Proteomic Analysis of Hepatocellular Carcinoma Tissues With Encapsulation Shows Up-regulation of Leucine Aminopeptidase 3 and Phosphoenolpyruvate Carboxykinase 2

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Abstract. Background/Aim: Cancer is the most fatal disease worldwide whose most lethal characteristics are invasion and metastasis. Hepatocellular carcinoma (HCC) is one of the most fatal cancers worldwide. HCC often shows encapsulation, which is related to better prognosis. In this study, proteomic analysis of HCC tissues with and without encapsulation was performed, in order to elucidate the factors which play important roles in encapsulation. Materials and Methods: Five HCC tissues surrounded by a capsule and five HCC tissues which broke the capsule were obtained from patients diagnosed with HCC who underwent surgical liver resection. Protein samples from these tissues were separated by two-dimensional gel electrophoresis (2-DE), and the protein spots whose expression was different between encapsulated and non-encapsulated HCC tissues were identified through gel imaging analysis software. The selected protein spots were analyzed and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: Two-DE analysis showed 14 spots whose expression was different between encapsulated and non-encapsulated HCC tissues. Of these, 9 were up-regulated and 5 were down-regulated in HCC tissues without encapsulation. The validation by Western blot confirmed that leucine aminopeptidase 3 (LAP3) and phosphoenolpyruvate carboxykinase mitochondrial (PCK2) were up-regulated significantly in HCC tissues with a capsule, compared to HCC tissues that broke the capsule. Conclusion: These findings suggest that LAP3 and PCK2 could be factors responsible for the maintenance of encapsulation in HCC tissues.

Although multidisciplinary therapies such as surgery, radiation and chemotherapy have made progress in the treatment of cancer, the latter is still one of the leading causes of death in many countries. Many types of cancer show strong invasive and metastatic capability. Cancer metastasis is composed of a number of steps. In principle, cancer cells show epithelial-to-mesenchymal transition (EMT) and migrate into surrounding tissues in the course of degrading extracellular matrix from cancer primary tissues. After intravasation, the cancer cells move to distant organs. Then, cancer cells extravasate from blood vessels, and invade the parenchyma and infiltrate adjacent tissues. Lastly, the cancer cells establish a metastatic tumor tissue with a wealth of tumor vasculature (1). So far, many types of anti-metastatic drugs, targeting multiple steps in the process of metastasis have been developed. Anti-matrix metalloproteinase (anti-MMP) drugs marimastat (2), prinomastat (3), BMS-275291, metastat (4)

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and neovastat (5) have been developed, while SU5416 and SU6668 are anti-angiogenesis drugs (6), and have been used in the phase II studies for several types of cancer. However, unfortunately all of these never yielded sufficient results, because any doctor does not choose them for the priority routine therapy for patients with advanced cancer (7).

In hepatocellular carcinoma (HCC), tissues are sometimes encapsulated with fibrous tissue (8). Ng et al. reported a pathologic study of 189 cases in which encapsulated tumors showed a much lower incidence of direct liver invasion ($p<0.0001$) compared to non-encapsulated ones, and it was apparent that a lower tumor invasiveness contributed significantly to the better prognosis of encapsulated HCCs (9, 10). Some groups reported that myofibroblasts are the cellular source of capsular collagen (11, 12). However, the detailed molecular mechanisms of capsule formation are still unclear. It would be highly beneficial to elucidate the molecules which induce the capsule formation in HCC tissues, and apply these mechanisms to inhibit cancer invasion and metastasis. As stated above, the prognosis of patients with encapsulated HCC is much better than of HCC patients without encapsulation. The encapsulated HCC tissues show lower invasion and metastasis, but in the malignant progression process HCC cells break the capsule and start invasion and metastasis. Therefore, it is important to identify the molecules which play the essential roles in maintaining the encapsulation (Figure 1).

The proteomic differential display method is a basic method to compare the protein expression profiles among different sample groups. The combinatorial technique of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) have assumed a large role in proteomic differential display (13).

In this study, we compared the expression patterns of intracellular proteins in encapsulated and non-encapsulated HCC tissues by means of 2-DE, and identified proteins whose expression was different in these two groups by means of MS. The purpose of the present study was to identify molecules which may be responsible for the maintenance of encapsulation of HCC tissues.

