

Up-regulation of 42 kDa Tubulin Alpha-6 Chain Fragment in Well-differentiated Hepatocellular Carcinoma Tissues from Patients Infected with Hepatitis C Virus

YASUHIRO KURAMITSU¹, MOTONARI TAKASHIMA^{1,2}, YUICHIRO YOKOYAMA^{1,3}, NORIO IIZUKA², TAKAO TAMESA², JUNKO K. AKADA¹, YUFENG WANG¹, TOSHIFUSA TODA⁴, ISAO SAKAIDA³, KIWAMU OKITA³, MASAOKI OKA² and KAZUYUKI NAKAMURA¹

Departments of ¹Biochemistry and Functional Proteomics, ²Surgery and Clinical Science, and ³Hepatology and Gastroenterology, Yamaguchi University Graduate School of Medicine, Ube, Japan; ⁴Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

Abstract. We performed proteomic differential display analysis of hepatitis C virus-associated 21 human hepatocellular carcinoma (HCV-HCC) tissues by using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). One of the numerous spots which was located next to three spots of glutamine synthetase showed stronger intensity in well-differentiated HCC tissues compared to non-cancerous tissues. Samples from 6 out of 21 patients showed up-regulation of this spot compared to non-cancerous tissues. After in-gel digestion, MALDI-TOF/MS identified the spot as tubulin alpha-6 chain. Two-dimensional immunoblot analysis confirmed that this spot was indeed tubulin alpha, and this spot was stronger in cancerous tissues than in noncancerous tissues. These results suggest that tubulin alpha-6 chain is one of the candidates for biomarkers for well-differentiated HCV-associated HCC.

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide, with a high incidence in many countries. Although the causes of HCC are recognized as infection with hepatitis virus, exposure to aflatoxin B1 and alcoholic drinking habit, infection with hepatitis C virus (HCV) is the most clearly established risk factor for HCC in developed countries, including Japan. On the other hand, in developing countries, infection with hepatitis B virus (HBV) is

the clearest risk factor for HCC. HCC cells progress through a multistep process based on histological changes. Although initially HCC tumour cells are well-differentiated, they dedifferentiate to become moderately or poorly differentiated, with high proliferation rates with the progression of the tumour. Therefore, it is very important to diagnose HCC at the stage of the well-differentiated form (1-4).

In recent studies, we have reported proteomic analysis for HCV-associated HCC tissues by the combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS), which is a proteomic method of high-throughput analysis of protein expression. These studies have identified diverse proteins that may be involved in the pathogenic mechanism of HCC (5-8). In these proteomic studies, we identified alpha enolase as being up-regulated in poorly differentiated HCV-associated HCC tissues (9), and glutamine synthetase isoforms as being up-regulated in well-differentiated HCV-associated HCC tissues (10). Both are expected to be biomarkers for poorly and well-differentiated HCV-associated HCC.

In the present study, we performed proteomic analysis for 21 pairs of HCV-associated HCC tissues and corresponding non-cancerous tissues to find proteins that might be involved in tumour differentiation and progression. Since in well-differentiated HCV-associated HCC tissues a spot of approximately 42 kDa in MW with a *pI* of 7.0 showed stronger intensity compared with that in non-cancerous samples, we further analyzed this spot.

Materials and Methods

Tissue samples. Twenty-one pairs of cancerous tissues and adjacent non-cancerous liver tissues were obtained from patients who had been diagnosed as having HCV-associated HCC and had undergone surgical liver resection at the Yamaguchi University School of Medicine from May 1997 to December 2000. The histological diagnosis of HCC was made from the formalin-fixed, paraffin-embedded tissues after surgery

Correspondence to: Yasuhiro Kuramitsu, MD, Ph.D., Department of Biochemistry and Functional Proteomics, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. Tel: +81 836222213, Fax: +81 836222212, e-mail: climates@yamaguchi-u.ac.jp

Key Words: Tubulin alpha-6 chain, HCV-HCC, proteomics, 2-DE, MS.

according to the World Health Organization criteria (Pathology and Genetics. Tumours of the Digestive System. WHO Classification of Tumours, Volume 2, Third Edition, 2000) in all cases. None was positive for hepatitis B surface antigen. Informed consent in writing had been obtained from all patients before surgery. The study protocol was approved by the Institutional Review Board for Human Use of the Yamaguchi University School of Medicine.

