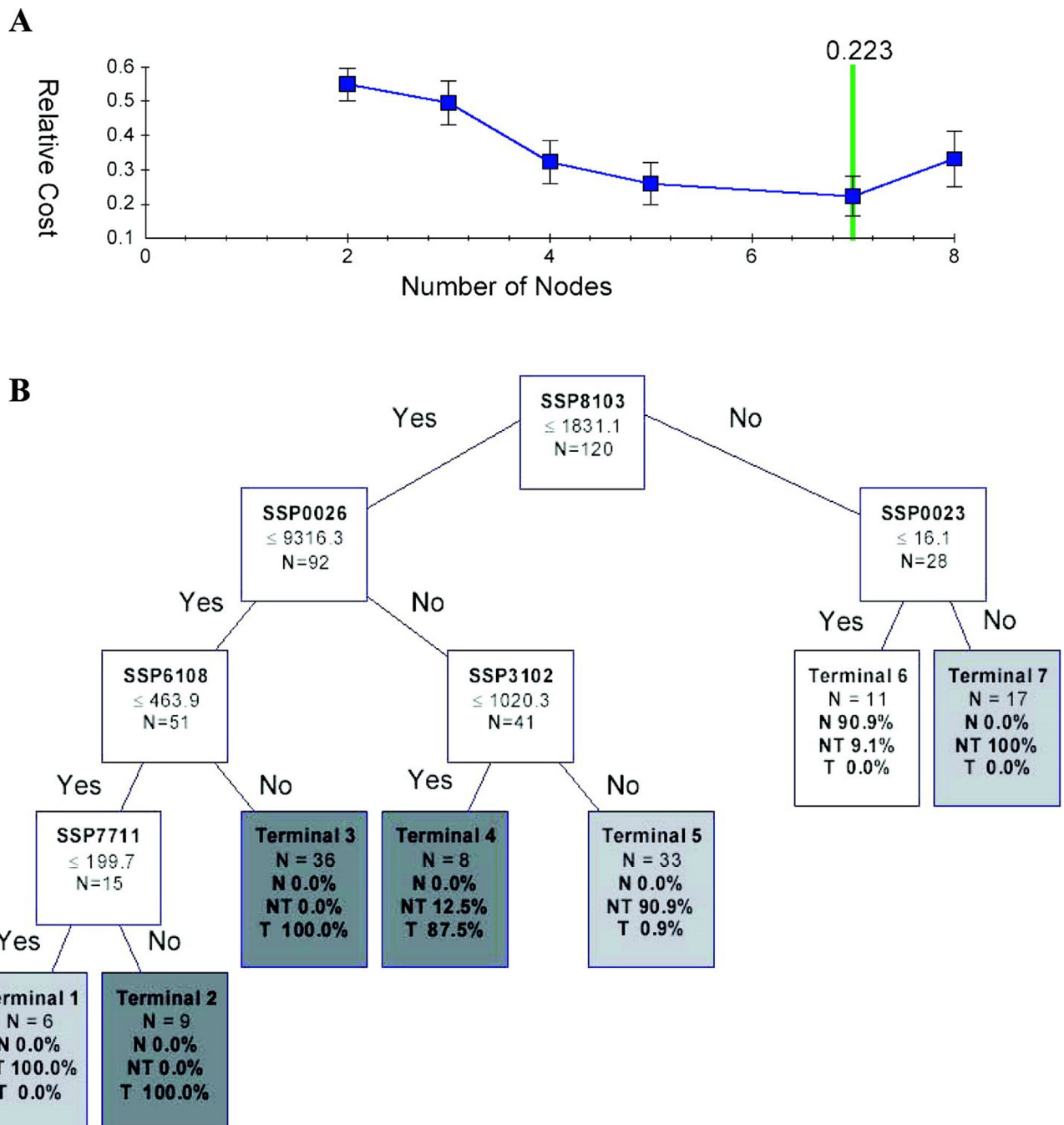


Fig. 2. The optimal classification tree generated by CART

(A) The cost value of decision trees with varying number of terminal nodes.

(B) The optimal decision tree is composed of 6 discriminative classifiers. The decision making process involves the evaluation of if-then rules of each node from top to bottom, which eventually reaches a terminal node with designated class outcome: tumor (T), non-tumor (NT) or normal (N).

