

## Degradation of Plectin with Modulation of Cytokeratin 18 in Human Liver Cells during Staurosporine-induced Apoptosis

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**Abstract.** *Background: Hepatoma cells are morphologically different from those of the normal liver. Intermediate filaments (IFs) are important in building the cellular architecture and maintaining the outline of cells. Plectin is a cross-linking protein that organizes the cytoskeleton into a stable meshwork, which can maintain the uniform size and shape of hepatocytes. Apoptosis might be the most possible pathway for creating plectin deficiency in the in vivo state. Materials and Methods: Apoptosis was induced by staurosporine (STS) treatment in liver cells. The protein expression of cytokeratin 18 (CK18) and plectin as well as the morphology of the liver cells and the distribution of CK18 and plectin in the cells was studied after STS treatment. Results: Plectin was cleaved in the liver cells during apoptosis and CK18 was modulated. Morphological changes were observed in the liver cells. Conclusion: By affecting the organization of IFs, plectin might play an important role in the pleomorphism of hepatoma cells and even the tumorigenesis of hepatoma.*

Cytokeratins (CKs) are the intermediate filament (IF) proteins of epithelial cells including hepatocytes. They are expressed in specific pairs in various epithelial cells and

constitute the largest family of the cytoskeleton (1). Hepatocytes have a very simple CK composition and express only one CK pair, CK8 (type II) and CK18 (type I) (2). CKs are required for the maintenance of hepatocyte integrity (3) and the altered expression of CK genes is known to be related to liver diseases, including chronic hepatitis, increased hepatocyte fragility and decreased bile secretion (4). Recently, the possible role of CK in tumorigenesis has also been reported since CKs were found to modulate cell adhesion, size, G1/S transition and protein synthesis of liver cells (5).

The assembly of the cytoskeleton involves the recruitment of a series of cell type-specific associated proteins. Some of these are integral membrane proteins, others act as cross-linking proteins and others comprise motor proteins (6). Intermediate filament associated proteins (IFAPs) connect IFs to microtubules and microfilaments and organize the cytoskeleton into a firm and stable meshwork, which can maintain the uniform size and shape of the cells. In hepatocytes, the main IFAPs are plectin and desmoplakin, which are members of the plakin gene family (7). The plectin possesses binding sites for keratin, vimentin, tubulin and actin and has been shown to interact with a variety of cytoskeletal structures, making it a cytoskeleton network integrator (8, 9). Based on immunogold electron microscopy, plectin has been visualized as thin (2-3 nm) and up to 200 nm long filaments connecting IFs to microtubules and/or microfilaments; the connection was seen as a striking millipede-like structure with an IF core and plectin sidearms (10). This IF-plectin complex comprised an extensive cross-linking of cellular components and provided a structural framework for integration of the cytoplasm. In addition, plectin has been reported as being involved in the assembly and disassembly of vimentin and CK5/14 in a dose-dependent manner (11).

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**Key Words:** Cytokeratin, plectin, hepatocellular carcinoma, staurosporine, apoptosis

Other evidence has supported the role of abnormal plectin expression in relating to human diseases. For example, epidermolysis bullosa simplex with muscular dystrophy is an autosomal recessive disorder caused by mutations of the human plectin gene that is located on chromosome 8q24.13-qtter (12). The resulting skin blistering is caused by a lack of plectin to connect keratin to hemidesmosomes while the muscular disorder may result from deficiencies in desmin attachment to the membrane (13,14). In prostate carcinoma, the expression of plectin is variable in the stroma around tumor nests (15).

The morphology of hepatoma cells is different compared with normal liver cells. It is reasonable to speculate that an alteration in function and structure of CK might cause the disorganization of the hepatic cytoskeleton that further induces the pleomorphism of hepatoma cells. Our previous study revealed the reduction of CK18 in human hepatocellular carcinoma (HCC) (16) which was demonstrated to be protein modulation and not a reduction in the mRNA level (17). A later study, using colchicine treatment, confirmed that in human liver cells, the stability of the CK18 network was related to an intact microtubule network (18).