Sample preparation. The liver tissues were suspended and homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM NaCl, 10 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1% NP40). After centrifugation at 15,000 ×g for 30 min at 4°C, the supernatants were taken and used as samples (6-10).

Two-dimensional gel electrophoresis (2-DE). Three hundred micrograms of protein was used for each electrophoresis. The first dimensional isoelectric focusing (IEF) was performed in an IPGphor3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 7 cm, immobilized, pH 3-10 linear gradient strips (Bio-Rad, Hercules, CA, USA) at 50 µA/strip. The strips were rehydrated with 125 µl sample containing 8 M urea, 2% CHAPS, 0.01% bromophenol blue, dithiothreitol (DTT), and immobilized pH gradient (IPG) buffer, for 14 h. IEF was then run in 3 steps, at 500 V for 1 h, 1000 V for 1 h, and 8000 V for 2 h. The second dimension was performed on SDS-polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) R250 (Nacalai Tesque, Kyoto, Japan) for 24 h. They were destained with 10% acetic acid in water containing methanol for 30 min, and again destained with 7% acetic acid (6-10).

Image analysis. The positions of the protein spots on the gels obtained using samples of non-cancerous and cancerous tissues were recorded with an Agfa ARCUS 1200 image scanner (Agfa-Gevaert N.V., Mortsel, Belgium) and were analyzed with Image Master 2D Platinum ver. 5.0 (GE Healthcare). Spots present at different intensities were excised from the gels for MS analysis (6-10).

In-gel digestion. The CBB dye was removed by rinsing twice in 60% methanol, containing 50 mM ammonium bicarbonate and 5 mM DTT, for 15 min, and twice in 50% acetonitrile, containing 50 mM ammonium bicarbonate and 5 mM DTT, for 7 min. The gel piece was dehydrated in 100% acetonitrile, and then re-swollen with an in-gel digestion reagent containing 10 µg/ml sequencing grade trypsin (Promega V5111, Madison, WI, USA) in 30% acetonitrile, containing 50 mM ammonium bicarbonate and 5 mM DTT. In-gel digestion was carried out overnight at 30°C (6-10).

Peptide mass fingerprinting (PMF). After the in-gel digestion, 1 µl of the reaction mixture was removed and mixed with 1 µl of the matrix solution (10 mg/ml alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 40% methanol, 0.1% trifluoroacetic acid (TFA)) on a MALDI target plate. The MALDI-TOF/MS for PMF was performed on a Shimadzu Biotech AXIMA-CFR mass spectrometer in a reflectron mode. The MS-Fit database search engine at the ProteinProspector web site (<http://prospector.ucsf.edu/>) was used for the protein identification (6, 7).

Two-dimensional immunoblot analysis. After 2-DE, fractionated proteins (50 µg) were transferred electrophoretically onto poly-vinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corporation, Bedford, MA, USA), and the membranes were blocked overnight at 4°C

in Tris-buffered saline (TBS) containing 5% skim milk. The primary antibody was a mouse anti-alpha tubulin monoclonal antibody (1:4000; Sigma-Aldrich, St. Louis, MO, USA). The membranes were then incubated for 1 h at 4°C, washed four times with TBS containing 0.05% Tween 20, and incubated for 1 h at 4°C with horseradish peroxidase-conjugated secondary antibody (1:5000; Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). The reaction was visualized with a chemiluminescence reagent (ImmunoStar Long Detection, Wako, Osaka, Japan) and scanned by using Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan) (10, 11).

