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Sequential Decrease in Tight Junctions as Revealed by 7H6 Tight Junction-associated Protein during Rat Hepatocarcinogenesis

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A sequential decrease in the number of hepatocyte tight junctions during the course of rat hepatocarcinogenesis was demonstrated by immunohistochemistry with a new 7H6 monoclonal antibody generated in our laboratory. Semiquantitative analysis by confocal laser scanning microscopy revealed that the expression of 7H6 antigen was reduced in hyperplastic foci, hyperplastic nodules and hepatocellular carcinomas (HCC) to 43%, 28% and 25%, respectively, compared to corresponding normal liver tissues. 7H6 antigen was scarce in HCC with a trabecular pattern, whereas it was expressed intensely at the apical and basolateral membrane of HCC with a glandular pattern. Immunoblot analysis of 7H6 expression in hepatocellular carcinomas showed a decrease roughly coincident with that shown by immunohistochemistry. These results indicated, for the first time, that tight junctions decrease progressively during carcinogenesis, leading to disruption of cellular polarity and cellular adhesiveness.

Key words: Tight junction — Hepatocarcinogenesis — Confocal laser scanning microscopy

The tight junction acts as an intercellular junctional apparatus and also plays a crucial role in maintaining the polarity of cell.[1-3] Since loss of cullular polarity and disruption of cell-cell adhesion are characteristic features of cancer cells,[4] it seems of great potential value to observe the changes in the tight junctions during carcinogenesis. A recent study has shown that the tight junction protein ZO-1[5] is homologous to the *Drosophila* disc-large tumor suppressor protein of septate junctions.[6] Since disc-large mutations have been shown to cause the loss of epithelial polarity and neoplastic growth in *Drosophila*,[7] it seems very likely that loss of tight junction expression in epithelial cells may occur during carcinogenesis. In this study, we examined the changes in tight junctions during the course of chemical hepatocarcinogenesis in rats using a novel monoclonal antibody 7H6[8] recently generated in our laboratory.

The results of the present histochemical and semiquantitative study clearly indicated that sequential decrease and aberrant localization of the tight junction occur in association with the progress of hepatocarcinogenesis in rats.

MATERIALS AND METHODS

Animals and induction of liver lesions Male Fischer 344 rats (Charles River Japan Inc., Kanagawa), weighing 150–180 g, were used. The rats were maintained on a basal diet (Oriental Yeast Co., Tokyo) and in rooms with temperature and light control. Liver lesions were induced in the rats according to the Solt-Farber protocol.[9] The rats were initially injected with diethylnitrosamine (DEN) (Wako Pure Chemical Ltd., Osaka) at a dose of 200 mg/kg. Two weeks later, they were fed a diet containing 0.02% 2-acetylaminofluorene (AAF) for 2 weeks and subjected to two-thirds partial hepatectomy for induction of liver lesions. Five or 6 rats each were killed under ether anesthesia at 4 weeks, 6 months and 1 year after the beginning of the treatment. The liver tissues were frozen in liquid nitrogen after brief perfusion with phosphate-buffered saline (PBS) through the portal vein.

Antibodies and immunostaining Details of the monoclonal antibody production and the characterization of the antibody, 7H6, have been reported elsewhere.[8] The culture supernatant of the hybridoma was used for immunostaining. Anti-ZO-1 antibody[5] (R26.4C, Chemicon International Inc., Temecula, CA, USA) was also used for staining the tight junctions. Frozen sections (approximately 6 μm thick) of the liver were mounted on poly-L-lysine-coated slides and fixed in cold acetone for 15 min. Sections were incubated either with 7H6 or R26.4C for 1 h at room temperature. After several washes with PBS, the second antibody, a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (DAKO Japan, Tokyo) or goat anti-rat Ig (DAKO) was diluted to 1:50 with PBS and applied for 1 h. Stained sections were examined with a fluorescence microscope (Nikon, Tokyo). To identify the hyperplastic foci, anti-rat glutathione S-transferase placental form (GST-P) antibody[10] was used as described previously.[11]

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Confocal laser scanning microscopy For semiquantitative analysis of 7H6 antigen expression in each liver lesion, sections stained with 7H6 were examined with a confocal laser scanning fluorescence imaging system, MRC500J (Bio-Rad, Watford, UK). Gain levels were adjusted to 8.0 so that we obtained a range of 0 (black) to 240 (white) levels of grey. Measurements of total pixel intensity of the fluorescence, representing the number of tight junctions per unit area, were performed directly from the monitor.

