

# Are gap junction gene connexins 26, 32 and 43 of prognostic values in hepatocellular carcinoma? A prospective study

I-Shyan Sheen, Kuo-Shyang Jeng, Po-Chuan Wang, Shou-Chuan Shih, Wen-Hsing Chang, Horng-Yuan Wang, Chung-Chu Chen, Li-Rung Shyung

**I-Shyan Sheen**, Division of Hepatogastroenterology, Chang Gung Memorial Hospital, Taipei, Taiwan, China

**Kuo-Shyang Jeng**, Departments of Surgery, Mackay Memorial Hospital, Taipei, Taiwan, China

**Po-Chuan Wang, Shou-Chuan Shih, Wen-Hsing Chang, Horng-Yuan Wang, Chung-Chu Chen, Li-Rung Shyung**, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan, China

**Kuo-Shyang Jeng**, Mackay Junior School of Nursing, Taipei, Taiwan, China

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**Correspondence to:** Kuo-Shyang Jeng, M.D., F.A.C.S., Department of Surgery, Mackay Memorial Hospital, No.92, Sec 2, Chung-San North Road, Taipei, Taiwan, China. issheen.jks@msa.hinet.net

**Telephone:** +886-2-25433535 **Fax:** +886-2-27065704

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## Abstract

**AIM:** To investigate the prognostic value of the expression of connexin (Cx) 26, 32 and 43 messenger RNA (mRNA) in hepatocellular carcinoma (HCC) tissues.

**METHODS:** Using a reverse-transcriptase polymerase chain reaction (RT-PCR), Cx 26, Cx 32 and Cx 43 mRNAs were determined in the liver tissues of 15 controls and in HCC tissues of 25 patients undergoing curative hepatic resection. The patients were followed up clinically.

**RESULTS:** Cx 26 and Cx 32 mRNAs were significantly lower in HCC tissues compared with controls (both  $P < 0.01$ ). By multivariate analysis, a lower level of Cx 26 and Cx 32 mRNA correlated significantly with a risk of HCC recurrence ( $P = 0.033$ ) and recurrence-related mortality ( $P = 0.031$ ,  $P = 0.031$ ). Cx 43 mRNA was higher in HCC tissues compared with controls but did not correlate with postoperative recurrence or recurrence-related mortality. Other significant predictors of HCC recurrence included cellular dedifferentiation ( $P = 0.033$ ), less encapsulation ( $P = 0.050$ ), vascular permeation ( $P = 0.046$ ), and daughter nodules ( $P = 0.046$ ). Significant variables related to recurrence-related mortality consisted of cell dedifferentiation ( $P = 0.031$ ), vascular permeation ( $P = 0.048$ ), and daughter nodules ( $P = 0.048$ ). The levels of Cx 26 and Cx 32 mRNAs correlated significantly with cell differentiation ( $P = 0.031$ ).

**CONCLUSION:** A low expression of Cx 26 and Cx 32 mRNAs in HCC tissues is predictive of postoperative recurrence of HCCs.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of death in Taiwan and many Asian countries, where hepatitis B and C have a high prevalence. During the last 10 years, efforts have been made worldwide toward early detection and curative surgical resection of HCC. However, despite diagnostic and therapeutic advances, postoperative recurrence is still common<sup>[1-7]</sup>. Predicting which patients are likely to have a recurrence is a challenge.

Gap junctional intercellular communication (GJIC) occurs via intercellular hemichannels, which are formed by six basic protein subunits expressed in neighboring cells. These subunits are called connexins (Cx)<sup>[8]</sup>. Six connexins locate around a central pore, through which adjacent cells directly exchange low weight molecules providing information or energy. GJIC plays important roles in embryonic development, metabolic cooperation, growth control, cell proliferation, and differentiation<sup>[9-12]</sup>. Many authors believed that disorders of GJIC were involved in carcinogenesis<sup>[12-21]</sup>.