Materials and Methods

Tumor samples. Five HCC tissues surrounded by a capsule and five HCC tissues with a broken capsule were obtained from patients diagnosed with HCC who underwent surgical liver resection at the Department of Surgery II, Yamaguchi University Hospital (Table I). None of the patients received any preoperative therapy. The study protocol was approved by the Institutional Review Board for Human Use of the Yamaguchi University School of Medicine and Health Sciences, University of Hokkaido. Written informed consent was obtained from all patients before surgery. The HCC tissue samples were handled by only K. S., S. H. and Y.K.

Sample preparation. HCC tissue samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% NP-40), and centrifuged at 15,000 g for 30 min at 4˚C. The supernatants were used as samples.

Two-dimensional gel electrophoresis. For two-dimensional gel electrophoresis, 80 μg of protein was applied. Samples were mixed with 200 μl of rehydration buffer (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare, Buckinghamshire, UK) and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). Isoelectric focusing (IEF) was performed using the following voltage program: rehydration for 10 h (no voltage), a stepwise increase from 0 to 500 V for 4 h, 500 to 1,000 V for 1 h, 1,000 to 8,000 V for 4 h, a linear increase from 8,000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. After IEF, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (BIO RAD, Hercules, CA, USA), run at 200 V. After 2-DE, the gels were fixed with 40% ethanol and 10% acetic acid for 2 h, and stained with Flamingo™ Fluorescent Gel Stain (BIO RAD) overnight (14).

Image analysis and spot selection. The protein spots on the gels were recorded using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA). Expression levels of the protein spots were quantified with Progenesis SameSpot
software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK), and the differences in expression between fully capsulated HCC tissues and HCC tissues with capsular invasion were analyzed statistically by ANOVA; p<0.05 was considered significant. After statistical analysis, the gels were re-stained with See Pico™ (Benebiosis Co., Ltd, Seoul, Republic of Korea), and the selected spots were cut and removed for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (15).

In-gel digestion. The See Pico dye was removed from the gel piece by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM diithiothreitol (DTT) for 15 min. The sample in the gel piece was reduced twice in 50% acetoniitrile (ACN), 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel piece was dehydrated in 100% ACN twice for 30 min, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml sequencing-grade-modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. This procedure for in-gel digestion was performed overnight at 30°C. The samples were lyophilized overnight with the use of Labconco Lyphlock 1L Model 77400 (Labconco, Kansas, MO, USA). Lyophilized samples were dissolved in 0.1% formic acid (16).

LC-MS/MS. Peptide sequencing of identified protein spots was performed using a QSTAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, USA). Proteins were identified by an ABSciex DS Sciex Analyst software and Mascot MS/MS Ions Search engine in a PC Workstation. According to the Mascot Search engine, the ion score is –10*Log(P), where P is the probability that the observed match is a random event. Individual ion scores >39 indicate identity or extensive homology (p<0.05). Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits.

Western blot analysis. Fifteen μg of the protein samples were used in Western blot analysis. Pre-cast gels (4-20% gradient polyacrylamide gels; Mini-PROTEAN TGX Gels, Bio-Rad, Hercules, CA, USA) were used for SDS-PAGE. The separated protein bands were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). Blocking was done with Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skimmed milk for 1h at room temperature. The primary antibodies used were: rabbit monoclonal antibody against RKIP (PEBP) (#13006 1:1000, CST, Beverly, MA, USA), rabbit polyclonal antibody against PCK2 (bs-5002R 1:1000, BIOSS Antibodies, Boston, MA, USA), rabbit polyclonal antibody against LAP3 (14612-1-AP 1:1000, Proteintech, Rosemont, IL, USA) and goat polyclonal antibody against actin (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membranes were incubated with each primary antibody overnight at 4°C, and after washing three times with TBS-T, were further incubated with secondary antibodies conjugated with horseradish peroxidase (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 1 h. Bands of RKIP, LAP3, PCK2 and actin were visualized by an enhanced chemiluminescence system (Clarity Western ECL Substrate; BIO-RAD) and Lumino Graph I (ATTO, Tokyo, Japan). Intensities of bands of RKIP, LAP3, PCK2 and actin were quantified by using the CS Analyzer software (ATTO).

The intensity ratio of RKIP, LAP3 and PCK2 to actin was then calculated in hepatoma tissues with or without encapsulation.

Results

Figures 2A and B show the 2-DE patterns of hepatocellular carcinoma tissues with and without encapsulation, respectively. More than 600 protein spots were detected. Nine spots (no 207, 219, 212, 615, 595, 252, 217, 191, 160) appear to be up-regulated, and 5 spots (no 233, 302, 293, 511, 586) appear to be down-regulated in hepatocellular carcinoma tissues without encapsulation vs encapsulated tissues (Table II). These 14 differentially expressed protein spots were picked up and identified by a QSTAR XL quadrupole time-of-flight mass spectrometer. The results of the identification for up-regulated and down-regulated protein spots in the HCC tissues with broken capsule are shown in Tables III and IV, respectively.

