Abstract

Proteomic Differential Display Analysis of
encapsulated and non-capsulated HCC tissues

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【Introduction】

Cancer development, invasion and metastasis is affected by factors as follow; 1) Mutations in oncogenes and tumor suppressor genes, 2) epigenetic modifications, 3) protein expression in the cancer microenvironment which is believed to exist around cancer tissues. The cancer microenvironment is a general term for the cells and substrates surrounding cancer tissues. Cancer-associated fibroblasts (CAFs) are among the stromal cells that comprise the cancer microenvironment. CAFs are derived from fibroblasts, epithelial cells, endothelial cells, and undifferentiated mesenchymal cells, but their interaction with cancer remains unclear.

Hepatocellular carcinoma (HCC) is known to frequently form a fibrous connective tissue capsule. The capsule is a one of cancer microenvironment which contains CAFs. Because the capsule exists between the tumor and the normal tissue, it is easy to identify the cancer microenvironment by the naked eyes. It is known that when HCC masses larger than 5 cm, the 5-year survival rate tend to be low. Therefore, the presence or absence of the capsule is an important factor predicting prognosis.

In recent years, proteomic analysis has been conducted to identify proteins that are targets for diagnosis and treatment of malignant tumors, but there are only a few reports that have observed the proteins in the microenvironment of the HCC capsule, and the details are unknown.

In this study, we comprehensively compared the cancer microenvironment of the capsular formation group and the capsular disruption group in HCC without cirrhosis by proteome analysis, and aimed to identify the proteins that may be involved in the capsular formation.

【Materials and Methods】

1. Hepatocellular carcinoma tissue specimens

   HCC tissues were collected from patients who had been diagnosed with HCV-related HCC and had consented to undergo surgical hepatectomy. Tissue was collected from each sample with a size of 7 × 7 × 7 mm centered on the limbic area of tumor. The collected HCC tissues were classified into two groups based on histopathological diagnosis: capsule formation group and capsule failure group. All tissues were free of liver cirrhosis.

2. Extraction of soluble proteins

   Tissue samples were dissolved in lysis buffer to extract soluble proteins. After homogenization in the lysis buffer, the tissues were stirred for an hour to dissolve completely. The protein lysate was then centrifuged at 21,500 × g for 30 minutes at 4°C, and the supernatant was used.

3. Proteome analysis

   1) Two-dimensional electrophoresis (2-DE)

      For 2-DE, 100 μg of protein extracted from each sample was used. For Isoelectric focusing (IEF) in the first dimension, samples were dissolved in 200 μL of IEF buffer

and added to the IPG Strip. The IPG strip was then placed in an isoelectric focusing apparatus and IEF was performed. For the second-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pre-cast polyacrylamide gel with a linear concentration gradient of 5~20% was used.

2) Fluorescent gel staining

After 2-DE, the gels were washed three times with ultrapure water, fixed with 40% ethanol and 10% acetic acid solution for 2 hours by shaking, and stained with fluorescent gel stain overnight.

3) Image analysis

The stained gels were scanned using the ProXPRESS™ 2D Proteomic Imaging System to detect the spots. The levels of protein expression spots were quantified for each gel using Progenesis SameSpots software. The fluorescence intensities of the spots were compared using average normalized volumes, and the sites with significant expression changes were automatically detected.

4) Spot picking

The gels were re-stained with See Pico™ dye, and the spots with significantly different expression between the two groups were picked out using a gel picker.

5) In-gel digestion

Gels of the excised protein spots were washed with 60% methanol, 50 mM ammonium bicarbonate, and 5 mM DL-dithiothreitol (DTT), and then the See Pico™ dye was removed. The decolorized gels were dehydrated and the proteins in the gels were digested in 30% acetonitrile containing 50 mM ammonium bicarbonate and 5 mM DTT with trypsin added to a concentration of 10 μg/mL at 30°C while re-swelling.