Connexin 32 (Cx 32) and connexin 26 (Cx 26) are the major gap junction proteins in hepatocytes. Some authors have reported that Cx 32 and Cx 43 mRNAs and their proteins were significantly decreased in HCC tissues and cell lines. In contrast, Cx 43 protein was increased in the hepatoma cell line SMMC-7721<sup>[22-25]</sup>.

To the best of our knowledge, little is known about the prognostic significance of connexin mRNA expression in the prediction of postresection recurrence of HCCs. We conducted this prospective study to investigate the correlation between connexin mRNA expression in HCC tissues and postoperative recurrence of the tumor.

## MATERIALS AND METHODS

### Study population

From July 1999 to August 2000, 25 (18 men and 7 women, with a mean age of  $56.4 \pm 12.6$  years) of 34 consecutive patients with HCC undergoing curative hepatectomy were enrolled in this prospective study. The 9 patients who were excluded had a previous hepatectomy, preoperative neoadjuvant ethanol injection, hepatic arterial chemoembolization (TACE), or surgical morbidity. The surgical procedures performed included 23 major resections (6 extended right lobectomies, 10 right lobectomies, 3 left lobectomies and 4 double segmentectomies) and 2 minor resections (single segmentectomy). Clinical details were available from medical records on all patients (Table 1). Connexins were assayed in both cancerous and noncancerous liver tissues obtained at the time of resection. A control group including 5 healthy volunteers, 5 individuals with chronic active hepatitis without HCCs, and 5 individuals with liver cirrhosis but no HCC also underwent liver biopsies during exploratory laparotomies for other reasons. The surgically removed liver samples were immediately transferred to the pathology laboratory, dissected, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until RNA extraction. Frozen and formalin-fixed samples of the dissected tumor and surrounding tissues were also studied by routine pathology. No obvious ischemic change

was observed in the surrounding tissues, suggesting that the time between removal and freezing of samples did not result in problematic artifacts.

After discharge, the patients were assessed regularly to detect tumor recurrence with abdominal ultrasonography (every 2-3 mo during the first 5 years, then every 4-6 mo thereafter), serum alpha fetoprotein (AFP) and liver biochemistry (every 2 mo during the first 2 years, then every 4 mo during the following 3 years, and every 6 mo thereafter), abdominal computed tomography (CT) (every 6 mo during the first 5 years, then annually), and chest X-ray and bone scans (every 6 mo). Hepatic arteriography was obtained if the other studies suggested possible recurrences. A recurrence was defined as detection of a tumor on any imaging study.

Clinicopathologic parameters analyzed included sex (male vs female), age, presence of liver cirrhosis, hepatitis B virus (HBV) infection (hepatitis B surface antigen-positive), hepatitis C virus (HCV) infection (anti-hepatitis C virus antibody-positive), serum AFP level (<20 ng/mL vs 20 to 1 000 ng/mL vs >1 000 ng/mL), cirrhosis, Child-Pugh class of liver functional reserve (A vs B), tumor size (<3 cm vs 3 to 10 cm vs >10 cm in diameter), tumor encapsulation (complete vs incomplete or absent), presence of daughter nodules, vascular permeation (including vascular invasion and/or tumor thrombi in either the portal or hepatic vein), and cell differentiation grade (Edmondson and Steiner grades I to IV) (Table 1).

**Table 1** Demographic, clinical and pathological variables in patients with HCC undergoing curative resections (n = 25)

Variables	Patients (n, %)
Age (mean, yr)	56.4±12.6
Male	18 (72)
Child- Pugh class A	
Serum AFP <20 ng/mL	3 (12)
20-10 <sup>3</sup> ng/mL	10 (40)
>10 <sup>3</sup> ng/mL	12 (48)
HBsAg (+)	12 (48)
Anti-HCV (+)	10 (40)
Diameter of HCC <3 cm	2 (8)
3-10 cm	10 (40)
>10 cm	13 (52)
Cirrhosis	20 (80)
Edmondson-Steiner's grade I	11 (44)
grade II	
grade III	14 (56)
grade IV	
Complete capsule	9 (36)
Vascular permeation	14 (56)
Daughter nodules	13 (52)
Multinodular HCC	5 (20)
Tumor necrosis	5 (20)
Tumor hemorrhage	1 (4)

AFP: serum alpha fetoprotein; HBsAg (+): positive hepatitis B surface antigen; Anti-HCV(+): positive hepatitis C virus antibody; Edmondson Steiner grade: differentiation grade.