Results

Identification of proteins on 2-DE gels. We have already reported proteomic analysis of cancerous and non-cancerous tissues of HCV-associated HCC, and showed that there were three spots of glutamine synthetase (GS) showing stronger intensity in the cancerous tissues of well-differentiated HCC than in the non-cancerous tissues (10). Besides these three spots (spots 1-3 in Figure 1B), a spot of approximately 42 kDa in MW with a pI of 7.0 (spot 4) showed stronger intensity in well-differentiated HCC tissues than in non-cancerous samples (Figure 1A). While the up-regulation of this spot was seen in 6/21 samples, in the samples of well-differentiated HCC tissues, up-regulation was found in 3/4. The spot was digested and analyzed by MS. This sample provided a good spectrum of amino acid sequences by MALDI-TOF/MS and the protein was identified as tubulin alpha-6 chain. Peptide sequences of tubulin alpha-6 chain were identified as (K)EIIDLVLDR(I), (K)YMACCLLYR(G), (R)LDHKFDLMYAK(R), (R)TIQFV DWCPTGFK(V), (R)AVFVDLEPTVIDEVR(T) and (R)NLDI ERPTYTNLNR(L) by MS spectra of trypsin-digested sample. The expression of this protein was confirmed by 2-D immunoblot analysis. The position of this spot was confirmed and the intensity of this spot was stronger in cancerous tissues than in noncancerous tissues (Figure 2).

Discussion

Up-regulation of tubulin alpha-6 chain in the well-differentiated HCV-associated HCC tissues was observed on the 2-DE gels, and immunoblot analysis with specific anti-alpha tubulin mAb confirmed that this spot was tubulin alpha-6 chain.

Tubulin forms microtubules by means of the polymerization of tubulin alpha and beta heterodimers. This assembly and disassembly of tubulin into microtubules is assisted by other factors such as the binding of microtubule-associated proteins. This dynamic state is characterized by growth randomly interrupted by pauses and shrinkage. Microtubules play critical roles in diverse cellular functions. They are essential components of all cells, and play roles as essential factors in intracellular signaling, cellular morphology and mitosis (12). Since microtubules play important roles in mitosis, microtubule-destabilizing agents and -stabilizing agents have been used for anticancer therapy.