6) Liquid chromatography-mass spectrometry (LC-MS/MS)

Peptide sequencing of protein spots after digestion in the gel was performed using a QSTAR XL quadrupole time-of-flight mass spectrometer. Proteins were identified using the ABSciex MDS Sciex Analyst software and the MASCOT MS/MS ion search engine. The function of the proteins was confirmed using Uniprot.

4. Western blot analysis

Western blot analysis was performed to confirm the reproducibility of the proteins whose expression was significantly reduced by proteome analysis. First, 15 μg of whole cell lysates were electrophoresed on Mini-PROTEAN TGX Gels and transferred to Polyvinylidene Fluoride (PVDF) membranes. Then, PVDF membranes were blocked with Tris-buffered saline with Tween-20 (TBS-T), containing 5% skimmed milk overnight at 4°C, and incubated with primary antibodies against each of the three proteins overnight at 4°C. The primary antibodies and concentrations were anti PCK2 antibody (rabbit polyclonal, 1:1000), anti LAP3 antibody (rabbit polyclonal, 1:1000), and anti PEBP antibody (rabbit

monoclonal, 1:1000). Then, all PVDF membranes were incubated with secondary antibody (HRP-conjugated anti-rabbit IgG (H+L) antibody, 1:10,000) for 1 hour at room temperature. Then, the cells were washed three times with TBS-T. The PVDF membrane was then immersed in chemiluminescent reagent for a few minutes and photographed with WSE-6200H LuminoGraph II. The images were then analyzed by Image Analysis Software CS Analyzer 4 to detect the protein expression.

Western blot analysis was performed to confirm the expression of actin as a loading control.The antibodies were stripped by warming in stripping solution and shaking strongly for 20 minutes at 55°C. After 5 washes with TBS-T, blocking was performed with TBS-T with 5% skim milk as described above. After three washes with TBS-T, anti actin antibody (rabbit polyclonal, 1:1,000) was added and incubated overnight at 4°C. The cells were washed three times with TBS-T and incubated with a secondary antibody (HRP-conjugated anti-rabbit IgG (H+L) antibody 1:10,000) for 1 hour at room temperature. The PVDF membrane was then washed three times with TBS-T, immersed in chemiluminescence reagent for a few minutes, photographed with WSE-6200H LuminoGraph II, and analyzed with Image Analysis Software CS Analyzer 4.

5. Statistical analysis
   1) 2-DE

      After 2-DE, the fluorescence intensities in the spots detected on the gel were quantified and the mean values of each spot were compared. ANOVA test was used for statistical analysis, and $p<0.05$ was considered as a significant difference.

   2) Western blot analysis

      The fluorescence intensities of the proteins detected by Western blot were quantified using Image Analysis Software CS Analyzer 4. The fluorescence intensities of the proteins in the capsule formation group and capsule failure group were averaged, and the obtained values were statistically analyzed using Student's T-test, and $p<0.05$ was considered a significant difference.

【Results and Discussion】

1. Proteome analysis using 2-DE and LC-MS/MS analysis

There were 14 spots that showed significant expression enhancement or reduction by 2-DE. These spots were subjected to LC-MS/MS to identify proteins, and proteins were identified in all spots. A total of 12 proteins, including HSPD1, HSPA1A, HSPA5, HSPA8, HSPA9, EIF5A1, PSMB9 and RRBP1 were identified as significantly up-regulated, and PCK2, LAP3, PBLD, and PEBP1 were down-regulated, respectively. HSC71 (enhanced expression) and LAP3 (decreased expression) were identified in two locations.

2. Western blot analysis

PCK2 and LAP3 were significantly down-regulated in the capsule failure group compared with capsule formation group, and PEBP1 was down-regulated in the capsule failure group compared with the capsule formation group, but there was no significant difference.

【Conclusion】

In this study, we used proteomic analysis to identify protein expression in tissues containing tumor marginal capsules from resected specimens of HCV-related HCC, and confirmed the reproducibility of protein expression by Western blot analysis. PCK2 and LAP3 were significantly down-regulated in the capsule failure group, suggesting that these proteins might be involved in HCC capsular formation.