#### Detection of connexin mRNA in liver tissues

We chose measuring connexin mRNA instead of measuring protein because RT-PCR was thought to provide better quantification method than immunohistochemistry.

**Extraction of RNA** Resected tissues were completely homogenized in 1 mL of RNA-bee™ (Tel-Test, Protech Technology Enterprises

Co., Ltd, Friendswood, TX, USA), 0.2 mL chloroform was added, and the mixture was shaken vigorously for 15 to 30 s. The samples were stored on ice for 5 min and then centrifuged at 12 000 g for 15 min. The supernatant was transferred to a new 1.5 mL Eppendorf tube, precipitated with 0.5 mL of isopropanol for 5 min at 4 °C, and centrifuged at 12 000 g for 5 min at 4 °C. The supernatant was removed and the RNA pellet was washed with 1 mL of isopropanol and shaken to dislodge the pellet from the side of the tube. The pellet was centrifuged again at 12 000 g for 5 min at 4 °C, the supernatant was removed, and the RNA pellet was washed once again with 750 mL/L ethanol. The pellet was resuspended in at least 1 mL of 750 mL/L ethanol and centrifuged at 7 500 r/min for 5 min at 4 °C, after which the ethanol was carefully removed. The RNA was allowed to dry in air and then dissolved in DEPC-H<sub>2</sub>O (50 to 100 µL) and stored at -80 °C.

**Reverse transcription** The RNA sample was heated at 55 °C for 10 min and chilled on ice. The following reagents were then added: (1) 4 µL 5×RT buffer containing Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl<sub>2</sub>, and 10 mmol/L DTT (dithiothreitol), (2) 3 µL 10 mmol/L dNTP (deoxyribonucleoside triphosphate), (3) 1.6 µL Oligo-d(T)<sub>18</sub> and 0.4 µL random hexamers (N) 6 (1 µg/µL), (4) 0.5 µL RNase inhibitor (40 U/µL), (5) 3 µL 25 mmol/L MnCl<sub>2</sub>, (6) 6 µL RNA in DEPC-H<sub>2</sub>O, and (7) 0.5 µL DEPC-H<sub>2</sub>O. The mixture was incubated at 70 °C for 2 min and then chilled to 23 °C to anneal the primer to the RNA. We then added 1 µL of M-MLV RTase (moloney murine leukemia virus reverse transcriptase, 200 U/µL, Promega) and the mixture was incubated for 10 min at 23 °C followed by 60 min at 40 °C. It was heated at 94 °C for 5 min, then chilled on ice. The cDNA was stored -20 °C.