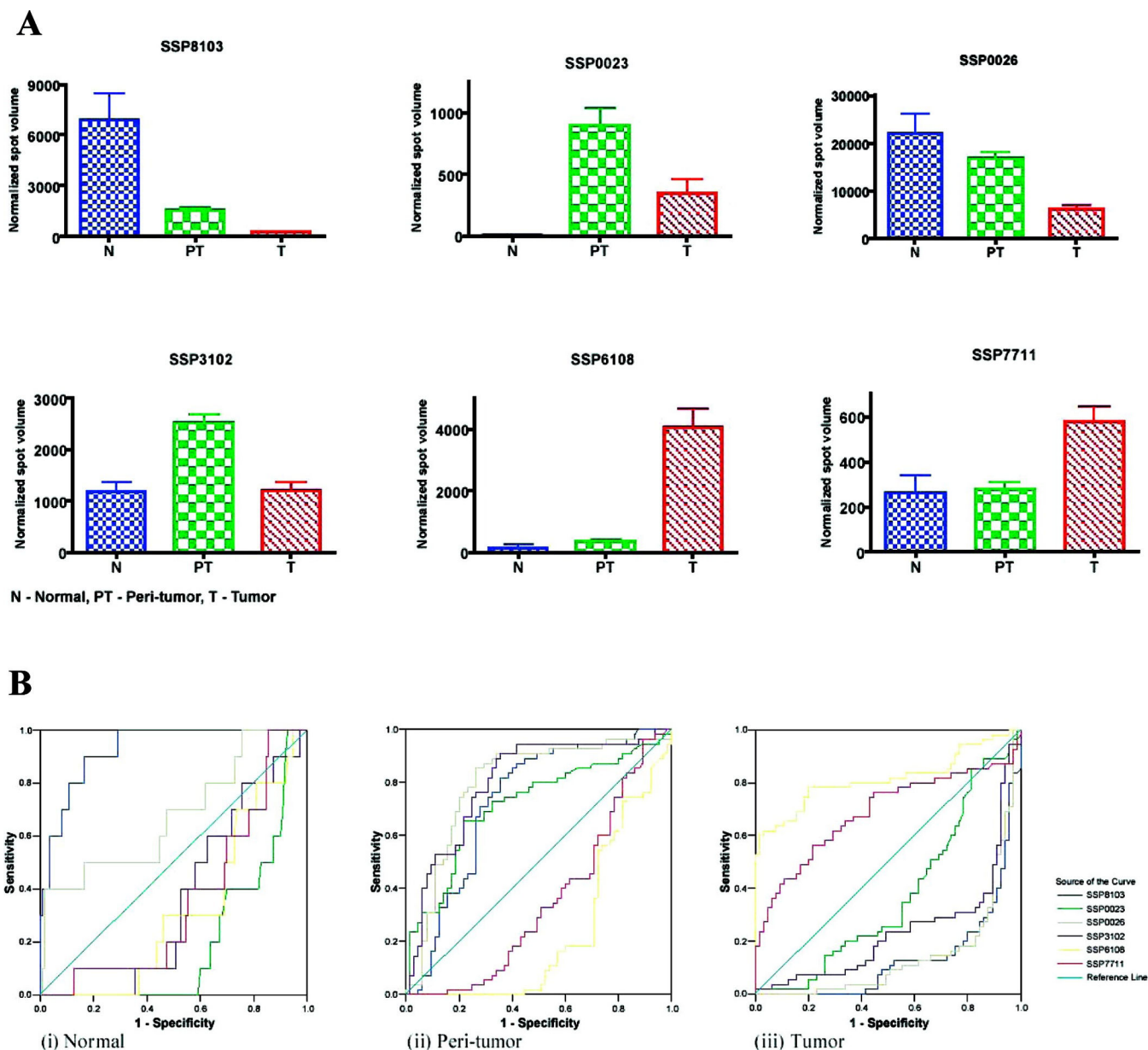


Fig. 3. Spot intensities and ROC curves of each six protein markers differentially expressed in HCC, non-tumor and normal liver tissues

(A) Spot intensities on 2-DE gel of the six protein markers were graphically presented in respective histogram charts. Each bar represents a mean \pm SD of the normalized spot intensity in ppm. Data were from the training set and the sample sizes were 55 (T), 55 (NT) and 10 (N). Statistical analysis was performed by student-T-test ($p < 0.05$) when compared T vs NT and T vs N.

