in vivo *22*: 457-462 (2008)

Possible Relation Between Histone 3 and Cytokeratin 18 in Human Hepatocellular Carcinoma

CHIN-CHIN HO[1*], CHIUNG-CHI CHENG[2*], YI-HSIANG LIU[3,4,5*], REN-JENG PEI[3,5], YUNG-HSIANG HSU[4], KUN-TU YEH[6], LU-CHANG HO[7], MING-CHUANG TSAI[5] and YIH-SHYONG LAI[5]

[1]*Department of Nursing, Central Taiwan University of Science and Technology, Taichung, Taiwan;*
[2]*Institute of Medicine, and Departments of* [5]*Pathology and* [7]*Surgery, Hospital and Medical College, Chung Shan Medical University, Taichung, Taiwan;*
[3]*Department of Pathology, Jen Ai Hospital, Taichung, Taiwan;*
[4]*Department of Pathology, Tzu Chi Hospital and University, Hualien, Taiwan;*
[6]*Department of Pathology, Changhua Christian Hospital, Changhua, Taiwan, R.O.C.*

Abstract. *Background: Previously we found some low molecular weight proteins identified as histone in hepatocelluar carcinoma. Our objective was to clarify whether the co-immunoprecipitation of histone and cytokeratin 18 was an artifact or not. Materials and Methods: Histone 3 and cytokeratin 18 were investigated in three cases of human hepatocellular carcinoma and one case of normal liver tissue. Nuclei of the tissues were isolated; the proteins inside the nuclei were analyzed by Western blot. Results: The results revealed histone was co-immunoprecipitated with cytokeratin 18 in hepatocellular carcinoma. It was speculated that modulation of the cytoskeleton in human hepatocellular carcinoma might disturb the organization of the nucleoskeleton. The unstable nucleoskeleton might further cause instability and fragility of nuclei, thus possibly exposing the histone and co-immunoprecipitating it with cytokeratin 18. Conclusion: The evidence might indicate that expression of histone 3 was highly related to modulation of cytokeratin 18 and might play an important role in tumorigenesis of hepatocellular carcinoma.*

Intermediate filaments provide flexible scaffolding that contributes to maintaining the structural integrity of cells and to resisting stresses that are applied to them (1). However, in human liver (including hepatoma), these filaments have rarely been investigated. In human liver parenchyma cells, intermediate filaments are formed by keratins. Human hepatocytes have a very simple keratin composition and express only one keratin pair: cytokeratin 8 (type II, molecular weight 52 kDa) and cytokeratin 18 (type I, molecular weight 45 kDa) (2). Keratins are required for the maintenance of hepatocyte integrity (3), and altered expression of keratin gene is known to be related to liver diseases, including chronic hepatitis, increased hepatocyte fragility and decreased bile secretion (4).

We have investigated the keratin molecules of cancer cells for many years; modulation of cytokeratin 18 (CK18) during tumor transformation in human hepatocellular carcinoma (HCC) was identified in our previous study (5). In addition to the modulation of CK18, a group of low molecular weight proteins (LMP) highlighted in the 12.4-18.4 kDa (mainly in the 14k Da) region were found in HCC tissues (5) and cell lines (6). The LMPs were also present in the transitional cell carcinoma of human kidney and urinary bladder (7), as well as in other carcinomas (8). These data suggested that LMPs played an important role in the carcinogenesis of human carcinoma. Considering the relationship between LMP and CK18, we found that there were several discriminations between them. First, anti-CK18 antibody could not recognize LMP on Western blot assay. This indicated that antigenicity of LMP was different from CK18. Second, the sequences of *N*-terminal residues of LMP were matched with those of the *N*-terminal residues of human histone. In our continuous studies, the LMP found in human HCC and other carcinoma was proven to be histone 3 (H3) and we also determined that the expression of H3 in human HCC was altered (9).

Histone has an important role in the condensation of DNA in chromatin. The amino acid side chains of histone

*All three authors contributed equally to this work.