Gel electrophoresis and immunoblotting Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out based on the discontinuous Tris-glycine system of Laemmli.[12] Whole homogenates of the normal and cancer tissues were mixed in an SDS sample buffer containing protease inhibitors (phenylmethylsulfonyl fluoride 1 m*M*, pepstatin A 1 μg/ml, leupeptin 2 μg/ml, aprotinin 4 μg/ml, antipain 10 μg/ml and soybean trypsin inhibitor 10 μg/ml) and immediately boiled for 3 min. The samples (containing 50 μg total protein per lane) were applied to a polyacrylamide gradient gel (4–20%) for electrophoresis. Part of the gel was stained with Coomassie Brilliant Blue R-250, while the rest was electrophoretically blotted onto nitrocellulose membrane for immunoblot analysis. Protein concentration was determined by a Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA).

The membrane was immunostained by incubation with 7H6 supernatant ($\times$10 dilution) followed by incubation with peroxidase-conjugated goat antiserum to mouse Ig ($\times$1000 dilution, DAKO Japan). The positive bands were detected by the ECL Western blotting detection system (Amersham International PLC, Amersham, UK).

RESULTS

In normal rat liver tissues, the 7H6 antigen was clearly visualized as two parallel lines bordering the bile canaliculi in longitudinal sections or as a pair of dots when tissues were cross-sectioned. The localization of 7H6 antigen along the bile canaliculi was consistent with our previous observation.[8]

Four weeks after the initial DEN injection, a large number of GST-P-positive hyperplastic foci emerged in the liver. The distribution of the 7H6 antigen was no longer uniform and many of the parallel lines along the bile canaliculi partially disappeared in these foci (Fig. 1). To confirm that 7H6 reacts with the tight junction protein, serial liver sections were stained with 7H6 and R26.4C. As shown in Fig. 1, the localizations of the 7H6 antigen and ZO-1 were similar.

Hyperplastic nodules, which compressed the surrounding liver tissue, were observed in the livers at 6 months after DEN treatment. Expression of the 7H6 antigen in these lesions was more irregular than that in the foci. Distended bile canalicular structures were often outlined by 7H6 antigen, but some bile canaliculi showed no reactivity for 7H6 at all (Fig. 3e).

HCC with a trabecular pattern showed a great decrease in expression of 7H6 antigen, which appeared as sparsely distributed spots in the vicinity of the cell mem-