(B) ROC curves of the 6 protein markers for their discriminative powers in the categories of normal livers (N), peri-tumor tissues (PT), and tumor tissues (T). For example, SSP8103 and SSP0026 showed preferential values for normal liver; SSP3102, SSP0023, SSP0026, SSP8103 for non-tumor, and SSP6108, SSP7711 for tumor, respectively. Data were from the training set.

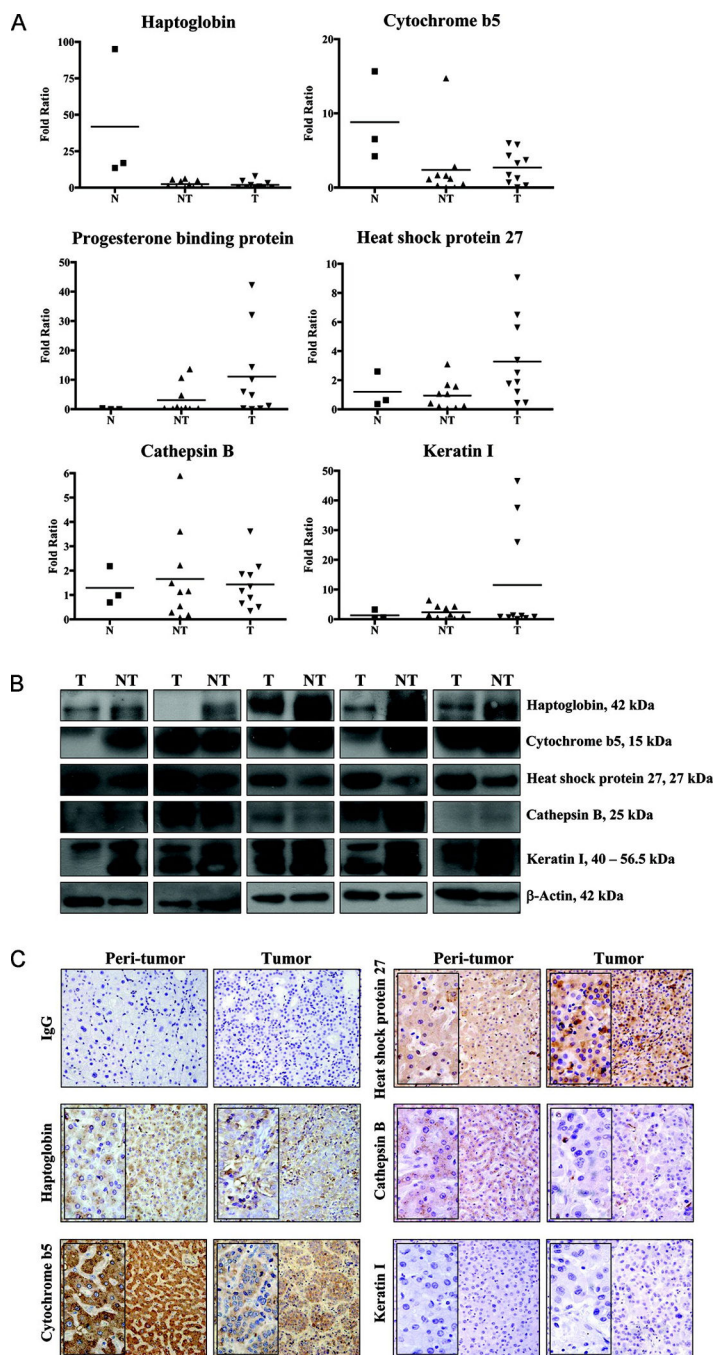


Fig. 4. Relative gene and protein expressions of the six protein markers in human liver tissues (A) Real time PCR was performed using total RNA isolated from normal human livers (N) (n = 3), non-tumor (NT) (n = 10) and tumor (T) (n = 10) liver tissues from HCC patients to examine the gene abundance. The relative gene expressions of haptoglobin, cytochrome b5, progesterone binding protein, heat shock protein 27, cathepsin B, and cytokeratin I were presented by fold ratio difference. The mean value of each category was also shown accordingly.

(B) Western blot was performed to reveal the protein levels of target proteins in peri-tumor (NT) and corresponding tumor (T) tissues of HCC patients. 10 pairs of NT and T clinical specimens were included and 5 representative sets of western blots are shown herein.