Correspondence to: Dr. Yih-Shyong Lai, Department of Pathology, Hospital and Medical College of Chung Shan Medical University, 110, Section 1, Chien-Kuo North Road, Taichung 408, Taiwan. Tel: +886 4 24730022 ext. 11623, Fax: +886 4 24753984, e-mail: yslaick18@yahoo.com.tw

Key Words: Cytokeratin, histone, modulation, hepatocellular carcinoma, immunoprecipitation.

are frequently modified by posttranslational additions of phosphate, methyl, or acetyl groups. The posttranslational modification of histone might be different between normal and cancer cells. For example, elevated levels of phosphorylated H1 and H3 might be responsible for the less condensed chromatin structure and aberrant gene expression observed in the oncogene-transformed cells (10). Aberrant acetylation or deacetylation of histone leads to many human disorders including cancer; the various groups of histone acetyltransferase and deacetylase are implicated in cancer progression and human diseases (11). The *in vitro* ADP-ribosylation of histone was found to be less active in nuclei from hepatoma than in nuclei from normal liver (12). These studies revealed that the histone modification in tumor cells and modulation of histone during tumor transformation was reasonable.

The nucleoskeleton is a permanent structure of cell nuclei, consisting of lamina and an inner network of 9- to 13-nm-thick "core filaments" covered with material referred to as the "diffuse nucleoskeleton". The core filaments morphologically resemble cytoplasmic intermediate filaments and they show positive immunoreactivity with antibodies against all subclasses of intermediate filaments (13). It was proposed that the nuclear matrix (or nucleoskeleton) was a critical, facilitating element in nuclear functions and played a key role in DNA replication. Chromatin in the interphase nucleus was found to be arranged in spatially separate chromosome-specific territories and it was anchored to the nuclear matrix that organized the overall nuclear structure (14). In our opinion, the organization of the nucleoskeleton might be an important factor in maintaining the architecture of hepatocyte nuclei; modulation of nucleokeratin might affect the integrity of hepatocyte nuclei and further influence the gene expression in human HCC.

Though we had confirmed the nature of the LMPs we found as H3, it was still not clear whether H3 was related to CK18 in human HCC. In this study, we investigated the possibility of co-immunoprecipitation of H3 and CK18 in human HCC.

Materials and Methods

Specimen and antibodies. Three specimens of human HCC and one of normal liver tissue were obtained by surgery and stored at –80˚C until analysis. The monoclonal anti-human H3 antibody was from Abcam (Cambridge, MA, USA), the monoclonal anti-human CK18 antibody was from ZYMED (Carlsbad, California, USA), the secondary antibody purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, USA) was horseradish peroxidase labeled anti-mouse IgG, anti-rabbit IgG antibody.

Extraction of CK18 and LMPs. Since LMPs were co-extracted with CK18 in HCC tissues *via* our previous studies, the same method was used to obtain LMP in this study. The extraction procedure mainly followed Katsuma *et al*.'s method (15). Unless otherwise indicated, the preparation was conducted at 4˚C. Briefly, the minced specimen was first incubated with CSK buffer (300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, 10 mM piperazine-1, 4-*bis* [2-ethansulphonic acid], 1.2 mM phenylmethylsulfonyl fluoride and 0.1 mM iodacetamide) containing 100 mM NaCl, for 5 minutes. After three washings, the pellets were treated with CSK buffer containing 250 mM $(NH_4)_2SO_4$ for 10 minutes and then with the same buffer containing bovine pancreatic DNase I (100 μg/ml) and RNase A (100 μg/ml) for 10 minutes. The pellets were rinsed with the same buffer containing DNase I, RNase A and $(NH_4)_2SO_4$ for 10 minutes at room temperature. Finally, the preparation was resuspended in CSK buffer adjusting its concentration to 1 mg/ml. Part of the extracted products were loaded onto sodium dodecyl sulfate polyacrylamide gel for electrophoresis.