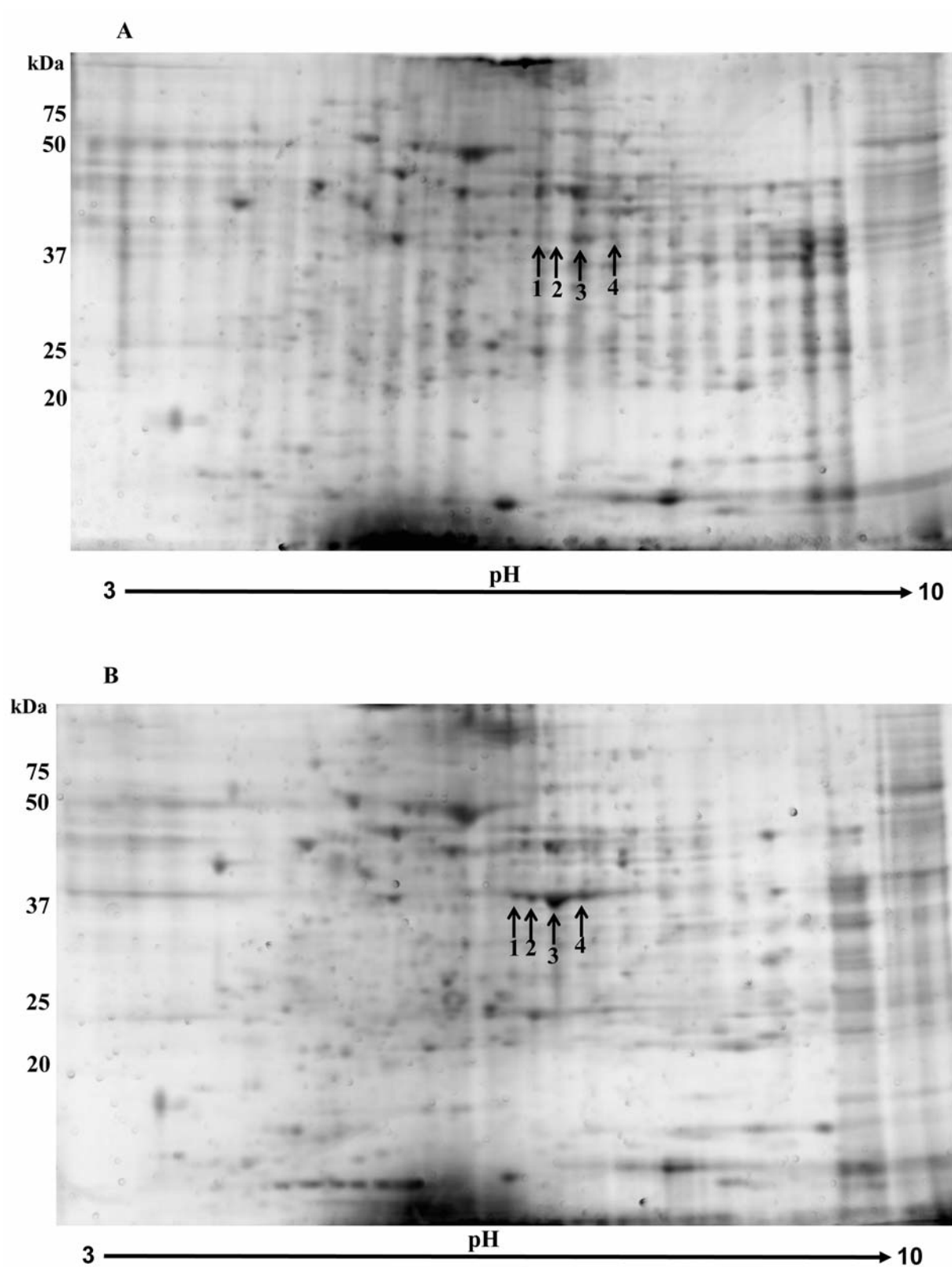