(C) Immunohistochemistry was performed to reveal the localizations and expressions of the target proteins in peri-tumor and corresponding tumor tissues of HCC patients. The primary antibodies targeting haptoglobin, cytochrome b5, heat shock protein 27, cathepsin B, cytokeratin I, or the respective IgG (negative control) were used at 1:100 dilution to reveal the corresponding immunoreactivities. A commercial antibody against progesterone membrane receptor component 1 is not available and thus not included. Representative micrographs of each antibody and selected IgG isotype control were shown. Magnification, $\times 200$ (insert $\times 400$).

Table 1

Demographics and Clinicopathological Features of HCC Patients

Characteristics	Training Set (n = 55)	Validation Set (n = 25)	<i>P</i> value
Gender (M:F)	48:7	20:5	0.405
Age	53.4 ± 5.8 (Median: 54)	57.0 ± 2.2 (Median: 59)	0.228
Hepatitis (HBV / HCV / negative)	48 / 0 / 7	20 / 1 / 4	0.534
Tumor Size (cm)	8.79 ± 0.61 (Median: 8.00)	7.42 ± 1.06 (Median: 7.00)	0.277
Tumor Stage (AJCC TNM)			
I–II	33	18	0.307
III–IV	22	7	
Tumor Recurrence	22	8	0.331
Child's Grade			
A	53	25	
B	2	0	0.340
C	0	0	

Note: Both tumor and non-tumor specimens from 80 HCC patients were randomly assigned for the training and validation purposes. There was no apparent difference between the 2 sample sets.

Table 2
Sensitivities and Specificities of Proteomics Expression Signature for Identification of HCC

	Sample Size (<i>n</i>)	Sensitivity / Specificity	Sample Classification (<i>N</i>)		
			Normal	Peri-Tumor	Tumor
Training Set					
Normal	10	100% / 90.9%	10	0	0
Peri-tumor	55	96.4% / 94.6%	1	53	1
Tumor	55	94.5% / 98.1%	0	3	52
Blind validation Set					
Peri-tumor	25	76.0% / 90.4%	1	19	5
Tumor	25	88.0% / 81.5%	0	3	22
Overall					
Normal	10	100% / 90.9%	10	0	0
Peri-tumor	80	90.0% / 83%	2	72	6
Tumor	80	92.5% / 92.5%	0	6	74

Note: "Peri-tumor" is non-malignant liver tissue adjacent to the tumor; "Normal" is residual liver graft tissue.

Table 3
MS/MS Characterization of The Six Protein Markers in Proteomic Signature Model of HCC.

Spot	Protein identity (<i>Gene name</i>)	MW (kDa)	pI	Mascot Score	Matched Peaks	Sequenced Peptides	Accession Numbers
SSP8103	Haptoglobin (<i>Hp2</i>)	41.743	6.23	173	1311.6 1439.6 895.5	TEGDGVYTLNDK TEGDGVYTLNNEK NPANPVQR	1006264A
SSP0026	Cytochrome b5 (<i>CYB5A</i>)	15.330	4.88	76	1186.6 1134.59 1511.73	YYTLEEIQK STWLILHHK FLEHPGGEEVLR	P00167
SSP0023	Progesterone receptor membrane component 1 (<i>PGRMC1</i>)	21.671	4.56	85	1644.8 1516.7	KFYGPEGPYGVFAGR FYGPEGPYGVFAGR	NP_006658
SSP6108	Heat shock 27kDa protein 1 (<i>HSPB1</i>)	22.782	5.98	73	961.4 1163.62 1104.53	GPSWDPPR LFDQAFGLPR QDEHGYISR	NP_001531
SSP3102	Lysosomal proteinase cathepsin B (<i>CTSB</i>)	23.077	5.19	164	1314.6 1634.70 1823.90	ICEFGYSPTYK HYGYNSYVSNSSEK GQDHCIESEVVAGIPR	AAA52125
SSP7711	Keratin I (<i>KRT1</i>)	66.038	8.15	68	1179.60 1475.70	YEELQITAGR FLEQQNQLQTK	NP_006112

Note: Protein identity was based on at >2 sequenced peptides from at least 2 separate MS/MS analysis. Internal standards: des-Arg1 Bradykinin, Angiotensin I and Glu1-Fibrino-peptide B (Monoisotopic MH⁺ m/z 904.4681, 1296.6853, 1570.6774, respectively) were used for mass calibration. The tandem mass spectra were collected in product ion mode on the peptide of interest. Measured peptide and fragment ion masses were used to search the NCBI and SWISS-PROT databases for protein identifications using the Mascot (www.matrixscience.com), with the following settings: mass tolerance at 50 ppm, one missed cleavage allowed, at least two matching peptides and searches limited to *Homo sapiens* species.