Immunoprecipitation. Protein A beads (from Sigma, USA) and the ligand (anti-CK18 and -H3 monoclonal antibodies) were coupled according to the brochure from Pharmacia. Four hundred micrograms of cellular extract were then incubated with the bound beads. The unbound protein molecules were washed off with 0.1 M phosphate buffer, pH 7.2. After centrifugation, the bound molecule-protein A beads were re-suspended with 2× sample buffer and then well mixed by means of vortex, heated for 10 min at 95˚C to half the molecule-protein A interaction and the molecules of interest were obtained (16). These immuno-precipitated proteins were analyzed by gel electrophoresis and Western blot.

Gel electrophoresis and Western blot assay. Equal amounts of protein samples were lysed in 2× sample buffer containing 0.5 M Tris-HCl, pH 6.8, 10% sodium dodecyl sulfate, 5% 2-mercaptoethanol and 30% glycerol. Ten micrograms of these products were separated by 12% sodium dodecyl sulfate polyacrylamide gel as described by Laemmli (18). Proteins were visualized by staining with Coomassie brilliant blue. After electrophoresis, proteins were transferred to PVDF (polyvinylidene difluoride) membranes (Millipore Corp., USA) and were incubated with anti-CK18 and anti-H3 monoclonal antibodies overnight at 4˚C. The PVDF membranes were incubated with horseradish peroxidase-labeled secondary antibody for 60 minutes at room temperature. The signals were detected by means of an enhanced chemiluminescence system (Amersham, USA) (19).

Isolation of nuclei from normal liver and hepatocellular carcinoma tissues. Single cells were separated from the normal liver and hepatocellular carcinoma tissues by gentle homogenization in phosphate-buffered saline (PBS). These separated cells were washed at 2˚C three times with isolation buffer [3.75 mM Tris-HCl pH7.4, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA, 20 mM KCl, 1% (v/v) thiodiglycol] in the presence of proteinase inhibitors and then lysed in 40 ml of ice-cold Triton X-100 lysis buffer (isolation buffer with 0.5% Triton X-100 and proteinase inhibitors) by gently agitating with a spatula. The released nuclei were washed again twice with 40-ml portions of Triton X-100 lysis buffer. Between each centrifugation at 120 × *g* for 5 min, the nuclei were re-suspended with a spatula to avoid shearing forces. After the last Triton X-100 wash, the

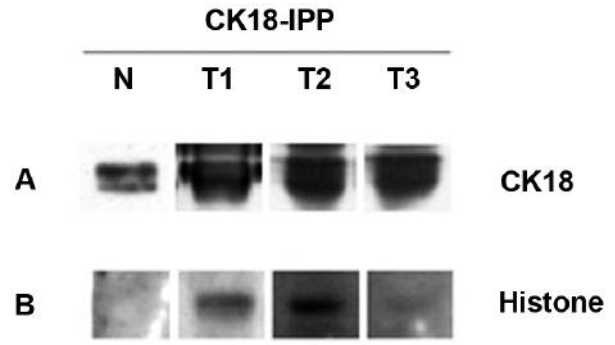

Figure 1. *Cell lysates were obtained from normal liver tissue (lane N) and HCC tissue (lanes T1, T2 and T3). These lysates were immunoprecipitated by protein A binding with anti-CK18 monoclonal antibodies. The immunoprecipitated proteins were transferred to a nitrocellulose membrane and reacted with monoclonal anti-CK18 (A) and anti-H3 (B) antibodies, respectively.*