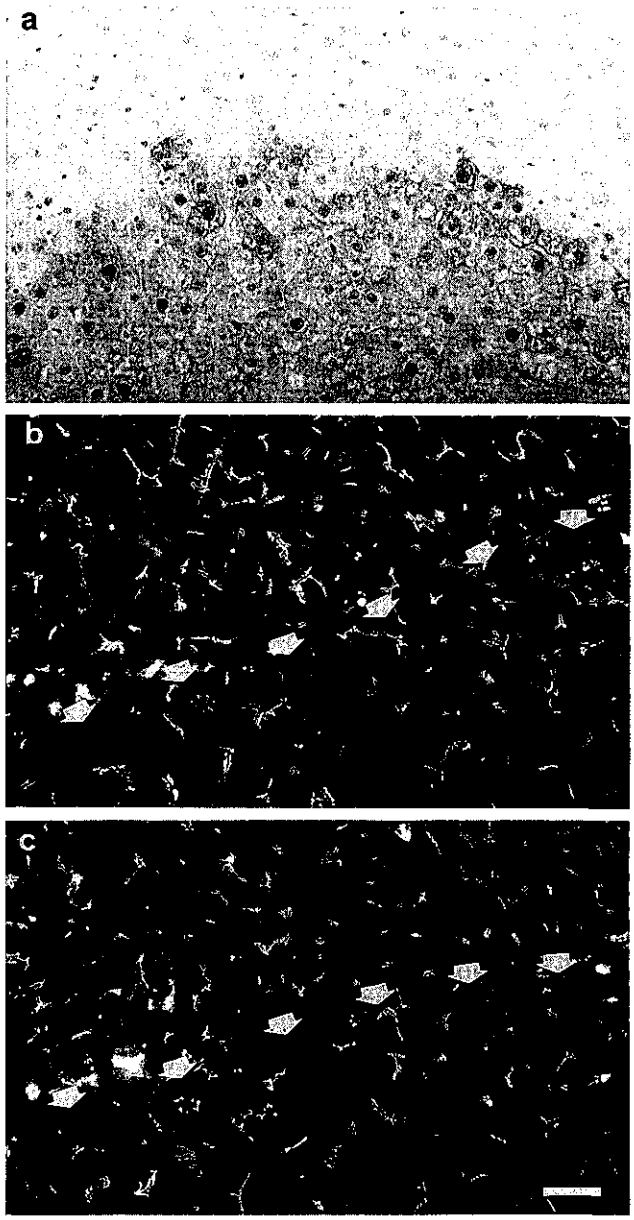

Fig. 1. Localization of tight junctions in GST-P-positive liver cell focus. Serial sections are immunostained with anti-GST-P antibody (a), 7H6 (b) and R26.4C (ZO-1) (c). Bar, 10 μm.