From the result of proteomic analysis, the differential expression of phosphatidylethanolamine-binding protein 1 (PEBP1), leucine aminopeptidase 3 (LAP3) and phosphoenolpyruvate carboxykinase mitochondrial (PCK2) was confirmed by using Western blotting (Figure 3). Although the expression of PEBP 1 in HCC tissues with encapsulation tended to be up-regulated compared to HCC tissues with a broken capsule, it was not significant (Figure 4A). On the other hand, the expression of LAP3 and PCK2 was up-regulated significantly in HCC tissues with encapsulation compared to HCC tissues with broken capsule (Figure 4B, C).

Discussion

The results of the present study showed that 14 protein spots were differentially expressed between encapsulated and non-encapsulated HCC tissues as identified by proteomic differential display analysis employing 2-DE and LC-MS/MS. The up-regulated proteins in non-encapsulated HCC tissues were identified as mitochondrial stress-70 protein (HSPA9), heat shock 70 kDa protein 1A (HSPA1A), heat shock cognate 71 kDa protein (HSPA8), mitochondrial 60 kDa heat shock protein (HSPD1), endoplasmic reticulum chaperone BiP (HSPA5), eukaryotic translation factor 5A-1 (EIF5A), proteasome subunit beta type-9 (PSMB9) and ribosome-binding protein 1 (RRPBP1). Heat shock cognate 71 kDa protein was identified from two spots. The down-regulated proteins in non-encapsulated HCC tissues were identified as mitochondrial phosphoenolpyruvate carboxykinase (PCK2), leucine aminopeptidase 3 (LAP3), phenazine biosynthesis-like domain-containing protein and phosphatidylethanolamine-binding protein 1 (PEBP1). Leucine aminopeptidase 3 was identified from two spots.

As up-regulated proteins in HCC tissues without encapsulation, the heat-shock proteins (HSPs) HSPD1, HSPA1A, HSPA5, HSPA8 and HSPA9 were identified. HSPD1 belongs to the HSP 60 kDa family, and HSPA1A, HSPA5, HSPA8 and HSPA9 belong to the HSP 70 kDa family.
Figure 2. 2-DE gel pattern of (A) HCC tissue without encapsulation, and (B) HCC tissue with encapsulation. Proteins (80 μg) were separated on precast polyacrylamide gel with a linear concentration gradient of 5-20%. After fixing, the gels were stained with Flamingo Gel Stain™.
HSPs are proteins which mount responses to cellular stress and play roles as molecular chaperones in cells (17). HSPs are classified into the HSP27, HSP40, HSP47, HSP60, HSP70, HSP110 and HSP150 families according to their molecular weight. The role of HSPD1 on the growth of cancer cells is highly controversial (18). HSPA1A and HSPA8 are molecular chaperone which are related to various intracellular process (19). HSPA5 is a molecular chaperone which is also known as binding immunoglobulin protein (BiP) (20). HSPA9, also called GRP75 or mortalin, is located in the mitochondria and inhibits cell division and is implicated in the control of cell proliferation and cellular aging (21). HSP 70 kDa family members have been reported to be related to malignancies. HSPA1A, HSPA8, HSPD1 and HSPD1 are known to function in various intracellular reactions and are up-regulated in cancer cells (22). Some of the HSP 70 kDa members are used as prognostic markers and therapeutic target molecules for cancer treatment (23). Takashima et al. reported that HSP70 kDa family members were up-regulated in HCV-associated HCC tissues compared to adjacent non-tumoral tissues (24). Jubran et al. reported that determination of HSPA9 and Hsp70 in blood could be a useful additive prognostic tool in guiding clinical utilization of RRBP1 to break encapsulation and proliferate.

EIF5A is a translation factor. EIF5A has two isoforms, EIF5A1 and EIF5A2. EIF5A1 is ubiquitously expressed in most cells. It is essential for cell proliferation and survival (26). In recent years, the role of EIF5A on cancers, diabetes mellitus, HIV-infection etc. have been identified and it has been exploited as a therapeutic target molecule (27). Upregulation of EIF5A1 in various cancer tissues and cells has been reported in multiple studies (28, 29). Since EIF5A1 is essential for cell proliferation and survival, the up-regulation of EIF5A1 in HCC tissues without encapsulation seems to be consistent with the characteristics of progressing HCC cells.