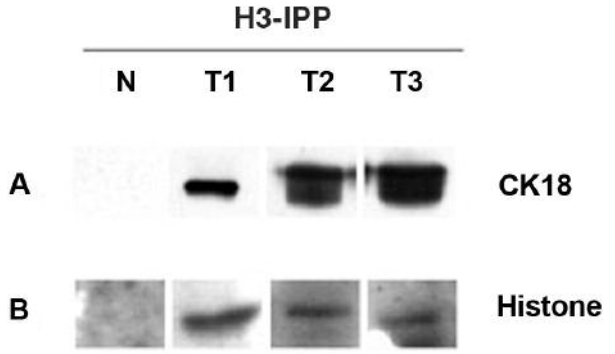

Figure 2. *Cell lysates were obtained from normal liver tissue (lane N) and HCC tissue (lanes T1, T2 and T3). These lysates were immunoprecipitated by protein A binding with anti-H3 monoclonal antibodies. The immunoprecipitated proteins were transferred to a nitrocellulose membrane and reacted with monoclonal anti-CK18 (A) and anti-H3 (B) antibodies, respectively.*

nuclei were subjected to strong shearing by 15 strokes in a tightly fitting Dounce homogenizer to remove cytoplasm (17). Finally, the isolated nuclei were smeared on a glass slide and visualized by Riu's stain. These isolated nuclei were further homogenized and the protein inside the nuclei was analyzed by gel electrophoresis and Western blot.

Riu's stain. The procedure followed the method of Riu (20). After smearing, the slides were dried and 0.8 ml of Riu's stain solution A was added (0.17% eosin Y and 0.05% methylene blue in methanol) for 30 seconds. Subsequently, 1.6 ml of Riu's stain solution B (0.14% methylene blue, 0.12% Azure I, 2.52% $Na_2HPO_4 \cdot 12H_2O$, 1.25% KH2PO4 in distilled water) was added and the two solutions were mixed. The slides were left for 90 seconds at room temperature. Finally, the slides were rinsed, dried and mounted with coverslips. Solutions A and B for staining were purchased from Nuto Pure Chemicals Co. Ltd. (Japan).

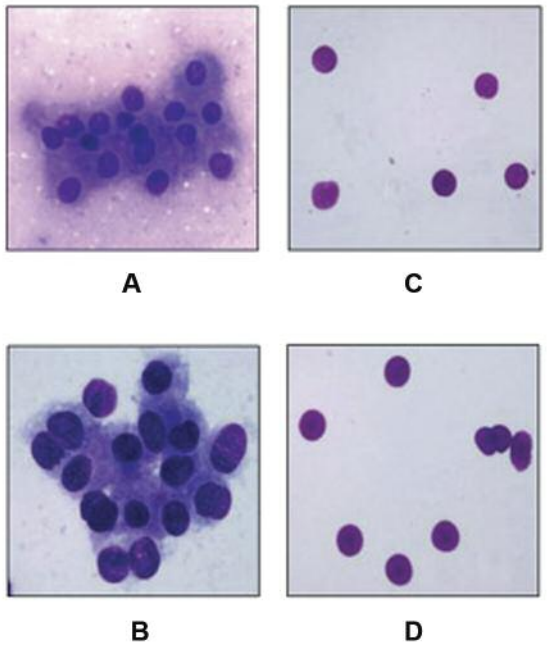

Figure 3. *By homogenization, the individual cells were separated from normal liver tissue (A) and HCC tissue (B) and visualized by Riu's stain (×1,000). The nuclei were further isolated from these individual cells. Isolated nuclei were visualized by Riu's stain and observed under a microscope. C: Nuclei of normal liver cell (×1,000); D: nuclei of HCC cell (×1,000).*

Results

H3 was co-immunoprecipitated with CK18 in human HCC. Cellular extractions were obtained from three cases of human HCC and one case of normal liver tissues, respectively, by the CSK buffer methods described in Materials and Methods (5). These extraction products were further immunoprecipitated by protein A binding with anti-CK18 monoclonal antibodies. The immunoprecipitated products were then analyzed by Western blot assay using anti-CK18 and anti-H3 monoclonal antibodies, respectively. In Figure 1A, the results of CK18 Western blotting can be seen: CK18 was recognized in normal liver (lane N) and HCCs (lanes T1, T2 and T3). This indicated that the CK18 existed both in normal liver and HCCs. In Figure 1B, the same samples were analyzed by H3 Western blotting. H3 was found in HCCs (lanes T1, T2 and T3) but not in normal liver (lane N). The data were identical to our previous study and indicated that H3 was co-immunoprecipitated with CK18 in HCCs but not in normal liver.