**Amplification of connexins 26, 32, 43, and GAPDH cDNA by PCR** First-strand cDNA synthesis was carried out using 2 µg of total RNA purified from 50 mg tissue. Reverse transcription was performed in a final volume of 20 µL containing 2 µg of random hexamer (Gene Tek Bioscience Inc., Taipei), and 1.5 mmol/L each of dATP, dCTP, dGTP, and dTTP. Each reaction mixture was incubated for 8 min at 23 °C with 20 U of rRNasin (RNase inhibitor; Promega, Madison, WI) followed by incubation with 200 U of moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Paisley, UK) for 60 min at 40 °C, followed by 5 min at 94 °C. PCR was performed in a final volume of 50 µL, by using 2 µL of cDNA solution in a mixture containing 0.4 mmol/L deoxynucleotide triphosphates, 40 pmol/L of both sense and antisense oligonucleotide primers for Cx 26, Cx 32 or Cx 43, 2.5 mmol/L MgCl<sub>2</sub>, 2.5U of Taq DNA polymerase (Promega) and 5 µL of 10x Taq DNA polymerase reaction buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl (pH9.0), 10 g/L Triton-X-100). PCR primer sequences of the sense and antisense oligonucleotides for Cx 26, Cx 32 and Cx 43 as well as the direction, size and reaction conditions are shown in Table 2. For example, Cx 26 anti-sense oligonucleotide (5'-CCGAAGTTCATGAAGGGAGA GAT-3') and its related scrambled sequence (5'-GGTCTTTTGG ACTTCCCTGAGCA -3') were synthesized (by Sigma-Genosys Ltd, Woodlands, TX, USA). GAPDH was used as a control, with the quantities of the other mRNA products reported as their intensity as a fraction of that of GAPDH mRNA. To eliminate any possibility of genomic DNA contamination, PCR amplification reaction was carried out on each sample from the RNA extraction. As another internal contamination control, PCR amplification was also carried out on a sample of reaction mixture in the absence of cDNA.

The intensity of bands was measured using Fujifilm Science Lab 98 (Image Gauge V3.12). The sensitivity of our assay was assessed using human hepatocytes. A HepG2 (hepatoblastoma) cell line served as a positive control for connexin mRNA expression. For negative controls, we used EDTA-treated water (filtered and vaporized).

### Statistical analysis

A statistical software package (SPSS for windows, version 8.0, Chicago, Illinois) was used. Student's *t*-test was used to analyze continuous variables and chi-square or Fisher's exact test was used for categorical variables. A Cox proportional hazards model was used for multivariate stepwise analysis to identify the significant factors predicting recurrence and mortality. Significance was taken as a *P* value <0.05.

**Table 2** Nucleotide sequences of the primer sets and specific oligonucleotide probes to each type of connexin 5'-noncoding mRNA

Type of connexin mRNA	Primer probes	Nucleotide sequences
Cx 26	Sense	5' CCGAAGTTCATGAAGGGAGAGAT
	Antisense	5' GGTCTTTTGGACTTCCCTGAGCA
Cx 32	Sense	5' CTGCTCTACCCGGGCTATGC
	Antisense	5' CAGGCTGAGCATCGGTGCTCTT
Cx 43	Sense	5' TACCATGCGACCAGTGGTGCGCT
	Antisense	5'GAATTCTGGTTATCATCGTCGGGGA

## RESULTS

### RT-PCR analysis of connexin transcripts in liver and HCC tissues

The mean values of Cx 26 mRNA and Cx 32 mRNA in HCC tissues were significantly lower than those in the control samples ( $P < 0.01$ ,  $P < 0.01$  respectively). There was no significant difference in Cx 43 mRNA between cancerous and control tissues (Table 3).

**Table 3** Correlation of Cx 26, Cx 32, and Cx 43 mRNAs in cancerous and noncancerous liver tissues of 25 patients with HCC and liver tissues of 15 control patients

Tissues	mRNA (mean value)		
	Connexin 26	Connexin 32	Connexin 43
HCC patients			
Cancerous liver <sup>1</sup>	0.745	0.775	0.241
Controls <sup>2</sup>	1.205	1.225	0.100

$P < 0.01$  for connexin 26 mRNA;  $P < 0.01$  for connexin 32 mRNA;  $P > 0.05$  for connexin 43 mRNA.

### Correlation of connexin mRNA expression and tumor recurrence

Patients were followed up for a median of 45 mo (range 38 to 51 mo). Fourteen (56%) had clinically detectable recurrences, of whom 6 died. A low Cx 26 mRNA correlated significantly with tumor recurrences by both univariate ( $P < 0.001$ ,  $P < 0.001$ ) and multivariate analyses ( $P = 0.033$ ,  $P = 0.033$ ). No such correlation was found with Cx 43 mRNA. By multivariate analyses, other significant predictors of recurrences included poor cell differentiation ( $P = 0.033$ ), less encapsulation ( $P = 0.050$ ), vascular permeation ( $P = 0.046$ ) and the presence of daughter nodules ( $P = 0.046$ ) (Table 4).