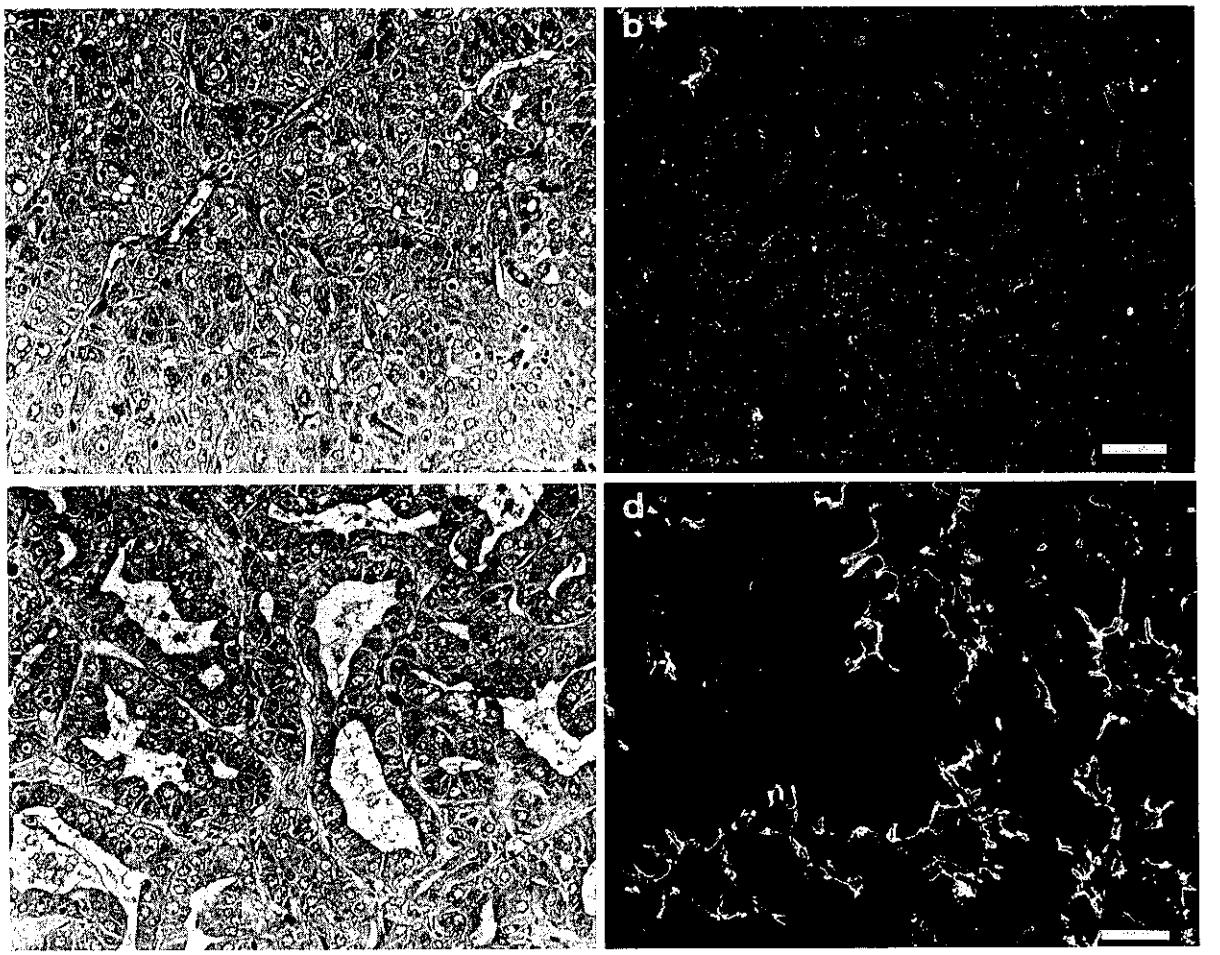

Fig. 2. Difference in localization of 7H6 antigen in hepatocellular carcinomas with a trabecular pattern (a, b), and a pseudoglandular pattern (c, d). Bar, 15 μm. a, c: H & E staining.

brane. On the other hand, HCC with a glandular (acinar) arrangement expressed relatively high levels of 7H6 antigen at the apical and basolateral portions of the glandular structure (Fig. 2).

To evaluate the levels of 7H6 antigen expression in the preneoplastic and neoplastic liver lesions, we carried out a semiquantitative analysis of fluorescence intensity per unit area of liver section with a confocal laser microscope imaging system. Immunofluorescence localization and the corresponding confocal laser microscope images are shown in Fig. 3. We observed a progressive decrease in fluorescence intensity with progression of the liver lesions. The results of the total pixel intensity count for 7H6 fluorescence in the liver lesions are shown in Table I. It was clearly demonstrated that the expression of 7H6 antigen sequentially decreased in the liver lesions during carcinogenesis, though there was no statistically significant difference between that in hyperplastic nodules and that in HCC.

Immunoblot analysis demonstrated the reduction in 7H6 antigen expression in the cancer tissues (Fig. 4). Immunoreactive proteins for 7H6, which were apparently of lower molecular weight than 7H6 antigen, were found in the lanes of cancer tissues.

DISCUSSION

The tight junction is an intercellular junction which plays a role not only in cell-cell adhesion but also in maintaining cellular polarity.[1-3] Recent studies have revealed the presence of several tight junction-associated proteins ZO-1,[5] cingulin,[13] ZO-2[14] and 7H6,[8] though little is known about the mutual association of each protein. It was found very recently that the tight junction