To rule out the possibility of this result being an artifact, the experiments were repeated using different antibodies. The cellular extractions were obtained from the same tissue samples again, and immunoprecipitated by protein A binding with anti-H3 monoclonal antibodies. The immunoprecipitated

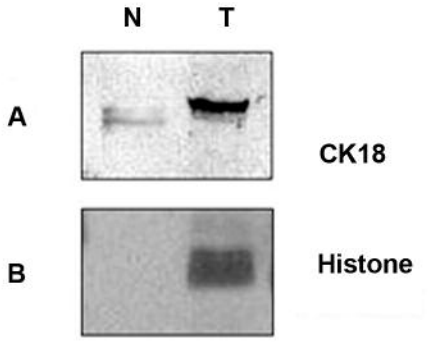

Figure 4. *The total protein of isolated nuclei were separated on 12% SDS-PAGE and transferred to nitrocellulose membrane and detected with anti-CK18 antibody (A) and anti-H3 antibody (B) on Western blot. N: Nuclear protein from normal liver tissues. T: nuclear protein from HCC tissues.*

products were then analyzed by Western blot assay using anti-CK18 and anti-H3 monoclonal antibodies, respectively. The results of CK18 Western blot are displayed in Figure 2A: CK18 was present in HCCs (lanes T1, T2 and T3) but not in normal liver (lane N). In Figure 2B, it is surprising that H3 appeared only in HCCs (lanes T1, T2 and T3). In contrast, the immunoprecipitated proteins of normal liver did not contain H3 (lane N).

By this experiment, we confirmed that in human HCC, H3 was co-immunoprecipitated with CK18; this phenomenon was not seen in the normal liver and it was not due to artifacts. Control studies ruled out the nonspecific binding between protein A and antibodies. The Western blot assay was negative when the beads were incubated only with cell lysates (data not shown).

Histone 3 of normal liver cells could not be immunoprecipitated by anti-H3 antibody. In this study, an interesting phenomenon was noted in that H3 of normal liver could not be immunoprecipitated by the anti-H3 antibody (Figure 2B lane N). Since histone is the nuclear protein inside the nuclei, we isolated the nuclei from normal liver and HCC tissues; the protein inside the nuclei was analyzed by gel electrophoresis and Western blot. By homogenization, the cells were washed and separated from intercellular stroma of the normal liver and HCC tissues. Using Riu's stain to confirm, the smear revealed individual cells have abundant cytoplasm with round or oval contour (Figure 3A and B). The nuclei were later isolated out of these cells and observed under a microscope. In Figure 3C (nuclei of normal liver cells) and 3D (nuclei of HCC cells), the pure nuclei were obtained and there was no contamination from other cellular components or extracellular matrix. These isolated nuclei were further homogenized and the total protein of nuclei was analyzed by gel electrophoresis and Western blot. In Figure 4A, both of the proteins that were obtained from the isolated nuclei of normal liver (N) and HCCs (T) were immunoreactive to the monoclonal anti-CK18 antibody on Western blot assay. However, CK18 was markedly up-regulated in HCC nuclei and its molecular weight was larger than that of normal liver nuclei. The most striking finding was that H3 existed in the HCC nuclei fraction (Figure 4B lane T) but not in normal liver nuclei (Figure 4B lane N).