PSMB9 is a component of the proteasome which contributes to the complete assembly of the 20S proteasome complex (30). Rouette et al. showed that expression of the immunoproteasome including PSMB9 is elevated in most cancer types (31). Even though it is still not clear why the immunoproteasome is up-regulated in cancer cells, its function may be increased in highly proliferating cancer cells.

RRBP1 is an endoplasmic reticulum membrane protein (32) which has essential roles for ribosomal function. One function is binding and the other is translocation of nascent proteins across the membrane of the rough endoplasmic reticulum (33). The up-regulation of RRBP1 in many types of cancer cells and tissues has been reported by many research groups (34-36). In line with these, cancer cells may utilize RRBP1 to break encapsulation and proliferate.

In addition, PCK2, LAP3, PEBP1 and PBLD were identified as downregulated proteins in HCC tissue without encapsulation. By means of Western blotting, the significant down-regulation of PCK2 and LAP3 was confirmed.

Kuhara et al. reported down-regulation of PBLD in HCC tissues while PBLD inhibited HCC progression via Ras and Rap1 (37). Li et al. found PBLD inhibited HCC cell growth and invasion in vitro and tumor growth in vivo, and they concluded that elevated PBLD expression might reduce HCC cell growth and invasion via inactivation of several tumorigenesis-related signaling pathways (38).
Figure 3. Western blot analysis of PEBP, LAP3, PCK2 and actin in HCC tissues with or without encapsulation. The protein expression of PEBP, LAP3 and PCK2 (bands of 21, 56 and 70 kDa, respectively) was reduced in HCC tissues with encapsulation compared to HCC tissues without encapsulation.

Table III. Identification of proteins up-regulated in HCC tissues without capsulation.

| Spot No. | Protein name                             | Accession No. | Theoretical pI | Theoretical Mr | Distinct peptides | Sequence coverage (%) | MS/MS search score | Change in spots (fold) |
|----------|------------------------------------------|---------------|----------------|----------------|---------------------|-----------------------|---------------------|-----------------------|
| 207      | Stress-70 protein, mitochondrial         | P38646        | 5.87           | 73,680         | 17                 | 27                    | 354                 | +2.0                  |
| 219      | Heat shock 70 kDa protein 1A             | P0DMV8        | 5.47           | 70,052         | 11                 | 20                    | 172                 | +1.7                  |
| 212      | Heat shock cognate 71 kDa protein        | P11142        | 5.37           | 70,898         | 45                 | 35                    | 612                 | +1.4                  |
| 615      | Eukaryotic translation factor 5A-1       | P63241        | 5.07           | 16,832         | 7                  | 30                    | 111                 | +3.3                  |
| 595      | Proteasome subunit beta type-9           | P28065        | 4.89           | 23,264         | 18                 | 30                    | 232                 | +2.4                  |
| 252      | 60 kDa heat shock protein, mitochondrial | P10809        | 5.70           | 61,055         | 28                 | 56                    | 479                 | +1.7                  |
| 217      | Heat shock cognate 71 kDa protein        | P11142        | 5.37           | 70,898         | 3                  | 6                     | 63                  | +2.6                  |
| 191      | Endoplasmic reticulum chaperone BiP      | P11021        | 5.07           | 72,333         | 30                 | 34                    | 522                 | +1.5                  |
| 160      | Ribosome-binding protein 1               | Q9P2E9        | 5.45           | 108,632        | 11                 | 13                    | 260                 | +4.3                  |

Table IV. Identification of proteins down-regulated in HCC tissues without capsulation.