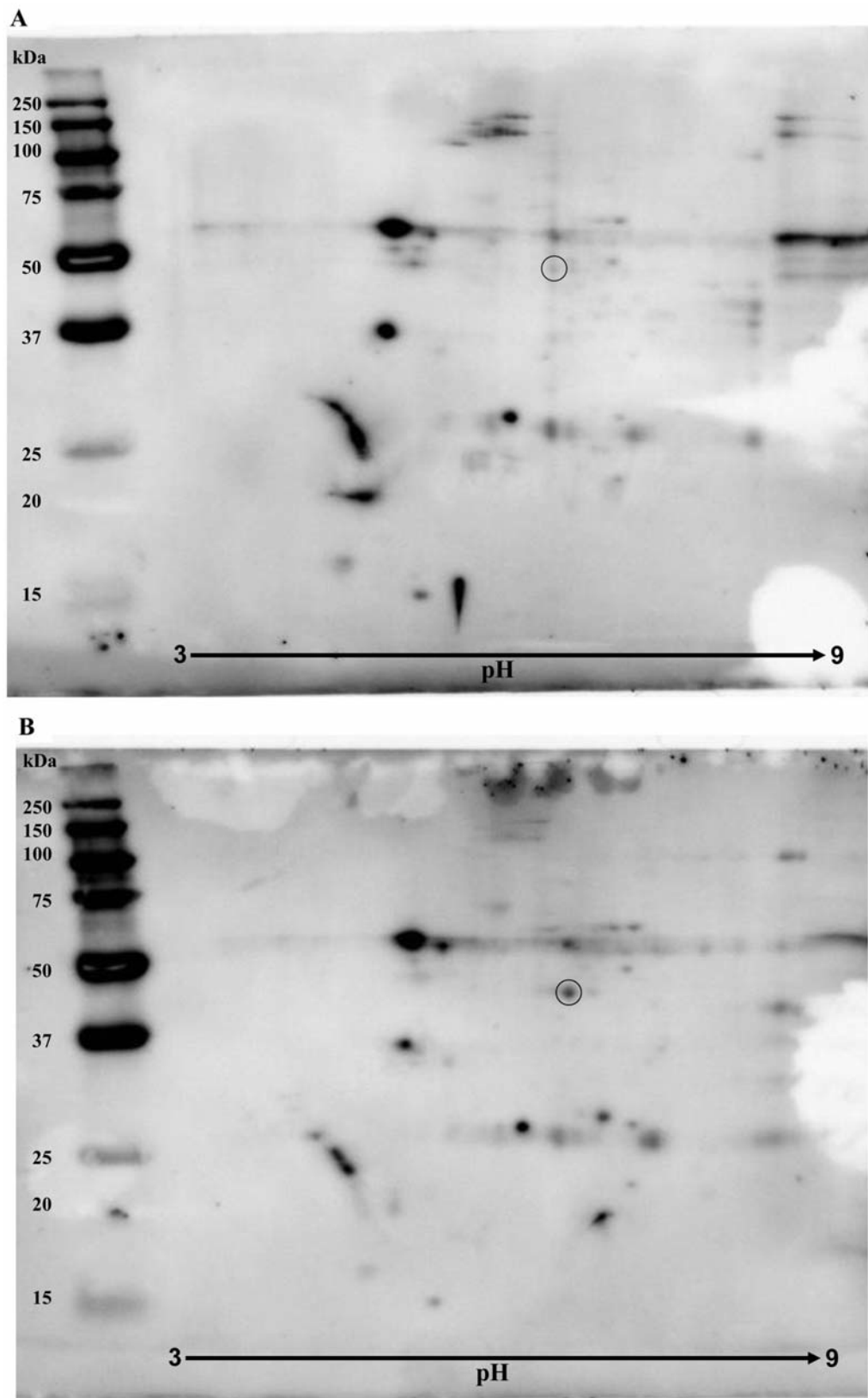