Discussion

In our previous studies, the LMPs found in human HCCs were confirmed to be H3 (9). Interestingly, we found that the H3 in human HCCs exhibited higher affinity to anti-CK18 antibody in the process of immunoprecipitation. It was doubted whether H3 could be co-immunoprecipitated with CK18 in HCCs. In this experiment, we confirmed that in human HCCs the H3 was co-immunoprecipitated with CK18; this phenomenon did not happen in tissue from the normal liver. We also confirmed the results were not artifacts. This implied that H3 in human HCC might share some common features with CK18 and adopted the structure recognizable for anti-CK18 antibody. This speculation is also supported by Wood *et al.*'s study. They showed that histone shared antigenic determinants with intermediate filaments since anti-neurofilament antibody did cross-react with histone (21). Nonetheless, the cross-reaction could not explain the fact that co-immunoprecipitation of H3 with CK18 did not occur in normal liver tissue.

Based on the evidence in this study, we raised a hypothesis to explain the co-immunoprecipitation of H3 with CK18 in human HCC: *in HCC, the higher order structure of the chromosomes and the organization of nuclei were unstable thus the nuclei were more fragile than that of normal hepatocytes. This phenomenon might cause nuclei in HCC to breakdown more easily and expose the histone proteins during the process of extraction.* In order to confirm this hypothesis, we isolated the nuclei from normal liver and HCC tissues. The proteins in these nuclei were then analyzed by gel electrophoresis and Western blot. Our results revealed that H3 was found in HCC but not in normal liver. We speculated that the normal liver nuclei might be removed intact during the total cell extract procedure so the H3 was not exposed, while the HCC nuclei were broken thus exposing H3 in the total cell extraction and co-immunoprecipitating it with CK18. The speculation was also identical to our previous studies since we found H3 was tightly bound to DNA so that it could not be recognized by antibodies and could not be immunoprecipitated in normal liver (9).

The structures of nuclei are supported by the nucleoskeleton. The nucleoskeleton was thought to be the

residual framework after removal of the nuclear envelope, chromatin and soluble components by sequential extractions (14). The organization of the nucleoskeleton is an important factor in maintaining the architecture of nuclei. Several studies had shown the existence of such a structure and mentioned that it might have a function in response to stress (22); or it might be involved in nuclear structure changes that occured during heat-shock response (23). In addition, nuclei assembled *in vitro* in the absence of lamins were fragile (24). In this study, we found CK18 was a part of the nucleoskeleton in normal liver and HCC nuclei since CK18 was seen in isolated nuclei by Western blot (Figure 4). This finding with that of Spencer *et al*. demonstrated the existence of intermediate filament proteins, including CK8, 18 and 19, in the nuclear matrix-intermediate filament fraction of human breast cancer cell line T-47D5 (25).

Besides the existence of CK18 in nuclei, we also noted that the CK18 was somewhat altered because we found up-regulation and modulation of CK18 in HCC nuclei (Figure 4). Regarding these studies, we had the concept that modulation of the nucleoskeleton (in this study, modulation of CK18 was proven) could affect the nuclear organization which in turn would disturb the integrity of the nuclei of HCC. This could further cause instability and fragility of HCC nuclei. Much literature had revealed similar concepts: for example, inappropriate expression of CK19 might be involved in the epigenetic activation of new cellular programs, through the reorganization of the cytoskeleton which in turn might perturb nuclear matrix functions (26). The cytoskeleton array is a dynamic system that transmits signals from the cell exterior to nuclear DNA. The composition and function of this mechanical signaling pathway was found to be altered in cancer cells (27).

The nucleoskeleton was also reported to connect to nuclear DNA and to regulate gene expression. For example, the activation of nucleolar transcription was connected with a spatial rearrangement of specific rDNA elements relative to the nucleoskeleton (28). In other studies, the nuclear matrix proteins play some roles in determining gene expression. For example, lamin binding to DNA and/or chromatin was implicated in replication (29). The nuclear matrix protein had linkage to intermediate filaments of the cytoskeleton. They were also implicated in transcription, regulation of gene expression and provided a mooring for certain hormone receptors (30). Based on these studies, we speculated that by attachment to DNA/histone, modulated CK18 might be involved in the regulation of gene expression in human HCC. The role of DNA attachment and determining gene expression in the nuclei of human HCC indicated that CK18 plays an important role in tumorigenesis of HCC.