### Correlation of connexin mRNA expression and recurrence-related death

A low level of Cx 26 mRNA and Cx 32 mRNA in HCC tissues was significantly correlated with death from recurrent tumors by both univariate ( $P < 0.001$ ,  $P < 0.001$ ) and multivariate analyses ( $P = 0.031$ ,  $P = 0.031$ ) (Table 5). By multivariate analyses, poor cell differentiation, vascular permeation, and daughter nodules correlated significantly with mortality ( $P = 0.031$ , 0.048, and 0.048

respectively), and less encapsulation correlated with mortality with a marginal significance ( $P = 0.053$ ).

**Table 4** Predictors of HCC recurrence

Variable	<i>P</i> values	
	UV	MV
Sex	1.000	-
Age (yr)	0.030	0.071
Tumor diameter (<3 cm, >10 cm)	0.250	-
Liver cirrhosis	0.009	0.067
Child-Pugh class	0.528	-
Serum AFP	0.744	-
HBsAg (+)	1.000	-
Anti-HCV (+)	1.000	-
Edmondson Steiner grade	<0.001	0.033
Capsule	<0.001	0.050
Vascular permeation	<0.001	0.046
Daughter nodules	<0.001	0.046
Tumor necrosis	0.046	-
Tumor hemorrhage	0.046	-
Connexin 26 mRNA	<0.001	0.033
Connexin 32 mRNA	<0.001	0.033
Connexin 43 mRNA	0.280	0.071

UV: univariate analysis; MV: multivariate analysis; AFP: serum alpha fetoprotein; HBsAg(+): positive hepatitis B surface antigen; Anti-HCV(+): positive hepatitis C virus antibody; Edmondson Steiner grade: differentiation grades I, II vs III, IV.

**Table 5** Correlation between clinical and pathological variables and recurrence-related mortality

Parameters	<i>P</i> values	
	UV	MV
Sex	0.888	-
Age (yr)	0.005	0.356
Tumor diameter (<3 cm, >10 cm)	0.324	-
Liver cirrhosis	0.030	0.324
Child-Pugh class	0.548	-
HBsAg (+)	0.956	-
Anti-HCV (+)	0.785	-
Edmondson Steiner grade	0.000	0.031
Capsule	0.000	0.053
Vascular permeation	0.000	0.048
Daughter nodules	0.039	0.048
Multinodular HCC	0.007	0.324
Tumor necrosis	0.373	-
Tumor hemorrhage	0.356	-
Connexin 26 mRNA	<0.001	0.031
Connexin 32 mRNA	<0.001	0.031
Connexin 43 mRNA	0.461	-

UV: univariate analysis; MV: multivariate analysis; AFP: serum alpha fetoprotein; HBsAg (+): positive hepatitis B surface antigen; Anti-HCV(+): positive hepatitis C virus antibody; Edmondson Steiner grade: differentiation grades I, II vs III, IV; n.s.: not significant.

### Correlation between connexin mRNA expression and clinical and histopathologic features

Cx 26 and Cx 32 mRNAs were significantly correlated with cell differentiation but not with gender, age, serum AFP level, chronic HBV or HCV carriage, tumor size, coexisting cirrhosis, encapsulation, vascular permeation, daughter nodules, tumor necrosis, or tumor hemorrhage (all  $P > 0.05$ ).

### DISCUSSION

Our study showed that a low value of Cx 26 and Cx 32 mRNAs in resected HCC tissues was significantly associated with an increased risk of postoperative recurrences and death. While increased Cx 43 mRNA was not significantly predictive of outcomes.