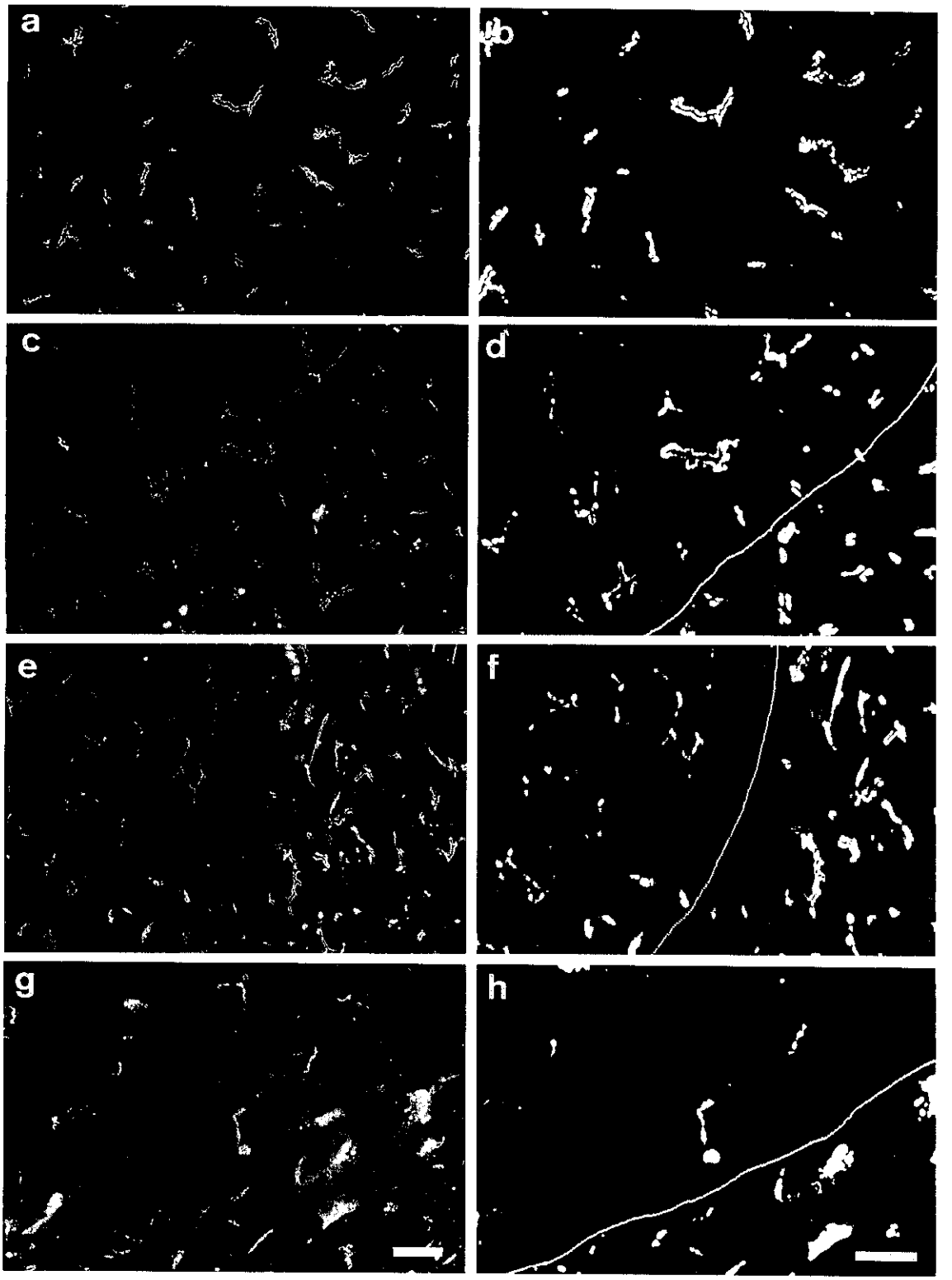

Fig. 3. Changes in localization of 7H6 antigen in the liver lesions that appeared during hepatocarcinogenesis (a, c, e, g) and the corresponding confocal laser scanning microscopic images (b, d, f, h). a, b, normal liver; c, d, liver cell focus; e, f, hyperplastic nodule; g, h, hepatocellular carcinoma. Bar, 20 μm.