Figure 1. 2-DE patterns of spots for four proteins in tissues from patients with hepatocellular carcinoma infected with HCV. Protein samples (300 μ g) were loaded onto the gel. A: Non-cancerous and B: cancerous tissues. Arrows indicate four strongly stained protein spots of 42 kDa and with pI 6.4-7.0. Spots 1-3 have been already reported as liver glutamine synthetase, including phosphorylated forms.



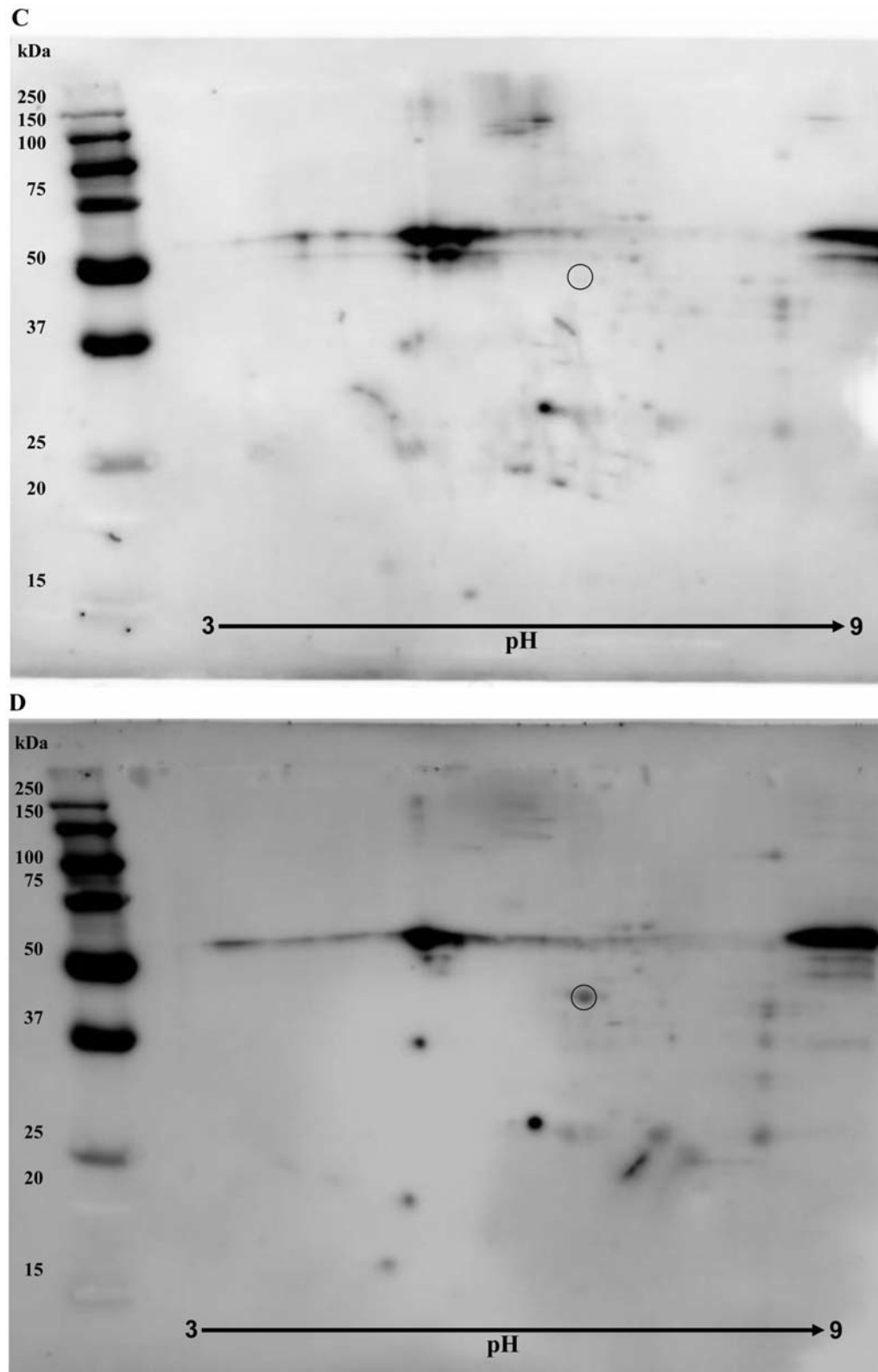


Figure 2. 2-D immunoblot analysis of alpha-tubulin in non-cancerous and paired cancerous tissues. For the 2-D immunoblot analysis, 50 μ g protein of non-cancerous (A, C) and cancerous (B, D) tissues was applied. Expression of alpha-tubulin was confirmed; the intensity of the spot was stronger in cancerous tissue than non-cancerous tissue. The spot (40 kDa, pI 7.0) marked by a circle was confirmed as being alpha-tubulin.

Microtubule-destabilizing agents, such as vinblastine and vincristine, inhibit polymerization of microtubules, resulting in the inhibition of mitosis; microtubule-destabilizing agents, such as taxanes, stabilize polymerized microtubules, arresting mitosis and apoptosis (13, 14).

Overexpression of tubulins in tumour tissues have been reported by many groups. Matos *et al.* reported beta-5-tubulin was up-regulated in HCC in the USA (15). He *et al.* showed tubulin beta-2C to be up-regulated in sentinel lymph nodes of colorectal cancer (16). Not only beta-tubulins, but also overexpression of alpha-tubulins in tumour tissues have been reported by many groups. In tissues of pulmonary sclerosing haemangioma, small cell lung cancer, renal cell carcinoma and oesophageal squamous cell carcinoma, overexpression of tubulin alpha proteins has been detected (17-20). Furthermore, overexpression of tubulin alpha 6 chain has been also reported in gastric cancer and ovarian cancer (21, 22). However, no report has shown increased fragment of alpha-tubulin in tumour tissues. The molecular weight of the up-regulated tubulin alpha-6 chain fragment in HCV-associated HCC tissues here was 42 kDa. Tubulin alpha-6 chain reported by Wu *et al.* (21) and Lim *et al.* (22) was of about 50 kDa. This would apparently seem to be different from our reported tubulin alpha-6 chain fragment. As yet, no report about digesting enzyme for tubulin alpha-6 chain has been published. Therefore, it is important to clarify the mechanism of overexpression of 42 kDa tubulin alpha-6 chain fragment in well-differentiated HCV-associated HCC tissues.