The effects of plectin on CK18 stability in human liver cells has been explored in our previous studies. By laser irradiation, the expression of plectin could be down-regulated which disturbed the organization of CK18 filaments in the human hepatoma cell lines HepG2 and J-5 (19). Using the small interference RNA (siRNA) technique to knockdown the plectin led to CK18 instability and pleomorphism in human liver cells (20). Thus we demonstrated that the stability of IFs was closely related to plectin in human hepatocytes.

The failure of cells to undergo appropriate apoptotic cell death is involved in degenerative diseases and malignancies. The apoptosis of liver cells has become the focus of many researchers since it became apparent that deregulation of the apoptotic program is pathophysiologically involved in liver diseases (21). HCC seems to escape immune surveillance and apoptosis induced by cytotoxic T-cells expressing CD95L (22). Another study showed that during apoptosis, CK8/18 was reorganized into granular structures enriched with phosphorylated CK8/18 that facilitated the rapid collapse of the cytoskeletal architecture (23). *In vivo*, plectin can be a substrate for caspase-8 during apoptosis and is required for the reorganization of the microfilament system (24). Recently, the pathway of colchicine-induced apoptosis resulting in cytoskeleton alteration has been demonstrated (25).

Plectin in human HCC has been less studied, but it might be an important factor for understanding the tumorigenesis of human HCC. Chemical (colchicine), physical (laser irradiation) and biological (siRNA) techniques have proven effective for exploring the roles of CK18 and plectin in human HCC, however, they produced an artificially created experiment state. In the *in vivo* situation, apoptosis might be

the most possible pathway for creating plectin deficiency. To understand the mechanism of plectin deficiency in human HCC, the possibility that plectin deficiency is triggered by an intracellular factor was investigated by inducing apoptosis in human liver cells.

## Materials and Methods

**Tissue samples and antibodies.** Four liver tissue samples, including one normal liver and three HCCs, were collected at surgical resection and stored at  $-80^{\circ}\text{C}$ . The normal liver tissue was obtained from a patient suffering a car accident. All the HCC cases were pathological grade II. The following commercial primary and secondary antibodies were used for the immunoblotting and immunofluorescence assays. Anti-CK18 monoclonal antibody was purchased from Zymed Laboratories, Inc. (South San Francisco, CA, USA). Anti-plectin monoclonal antibody and anti-GAPDH antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All of the secondary antibodies including anti-goat IgG, anti-mouse IgG and anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). They were conjugated to horseradish peroxidase (HRP) for immunoblotting analysis and fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG and rhodamine-conjugated anti-mouse IgG for immunofluorescence staining.

**Cell culture and apoptosis induction.** Chang human normal liver cells were obtained from The American Type Culture Collection (ATCC) number CCL-13. The cells were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 unit/ml penicillin and streptomycin and 200 mM L-glutamine. The medium was replaced every two days. The cells were subcultured: for the immunoblotting analysis,  $3 \times 10^5$  cells were seeded into 6-well tissue plates, and for the immunofluorescence assay,  $3 \times 10^4$  cells were grown on coverslips in 24-well tissue plates. At approximately 80% confluence, the medium was exchanged with new test medium containing 1  $\mu\text{M}$  staurosporine (STS) in dimethyl sulfoxide (DMSO) and the cells were incubated for four hours for apoptosis induction. Medium without STS was used as the negative control.

**Quantification of apoptotic cells.** The STS-treated cells on coverslips were washed with ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM  $\text{Na}_2\text{HPO}_4$  and 1.5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4) and fixed with 3% paraformaldehyde in PBS for 20 minutes at room temperature. After washing in PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for two minutes followed by three washes with cold PBS. The cells were then stained with 1  $\mu\text{g}/\text{ml}$  4,6-diamidino-2-phenylindole (DAPI) in 1% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. After staining, the coverslips were washed three times with cold PBS, mounted on slides and observed under a fluorescence microscope (BX51, Olympus Corporation, Japan). The fragmental nuclei of apoptotic cells were calculated in a total of 200 cells.