Table I. Relative 7H6 Antigen Content in Normal Liver and Liver Lesions

Lesions	No. of lesions examined	Pixel intensity/unit area (%)
Normal liver	8	26209±1155 (100)
Foci	8	11269±3806 (43)
Nodules	5	7338±2134 (28)
HCCs	4	6552±5892 (25)

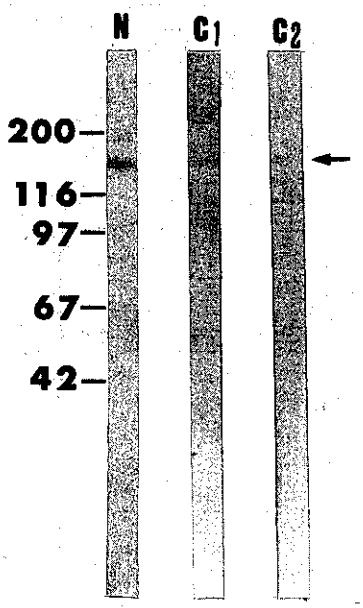

Fig. 4. Immunoblot analysis of 7H6 antigen in normal liver tissue (N) and hepatocellular carcinoma tissues (C_1 and C_2). The arrow indicates a 155 kD band of 7H6 antigen.

protein ZO-1 is homologous to the *Drosophila* disc-large tumor suppressor protein of septate junction.[6] Since disc-large mutation results in loss of epithelial polarity and in neoplastic growth,[7] the role of the tight junction protein of vertebrate cells during carcinogenesis has attracted much attention.

In the present study, we analyzed the changes in tight junctions during the course of chemical hepatocarcinogenesis in rats using the 7H6 anti-tight junction antibody, which was recently established in our laboratory. The results clearly showed that the tight junctions decrease and change their localization in association with the progress of carcinogenesis. The localization of 7H6 antigen was confirmed to be similar to that of ZO-1 in terms of immunohistochemistry in normal and preneoplastic rat liver tissues (Fig. 1).

We attempted a semiquantitative analysis of the tight junctions using confocal laser scanning microscopy; the results showed a sequential decrease in tight junctions with progression of liver lesions to hepatocellular carcinomas (Table I).

The reason why there was no statistically significant difference between the expression of 7H6 antigen in hyperplastic nodules and that in HCC is probably that HCC with a glandular pattern expressed more intense immunoreactivity to 7H6 than the normal rat liver, though the localization was abnormal. This suggests that the level of expressed tight junction protein may be related to some aspect of tumor morphology.

The semiquantitative data from confocal laser microscopic analysis are supported by the results of the immunoblot analysis (Fig. 4), which showed that the expression of the 155 kD 7H6 antigen in HCC is reduced compared with that in normal liver tissue, though the nature of the low-molecular-weight bands seen in the lanes of carcinoma tissue was not evident.

The results of the present study were consistent with the results of Pauli *et al.*,[15] who demonstrated that the strands of the tight junction seen in freeze-fracture replicas decreased during experimental bladder carcinogenesis, but were inconsistent with those of Citi *et al.*,[16] in that the expression of the tight junction protein cingulin is higher in human colon carcinoma tissues than in normal colon. This probably stems from difference in carcinoma tissues studied. As discussed above, the expression of 7H6 antigen in HCC with a glandular (acinar) pattern was much higher than that in HCC with a trabecular pattern (Fig. 2). It is of interest to study whether carcinomas with a differentiated gland-like arrangement maintain relatively high levels of tight junction protein expression or not.

With respect to the disrupted cell-cell adhesion in cancer cells, involvement of other junctional apparatuses must be considered.[17-19] Our previous study on the expression of gap junction proteins CX32 and CX26 during hepatocarcinogenesis in rats revealed that CX32 decreased sequentially in liver lesions, whereas CX26 decreased only in cancer tissues.[11]

In conclusion, the results of this study clearly showed that sequential impairment in tight junctions occurs during the carcinogenic process, resulting finally in the acquisition of malignant phenotypes, such as loss of cellular polarity and cellular adhesiveness, which are ubiquitously seen in cancer cells.

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