References

- El-Serag HB and Mason AC: Rising incidence of hepatocellular carcinoma in the United States. *N Eng J Med* 340: 745-750, 1999.
- Tanaka E and Kiyosawa K: Natural history of acute hepatitis C. *Gastroenterol Hepatol* 15: E97-E104, 2000.
- Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hürter D, Nawrocki M, Kruska L, Hensel F, Petry W and Häussinger D: Prognosis of chronic hepatitis C: results of a large, prospective cohort study. *Hepatology* 28: 1687-1695, 2003.
- Okuda K: Hepatocellular carcinoma. *J Hepatol* 32: 225-237, 2000.
- Kuramitsu Y and Nakamura K: Current progress in proteomic study of hepatitis C virus-related human hepatocellular carcinoma. *Expert Rev Proteomics* 2: 589-601, 2005.
- Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Toda T, Saiakida I, Okita K, Oka M and Nakamura K: Proteomic profiling of heat-shock protein 70 family members as biomarkers for hepatitis C virus-related hepatocellular carcinoma. *Proteomics* 3: 2487-2493, 2003.
- Yokoyama Y, Kuramitsu Y, Takashima M, Iizuka N, Toda T, Terai S, Sakaida I, Oka M, Nakamura K and Okita K: Proteomic profiling of proteins decreased in hepatocellular carcinoma from patients infected with hepatitis C virus. *Proteomics* 4: 2111-2116, 2004.
- Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, Sakaida I, Okita K, Oka M and Nakamura K: Proteomic analysis of autoantibodies in patients with hepatocellular carcinoma. *Proteomics* 6: 3894-3900, 2006.
- Takashima M, Kuramitsu Y, Yokoyama Y, Iizuka N, Fujimoto M, Nishisaka T, Sakaida I, Okita K, Oka M and Nakamura K: Overexpression of alpha enolase in hepatitis C virus-related hepatocellular carcinoma: association with tumor progression as determined by proteomic analysis. *Proteomics* 5: 1686-1692, 2005.
- Kuramitsu Y, Harada T, Takashima M, Yokoyama Y, Hidaka I, Iizuka N, Toda T, Fujimoto M, Zhang X, Sakaida I, Okita K, Oka M and Nakamura K: Increased expression, and phosphorylation of liver glutamine synthetase in well-differentiated hepatocellular carcinoma tissues of patients infected with hepatitis C virus. *Electrophoresis* 27: 1651-1658, 2006.
- Tanaka T, Kuramitsu Y, Fujimoto M, Naito S, Oka M and Nakamura K: Down-regulation of two isoforms of ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) correlates with high metastatic potentials of human SN12C renal cell carcinoma cell clones. *Electrophoresis* 29: 2651-2659, 2008.
- Wade RH: On and around microtubules: an overview. *Mol Biotechnol* 43: 177-191, 2009.
- Yue QX, Liu X and Guo DA: Microtubule-binding natural products for cancer therapy. *Planta Med* 76: 1037-1043, 2010.
- Perez EA: Microtubule inhibitors: Differentiating tubulin-inhibiting agents based on mechanisms of action, clinical activity, and resistance. *Mol Cancer Ther* 8: 2086-2095, 2009.
- Matos JM, Witzmann FA, Cummings OW and Schmidt CM: A pilot study of proteomic profiles of human hepatocellular carcinoma in the United States. *J Surg Res* 155: 237-243, 2008.
- He ZY, Wen H, Shi CB and Wang J: Up-regulation of hnRNP A1, ezrin, tubulin β -2C and annexin A1 in sentinel lymph nodes of colorectal cancer. *World J Gastroenterol* 16: 4670-4676, 2010.
- Cho SJ, Jin LJ, Kim BY, Cho SI, Jung WY, Han JH, Ha SY, Kim HK and Kim A: Increased expression of matrix metalloproteinase 9 and tubulin-alpha in pulmonary sclerosing hemangioma. *Oncol Rep* 18: 1139-1144, 2007.
- Jeong HC, Kim GI, Cho SH, Lee KH, Ko JJ, Yang JH and Chung KH: Proteomic analysis of human small cell lung cancer tissues: up-regulation of coactosin-like protein-1. *J Proteome Res* 10: 269-276, 2011.
- Seliger B, Dressler SP, Wang E, Kellner R, Recktenwald CV, Lottspeich F, Marincola FM, Baumgärtner M, Atkins D and Lichtenfels R: Combined analysis of transcriptome and proteome data as a tool for the identification of candidate biomarkers in renal cell carcinoma. *Proteomics* 9: 1567-1581, 2009.
- Qi Y, Chiu JF, Wang L, Kwong DL and He QY: Comparative proteomic analysis of esophageal squamous cell carcinoma. *Proteomics* 5: 2960-2971, 2005.
- Wu C, Luo Z, Chen X, Wu C, Yao D, Zhao P, Liu L, Shi B and Zhu L: Two-dimensional differential in-gel electrophoresis for identification of gastric cancer-specific protein markers. *Oncol Rep* 21: 1429-1437, 2009.
- Lim R, Lappas M, Ahmed N, Permezel M, Quinn MA and Rice GE: 2D-PAGE of ovarian cancer: analysis of soluble and insoluble fractions using medium-range immobilized pH gradients. *Biochem Biophys Res Commun* 406: 408-413, 2011.

Received June 15, 2011

Revised July 21, 2011

Accepted July 22, 2011