**Total protein extraction from tissues.** The total protein was extracted from the surgically resected liver and hepatoma tissues. The tissue samples (less than 1 g) were immersed in liquid nitrogen and minced to powder then lysed in lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Calbiochem,

EMD Chemicals Inc., Darmstadt, Germany) vortexed for 30 seconds then left to stand for 30 minutes on ice. After grinding by pestle, the unbroken cells and connective tissue were removed from the homogenate by centrifugation at 500 xg for 10 minutes at 4°C. The supernatants were stored at -80°C until required.

**Total protein extraction from apoptotic cells.** The STS-treated adherent cells on the 6-well tissue plates were briefly washed with ice-cold PBS and were scraped into PBS and then centrifuged at 2000 rpm for 10 minutes at 4°C. The cell pellet was frozen in liquid nitrogen, thawed and then resuspended in lysis buffer containing protease inhibitors (as above). After vortexing for 30 seconds and homogenizing using a 27-G syringe, the unbroken cells were discarded from the homogenate by centrifugation at 500 xg for 5 minutes at 4°C. The samples were kept on ice at all times and the supernatants were stored at -80°C until use.

**Immunoblot analysis.** Protein quantification was performed with bicinchoninic acid (BCA) protein assay before electrophoresis. Equal amounts of protein extracted from the normal liver and hepatoma tissues and the apoptotic cells (10 µg for CK18 electrophoresis, 40 µg for plectin electrophoresis) were suspended in sample buffer (0.5M Tris-HCl, pH 6.8, 10% sodium dodecyl sulfate, 5% 2-mercaptoethanol and 30% glycerol). After heating for 5 minutes at 95°C, the samples were electrophoresed on 6% (for plectin electrophoresis) and 10% (for CK18 electrophoresis) sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) as described by Laemmli (26). After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the semi-dry transfer method (Bio-Rad Laboratories, Inc., CA, USA). The membrane was blocked with a blocking buffer containing 5% (w/v) nonfat dry milk by incubate for 1 hour at room temperature with agitation. After blocking, the membrane was incubated with monoclonal anti-CK18, anti-plectin and anti-GAPDH antibodies overnight at 4°C. GAPDH was used as an internal control. After that, the membranes were incubated with the secondary antibody, HRP-conjugated anti-mouse IgG and anti-goat IgG, for 60 minutes at room temperature and visualized using an enhanced chemiluminescence reagent (NEN, PerkinElmer, Inc., Waltham, MA, USA).

**Immunofluorescence assay.** The Chang liver cells with or without STS treatment were grown on 24-well tissue plates (3×10<sup>4</sup> cells per well) as described above. Before staining, the cells were washed with ice-cold PBS and fixed for 20 minutes with 3.7% paraformaldehyde in PBS at room temperature. Thereafter, the cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 2 minutes at room temperature. The cells were washed three times in PBS and double stained for 60 minutes with primary antibodies (mouse anti-CK18 and goat anti-plectin) at room temperature, and then washed with PBS again. After this, the cells were incubated with rhodamine-conjugated anti-mouse IgG and FITC-conjugated anti-goat IgG secondary bodies for 30 minutes at room temperature. Finally, the unbound antibodies were removed by washing twice for 10 minutes in PBS (27). The images were evaluated under a fluorescence microscope (Olympus BX51).

## Results

**CK18 and plectin in human HCC.** After electrophoresis, the expression of CK18 in the HCC tissue samples differed from that in the normal liver tissue (Figure 1A). Notably, the

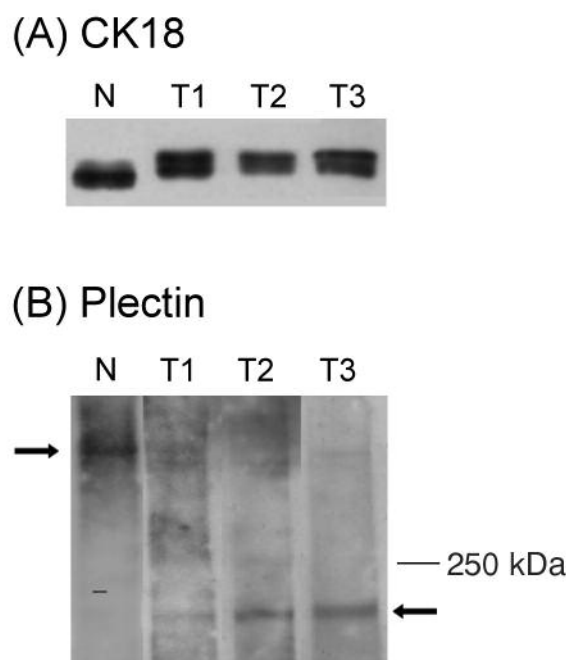


Figure 1. Immunoblot analysis of normal liver and hepatoma tissues. Total proteins extracted from liver (N) and hepatoma (T1 to T3) tissues were analyzed using monoclonal anti-CK18 (A) and anti-plectin (B) antibodies.