Table 4
Individual Diagnostic Performance of Biomarkers to Discriminate Tumor, Non-tumor, and Normal Liver Tissues

Test criteria	Classifiers in each tissue type							
	Tumor			Non-Tumor			Normal	
	SSP6108 (HSP27)	SSP7711 (keratin I)	SSP0023 (progesterone receptor)	SSP0026 (cytochrome b5)	SSP3102 (cathepsin B)	SSP8103 (haptoglobin)	SSP0026 (cytochrome b5)	SSP8103 (haptoglobin)
AUC	0.820	0.697	0.720	0.783	0.801	0.724	0.733	0.950
Cut-off point	6.298	5.675	5.630	9.190	7.176	6.697	9.171	7.735
Sensitivity	0.782	0.764	0.667	0.778	0.852	0.736	0.800	0.900
Specificity	0.797	0.562	0.779	0.738	0.672	0.708	0.523	0.870
False positive rate, FPR	0.203	0.438	0.221	0.262	0.328	0.292	0.477	0.130
False negative rate, FNR	0.218	0.236	0.333	0.222	0.148	0.264	0.200	0.100
Accuracy	0.790	0.663	0.723	0.756	0.756	0.563	0.546	0.958
Youden's Index	0.579	0.326	0.446	0.516	0.524	0.444	0.323	0.770
Positive likelihood ratio, LR+	3.852	1.744	3.018	2.970	2.598	2.521	1.677	6.923
Negative likelihood ratio, LR-	0.225	0.420	0.428	0.301	0.220	0.373	0.382	0.115
Positive predictive value, PV+	0.768	0.600	0.706	0.712	0.852	0.672	0.132	0.281
Negative predictive value, PV-	0.810	0.735	0.735	0.800	0.846	0.459	0.966	0.990

Table 5
Pearson Correlation of Protein Markers with Clinicopathological Data in HCC

Characteristics	Protein Markers*					
	SSP0026 (cytochrome b5)		SSP0023 (progesterone receptor)		SSP7711 (keratin I)	
	Coefficient	P value	Coefficient	P value	Coefficient	P value
Ishak's Score**	-0.143	0.505	-0.271	0.200	-0.652	0.001
Edmonson Grade	-0.117	0.405	-0.081	0.565	-0.247	0.080
Total Bilirubin (umol/l)	0.185	0.185	0.015	0.915	0.026	0.857
Albumin (g/l)	-0.097	0.488	0.121	0.388	-0.108	0.444
SGOT (U/L)	0.188	0.187	-0.048	0.738	0.136	0.340
SGPT (U/L)	0.026	0.856	0.003	0.985	0.158	0.267
AFP	-0.390	0.000	-0.152	0.128	-0.064	0.524
HBsAg	-0.026	0.798	0.100	0.313	-0.087	0.382
Serum Total Protein	-0.000	0.998	0.223	0.024	-0.052	0.604

* SSP8103, SSP6108 and SSP3102 that showed no significant correlation ($P < 0.05$, 2-tailed) were not presented.

** Ishak score: 0, none fibrosis; 1-2, portal fibrotic expansion; 3-4, bridging fibrosis; 5-6, cirrhosis

Table 6

The Biological Functions of the Protein Markers in the HCC Signature Model

Protein markers	General Functions*	Implications in cancer	References
Haptoglobin	binding of free plasma hemoglobin; iron homeostasis; hemoglobin clearance	breast, kidney, liver ovary	43-45
Cytochrome b5	stimulation of cytochrome P450 reactions; cytochrome P450-related drug metabolism	liver	46-48
Progesterone receptor	distant homolog of cytochrome b5; having similar functions as cytochrome b5	breast	49-51
Heat shock protein 27	stress resistance; molecular chaperone; drug resistance; cytoprotective functions	breast, liver, tongue ovary	52-54
Cathepsin B	cysteine protease; protein catabolism; protein degradation	colon, lung, stomach	55,56
Keratin I	a type of intermediate filament; structural protein; scaffolding functions	liver	57,58

* This table describes the general functions of these protein markers and their implications in cancer malignancies. Readers should refer to the text regarding to the cancer- or liver-related functions of these protein markers