Several factors are involved in the stability of the cytoskeleton in hepatocytes. Our previous studies confirmed that in human liver cells, the stability of the cytokeratin network was related to the intact microtubule network (31). In addition, plectin was an important factor in the stability of cytokeratin, since we found the stability of CK18 is affected by plectin deficiency in human HCC (32). Based on these studies, we speculated that the cytoskeleton (CK18) of human HCC was modulated and unstable, which might influence the stability of nucleoskeleton and further cause the instability and fragility of nuclei. After that, the histone (H3) might be exposed from the nuclei and be co-immunoprecipitated with CK18 in HCC.

In this study, we determined that H3 was co-immunoprecipitated with CK18 in human HCC. The evidence might indicate that expression of H3 was highly related to modulation of CK18 and might play an important role in tumorigenesis of human HCC.

Acknowledgements

We are very grateful to Ing-Jen Wu for invaluable technical assistance.

References

1 Fuchs E and Cleveland DW: A structural scaffolding of intermediate filaments in health and disease. Science *279*: 514-519, 1998.

2 Van Eyken P and Desmet VJ: Cytokeratins and the liver. Liver *13*: 113-122, 1993.

3 Loranger A, Duclos S, Grenier A, Price J, Wilson-Heiner M, Baribault H and Marceau N: Simple epithelium keratins are required for maintenance of hepatocyte integrity. Am J Pathol *151*: 1673-1683, 1997.

4 Omary MB and Ku NO: Intermediate filament proteins of the liver: emerging disease association and functions. Hepatology *25*: 1043-1048, 1997.

5 Su B, Pei RJ, Yeh KT, Hsu YH and Lai YS: Could the cytokeratin molecule be modulated during tumor transformation in hepatocellular carcinoma? Pathobiology *62*: 155-159, 1994.

6 Liu YH, Pei RJ, Yeh CC, Lee KY, Yeh KT, Hsu YH, Ho CC and Lai YS: The alteration of cytokeratin 18 molecule and its mRNA expression during tumor transformation in hepatoma. Res Commun Mol Pathol Pharmacol *96*: 243-253, 1997.

7 Yeh CC, Pei RJ, Liu YH, Su B, Lee KY, Yeh KT, Hsu YH, Ho CC, Ho HC and Lai YS: The expression of cytokeratin 18 in transitional cell carcinoma comparing with hepatoma. Res Commun Mol Pathol Pharmacol *105*: 3-10, 1999.

8 Pei RJ, Liu YH, Su B, Yeh CC, Lee KY, Yeh KT, Hsu YH, Ho CC and Lai YS: Do the CK18 related proteins change in general in epithelial cancers? Res Commun Mol Pathol Pharmacol *108*: 253-260, 2000.

9 Liu YH, Pei RJ, Cheng CC, Ho HC, Ho CC, Yeh KT, Lee KY and Lai YS: HCC CKs are altered histones during tumor transformation in hepatoma. Res Commun Mol Pathol Pharmacol *112*: 27-38, 2002.

10 Chadee DN, Hendzel MJ, Tylipski CP, Allis CD, Bazett-Jones DP, Wright JA and Davie JR: Increased Ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts. J Biol Chem *274*: 24914-24920, 1999.