CK18 of the normal liver tissue gave one band while the modulated CK18 in the tumor tissues had two bands and higher molecular weight. Simultaneously, different expression of plectin was found in the HCC tissues (T1-3) and normal liver tissue (Figure 1B). The zymogenic plectin was expressed at approximately 300 kDa in the normal liver tissue while in contrast, degraded plectin at approximately 200 kDa was markedly present in the HCC tissues and the expression of zymogenic plectin was down-regulated.

**Degradation of plectin during apoptosis in Chang liver cells.** Scant fragmental nuclei cells were found in the control group (Figure 2A) and approximately 70% of apoptotic cells were found in the STS-treated group (Figure 2). Zymogenic plectin was seen at approximately 300~400 kDa bands in the untreated liver cells while degraded plectin appeared at 200 kDa in the STS-treated cells (Figure 3A). Similarly, zymogenic CK18 was found in the untreated cells while two bands, zymogenic CK18 and cleaved CK18, appeared after STS treatment of the liver cells (Figure 3B).

**Cell morphology in Chang liver cells.** As shown in the immunofluorescence assay (Figure 4), the plectin of the untreated cells was mainly distributed as a mesh structure in the perinuclear region and extended to the membrane with a granular pattern. The CK18 exhibited fine filament networks in

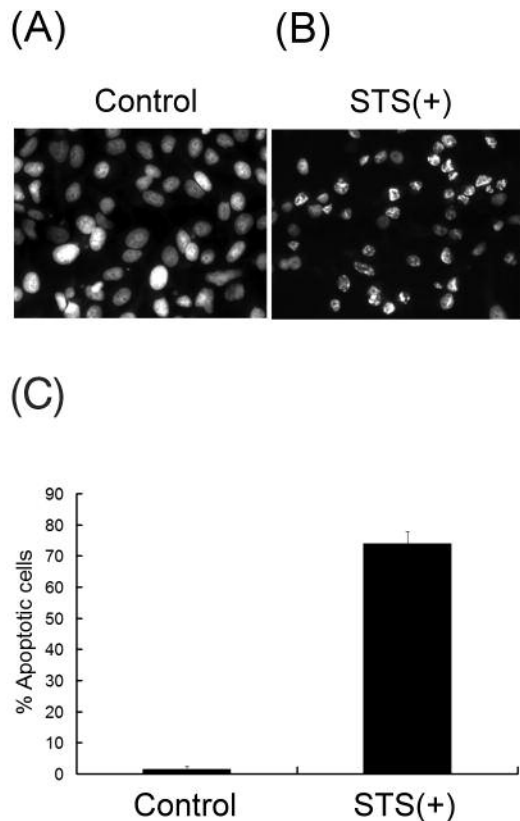


Figure 2. Induction of apoptosis in Chang liver cells. DAPI staining demonstrating (A) the regular shape of Chang liver cell nuclei in untreated control cells and (B) the typical morphological features of apoptotic cells including prominent nuclear fragmentation and condensation of DNA after 1  $\mu$ M STS-induced apoptosis. (C) Apoptosis induction was calculated at approximately 70% of 200 cells.

the cytoplasm and was also abundant in the perinuclear region, the mesh-like distributions extended towards the cell membrane (upper panel). When the cells were treated with STS, the plectin mesh collapsed, concentrated in the perinuclear region and showed a granular pattern (lower panel). The characteristics of the CK18 networks also displayed a disrupted pattern and granular structure after apoptosis induction.