| Spot No. | Protein name                             | Accession No. | Theoretical pI | Theoretical Mr | Distinct peptides | Sequence coverage (%) | MS/MS search score | Change in spots (fold) |
|----------|------------------------------------------|---------------|----------------|----------------|---------------------|-----------------------|---------------------|-----------------------|
| 233      | Phosphoenolpyruvate carboxykinase        | Q16822        | 6.63           | 70,699         | 19                 | 33                    | 251                 | –1.3                  |
|          | mitochondrial                            |               |                |                |                     |                       |                     |                       |
| 302      | Leucine aminopeptidase 3                 | P28838        | 8.03           | 56,166         | 37                 | 44                    | 440                 | –1.5                  |
| 293      | Leucine aminopeptidase 3                 | P28838        | 8.03           | 56,166         | 44                 | 57                    | 745                 | –2.1                  |
| 511      | Phenazine biosynthesis-like domain-containing protein | P30039 | 7.10           | 31,785         | 40                 | 76                    | 381                 | –1.4                  |
| 586      | Phosphatidylethanolamine-binding protein 1| P30086        | 7.01           | 21,057         | 10                 | 47                    | 176                 | –2.1                  |
Figure 4. The intensity of (A) PEBP (B) LAP3 bands and (C) PCK2 bands, normalized by actin, in HCC tissues with or without encapsulation. The intensity of LAP3 and PCK2 bands were normalized by actin and were significantly reduced in HCC tissues without encapsulation compared to HCC tissues with encapsulation (p<0.05 and p<0.005, respectively by student’s t-test). A value of p<0.05 was considered statistically significant (n=5). N.S.: not significant.
Pebp1, also known as Raf kinase inhibitory protein (RKIP), is an inhibitor of the MEK phosphorylation by Raf-1. PEBP1 inhibits the Raf-1/MEK/ERK pathway by direct inhibition of Raf-1 and MEK (39), and has anti-metastatic and pro-apoptotic properties in cancer cells (40). Although Western blotting could not show significant down-regulation of PEBP1 in encapsulated HCC tissues compared to HCC tissues without encapsulation, there is a possibility that PEBP1 has a role in the maintenance of encapsulation in HCC tissues.

Significant down-regulation of PCK2 in the HCC tissues without encapsulation was confirmed by Western blotting. PCK2 is a kinase located in the mitochondria. PCK2 phosphorylates oxaloacetic acid and produces phosphoenolpyruvic acid. This phosphoenolpyruvic acid goes through the mitochondrial membrane, and produces glucose (glyconeogenesis) (41). In the central part of cancer tissues, the supply of glucose is not enough for proliferating cancer cells. PCK2 plays an important role in the supply of glucose in cancer cells by using gluconeogenesis. Although some reports showed that the activation of PCK2 increases the proliferative ability of cancer cells (41), the expression of PCK2 was reported to be down-regulated in ovarian cancer cells (42), aflatoxin-induced HCC cells (43), pancreatic cancer cells (44), renal cell carcinoma tissues (45), and osteosarcoma tissues (46). The most interesting report about PCK2-expression is a quantitative proteomic differential display by using isobaric tags for relative and absolute quantification (iTRAQ) for HCC tissues and adjacent non-cancerous tissues. The result showed down-regulation of PCK2 in HCC tissues compared to adjacent non-cancerous tissues. Although they compared PCK2 expression between HCC cancerous and adjacent non-cancerous tissues, our result, where PCK2 was down-regulated in HCC tissues without encapsulation, has a similar result (47).

Significant down-regulation of LAP3 in the HCC tissues without encapsulation was also confirmed by Western blotting. LAP3 is a cell surface aminopeptidase. LAP3 catalyzes the hydrolysis of leucine residues from the protein amino termini (48). It has been reported that LAP3 is related to invasion and metastasis of cancer cells by controlling signal transduction. Fang et al. reported that LAP3 up-regulates fascin, which phosphorylates the HSP27-NF-κB signalling pathway and promotes the metastatic ability of cancer cells. Furthermore, they showed that LAP3 up-regulated matrix metalloproteinase-2 (MMP-2) and MMP-9, and high LAP3 expression was correlated with the grade of malignancy (49). Tian et al. clarified that LAP3 promoted motility and invasiveness of HCC cells (48). Wang et al. reported that vimentin, a mesenchymal marker which strongly relates to EMT, was up-regulated in breast cancer tissues, and LAP3 induced expression of vimentin (50). Although the LAP3-vimentin axis seems to be a promoter of invasion and metastasis, Kajita et al. reported that vimentin accumulated in normal cells specifically at the interface with transformed cells and extruded transformed cells from the epithelium. They called this process cell competition. It is not clear how cell competition starts, but vimentin induced by LAP3 may have an important role in encapsulation of HCC tissues (51).

In conclusion, in HCC tissues with a broken capsule, the protein levels of LAP3 and PCK2 were significantly down-regulated. The role of both molecules on the maintenance of encapsulation of HCC tissues is not clear at present. However, further investigation of LAP3 and PCK2 may elucidate their important function for cancer tissue encapsulation, and this could be used as an anti-metastatic strategy to induce encapsulation for cancer tissues and prevent metastasis.

**Conflicts of Interest**

The Authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Author’s Contributions**

Y.K., K.N., H.N. and M.K. designed the research; Y.K., K.K., T.K. and K.T. performed the experiments; K.S. and H.N. provided the HCC tissues; K.K., Y.K. and B.B. wrote the article.

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