11 Timmermann S, Lehrmann H, Polesskaya A and Harel-Bellan A: Histone acetylation and disease. Cell Mol Life Sci *58*: 728-736, 2001.
12 Lea MA: Nuclear proteins of tumors. Int J Biochem *15*: 767-770, 1983.
13 Philimonenko VV, Flechon JE and Hozak P: The nucleoskeleton: a permanent structure of cell nuclei regardless of their transcriptional activity. Exp Cell Res *264*: 201-210, 2001.
14 Martelli AM, Falcieri E, Zweyer M, Bortul R, Tabellini G, Cappellini A, Cocco L and Manzoli L: The controversial nuclear matrix: a balanced point of view. Histol Histopathol *17*: 1193-1205, 2002.
15 Katsuma Y, Marceau N, Ohta M and French SW: Cytokeratin intermediate filaments of rat hepatocytes: different cytoskeletal domains and their three-dimensional structure. Hepatology *8*: 559-568, 1988.
16 Merisko EM, Farquhar MG and Palade GE: Coated vesicle isolation by immunoadsorption on *Staphylococcus aureus* cells. J Cell Biol *92*: 846-857, 1982.
17 Tolstonog GV, Sabasch M and Traub P: Cytoplasmic intermediate filaments are stably associated with nuclear matrices and potentially modulate their DNA-binding function. DNA Cell Biol *21*: 213-239, 2002.
18 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*: 680-685, 1970.
19 Towbin H, Staehelin T and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA *76*: 4350-4354, 1979.
20 Riu CH: On the studies of the methods of staining blood film (Romanowsky's system). J Niigata Med Assoc *70*: 635-643, 1956.
21 Wood JN, Lathangue NB, McLachlan DR, Smith BJ, Anderton BH and Dowding AJ: Chromatin proteins share antigenic determinants with neurofilaments. J Neurochem *44*: 149-154, 1985.
22 Wasser M and Chia W: The EAST protein of drosophila controls an expandable nuclear endoskeleton. Nat Cell Biol *2*: 268-275, 2000.
23 Neri LM, Riederer BM, Marugg RA, Capitani S and Martelli AM: Analysis by confocal microscopy of the behavior of heat shock protein 70 within the nucleus and of a nuclear matrix polypeptide during prolonged heat shock response in HeLa cells. Exp Cell Res *221*: 301-310, 1995.
24 Stuurman N, Heins S and Aebi U: Nuclear lamins: their structure, assembly, and interactions. J Struct Biol *122*: 42-66, 1998.
25 Spencer VA, Coutts AS, Samuel SK, Murphy LC and Davie JR: Estrogen regulates the association of intermediate filament proteins with nuclear DNA in human breast cancer cells. J Biol Chem *273*: 29093-29097, 1998.
26 Barboro P, Alberti I, Sanna P, Parodi S, Balbi C, Allera C and Patrone E: Changes in the cytoskeletal and nuclear matrix proteins in rat hepatocyte neoplastic nodules in their relation to the process of transformation. Exp Cell Res *225*: 315-327, 1996.
27 Spencer VA and Davie JR: Signal transduction pathways and chromatin structure in cancer cells. J Cell Biochem Suppl *35*: 27-35, 2000.
28 Weipoltshammer K, Schofer C, Almeder M, Philimonenko VV, Frei K, Wachtler F and Hozak P: Intranuclear anchoring of repetitive DNA sequences: centromeres, telomeres, and ribosomal DNA. J Cell Biol *147*: 1409-1418, 1999.
29 Hozak P, Sasseville AM, Raymond Y and Cook PR: Lamin proteins form an internal nucleoskeleton as well as a peripheral lamina in human cells. J Cell Sci *108(Pt 2)*: 635-644, 1995.
30 Vemuri MC, Raju NN and Malhotra SK: Recent advances in nuclear matrix function. Cytobios *76*: 117-128, 1993.
31 Liu Y, Su B, Pei R, Yeh C, Yeh K, Ying Lee K, Hsu Y, Ho C and Lai Y: The stability of cytokeratin 18 in human liver cells during colchicine-induced microtubule disruption. Food Chem Toxicol *39*: 85-89, 2001.
32 Cheng CC, Liu YH, Ho CC, Chao WT, Pei RJ, Hsu YH, Yeh KT, Ho LC, Tsai MC and Lai YS: The influence of plectin deficiency on stability of cytokeratin18 in hepatocellular carcinoma. J Mol Histol *39(2)*: 209-216, 2008.

Received January 31, 2008
Revised April 15, 2008
Accepted April 21, 2008