## Discussion

Abnormal regulation of the cytoskeleton as well as its associated proteins has been investigated in several neoplasms, for example, down-regulation of CK19 in oral squamous cell carcinoma (28), down-regulation of microfilaments and their binding protein gelsolin in breast cancer (29), and up-regulation of tenascin-C and vimentin in breast cancer (30). In addition, the relationship between cytoskeletal structure and the pleomorphism of cancer cells was identified by electron microscopy when it was confirmed

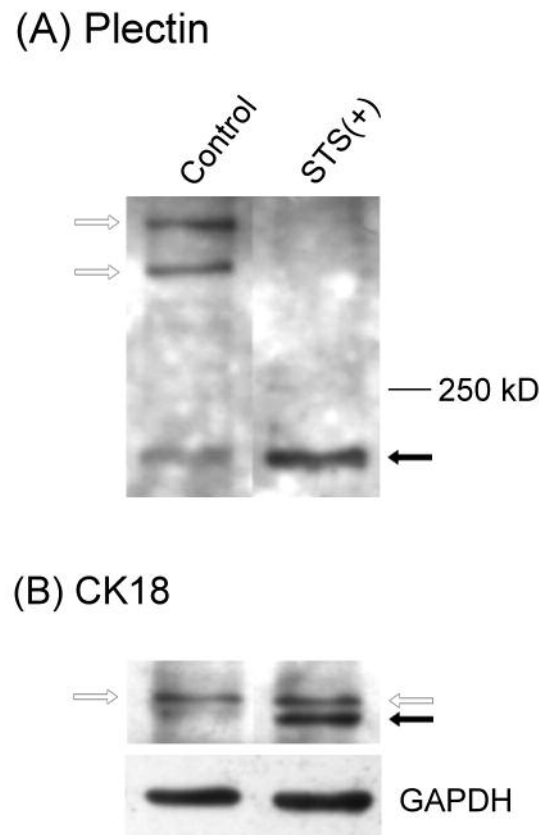


Figure 3. Plectin cleavage by STS-induced apoptosis in Chang liver cells. Immunoblot analysis of (A) plectin and (B) CK18 with or without STS treatment. GAPDH was used as an internal control. Blank arrows, zymogenic (A) plectin, (B) CK18; black arrow (A) degraded plectin, (B) cleaved CK18.

that alterations in cell shape of the human prostate cancer cell line DU145 are regulated by microfilaments (31). The involvement in the morphological change, invasion and carcinogenesis of cancer cells by these abnormal regulations of the cytoskeleton and their associated proteins was also raised in these studies.

In the present study, plectin was found to be down-regulated and cleaved in human HCC tissues (Figure 1B). By the STS treatment, apoptosis induced in the normal liver cells (Figure 2) mimicking the *in vivo* situation and degradation of the plectin was triggered (Figure 3A). The CK18 was modulated (Figure 3B) and the organization of the CK18 was also destroyed and the cell morphology transformed (Figure 4), indicating that, consistent with our previously study, the stability of CK18 might be affected by deficiency of plectin. Thus, in addition to extracellular forces (colchicines treatment, laser irradiation or siRNA knockdown), plectin expression might be down-regulated by an intracellular factor (apoptosis) in native state cells.

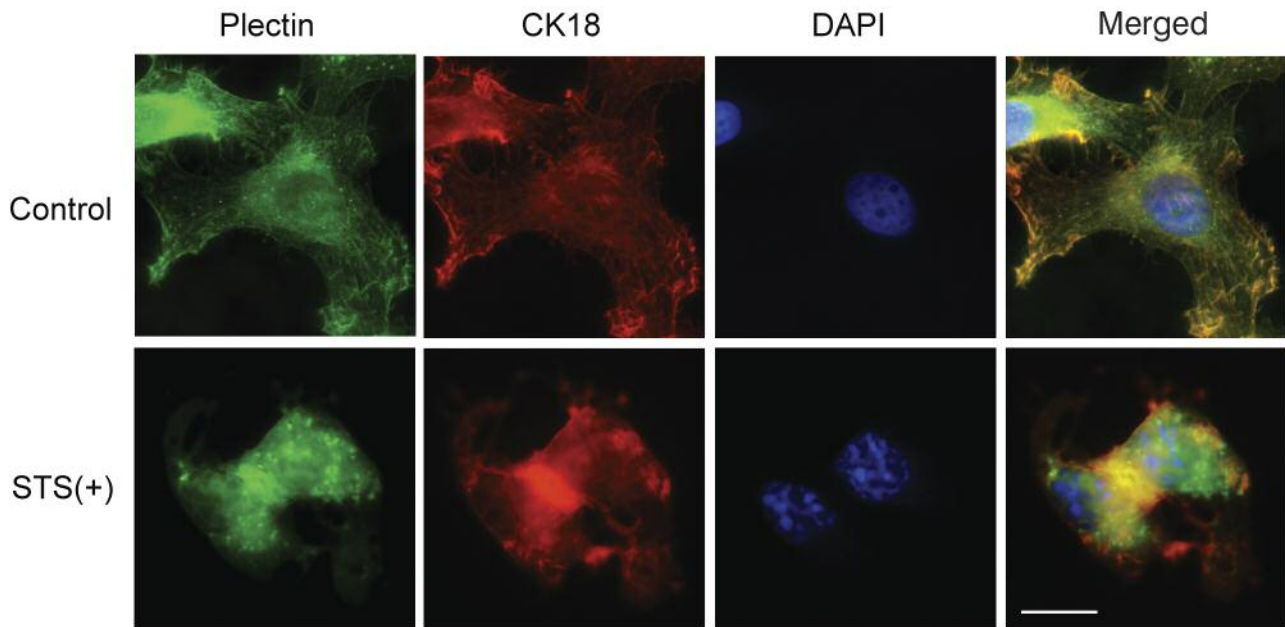


Figure 4. Immunofluorescence assay of plectin and CK18 in Chang liver cells after apoptosis induction. In control untreated cells (upper panel), the plectin was mainly distributed as mesh structure in the perinuclear region and extended to the membrane with a granular pattern. CK18 exhibited fine filament networks in the cytoplasm, abundant in the perinuclear region, the mesh-like distributions extended toward cell membrane. DAPI staining shows uniform shape of DNA. The merged image displays CK18 colocalized with plectin. In Chang liver cells treated with 1  $\mu$ M STS for 4 hours (lower panel), the plectin was concentrated around the nuclei with a granular pattern and CK18 filament networks were disrupted and reorganized in a granular pattern. DAPI staining shows fragmentation of nuclei and condensation of DNA and the merged image shows dramatic morphological change with collapse of the plectin and CK18 structures. Scale bar=20  $\mu$ m.

Plectin is a substrate for endogenous kinase or protease. Since protein kinase A and C can regulate the interaction of plectin with lamin B and vimentin, plectin may be a major effector of the phosphorylation-dependent regulatory system involved in the spatial organization and anchorage of the cytoskeleton (32). In rat hepatocytes, plectin is a naringin-sensitive phosphoprotein, which plays a role in the cytoskeletal disruption and apoptosis induced by algal toxins (33). Liver cell plectin is also the substrate of  $\mu$ -calpain and the degradation of plectin could be an important event in the destabilization of hepatoma cells (34). In the human breast carcinoma cell line MCF7, plectin is a major early *in vivo* substrate for caspase 8 during CD95- and tumor necrosis factor receptor-mediated apoptosis (24). These observations could further verify the *in vivo* degradation of plectin consistent with our findings.

Deficiency or down-regulation of plectin in cells might abolish the function of linking among the cytoskeletal elements, resulting in disorganization of the cytoskeleton and cellular pleomorphism. As similar phenomenon was also demonstrated in plectin knockout cells of mice by other investigators. They showed that in the absence of plectin, the keratin networks are less delicate, the mesh size is increased, and the individual filaments appear bundled and straighter

(35). Another investigator suggested that plectin may play a role in the reorganization of the actin cytoskeleton during death receptor-mediated apoptosis (36). In our opinion, down-regulation of plectin could cause disorganization of IFs and result in the pleomorphism of hepatoma cells. By affecting the organization of the cytoskeleton, plectin deficiency might be an important issue in the tumorigenesis of human HCC.

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Received March 3, 2008

Revised May 2, 2008

Accepted May 9, 2008