Most tumors studied had a reduction in either homologous or heterologous GJIC. This presumably contributed to neoplastic progression by allowing tumor cells to escape intercellular signals involved in regulation of cell proliferation, differentiation, and apoptosis<sup>[8,10,12,13]</sup>. Cx 32 and Cx 26, the components of hepatocyte gap junctions, were also found in a variety of other cell types. Cx 43 (or  $\alpha 1$ ) was prominent in the liver capsule and between other types of liver cells, including Ito (fat-storing) cells, cholangiocytes, and endothelial cells lining the venules. We speculated that, because the Cx 32 and Cx 26 genes were expressed in normal liver tissues, they might serve as tumor suppressor genes. Other authors have suggested Cx 32 and Cx 43 for this role<sup>[16]</sup>. Eghbali *et al.*<sup>[18]</sup> found that transfection of tumor cells with Cx 32 cDNA retarded tumor growth *in vivo*. They demonstrated that the growth rate of tumor cells correlated negatively with the strength of intercellular communication.

Since Lowenstein *et al.*<sup>[12]</sup> used electrophysiologic techniques to demonstrate a lack of communication between rat liver cancer cells, an interest has been attracted to changes in cell-to-cell communications in carcinoma tissues. In studies of rat hepatocarcinogenesis, several laboratories have found that a significant decrease in the major liver gap junction protein Cx 32 at the mRNA or protein level occurred in preneoplastic nodules and HCCs induced by chemicals<sup>[14,17,19,21]</sup>. Loss of GJIC due to down-regulated expression of Cx genes appears to be an important event in cell transformation and is associated with uncontrolled tumor cell growth. Transformed cells *in vitro* and *in vivo* had a decreased GJIC capacity between themselves or with surrounding normal cells<sup>[18,20]</sup>. Disruption of GJIC activity could contribute to the multi-step process of carcinogenesis.

Oyamada *et al.*<sup>[25]</sup> reported that GJIC was altered in human HCCs by molecular mechanisms different from those in rat hepatocarcinogenesis. It is possible that defects in post-translational processing of Cx 32 and Cx 43 proteins may be an obstacle in their transportation to cell membranes. Some studies suggested that multiple mechanisms could likely contribute to block of GJIC, including decreased expressions of gap junction proteins and abnormal pathways of signal transduction resulted from decreased levels of intracellular  $Ca^{2+}$ , phosphorylation of the tyrosine of Cx proteins<sup>[19]</sup>. Other potential mechanisms included control of cell recognition and gating of established gap junctions<sup>[19,20,24]</sup>.

Different authors have proposed various mechanisms by which changes in connexin mRNA might contribute to the biological behaviors of HCC. These include rapid proliferation of tumor cells, changes in the interaction between host and tumors, aberrant localization of connexins, and change in connexin expression during tumor differentiation. Whether the rapid growth of preneoplastic tissues is the cause or the effect of a decrease in connexin mRNA remains unknown.

Some authors have found a great decrease in the number of gap junctions after partial hepatectomy. A reciprocal correlation between the expression of Cx32 and the mitotic activities of hepatocytes during liver regeneration was noted. Based on their findings of a significant reduction of Cx 32 expression in S-phase cells, it seems that quantitative changes in gap junction expression may play an important role in the control of proliferation of liver cells. Since neoplastic liver tissues had a higher proliferation rate than the surrounding tissues, it might have fewer gap junctions. It was found that the partial loss of gap junctions provided a selective advantage for those preneoplastic liver cells which developed into rapidly proliferating tumor cells. It is possible that preneoplastic cells with a reduced number of gap junctions might have an increased capability of proliferating and are therefore more likely to develop into HCC<sup>[14]</sup>.

With regard to changes in the interaction between host and tumors, Krutovskikh *et al.*<sup>[24]</sup> highlighted the role such an interaction might play in natural host resistance against neoplastic growth, with an emphasis on the underlying mechanisms of both connexin function and impairment.

Some other authors attributed the possible contribution of connexins to aberrant localization of the gap junction proteins. Some studies showed that the levels of Cx 32 and Cx 43 mRNAs were not decreased in HCCs compared with normal liver tissues, but the proteins were aberrantly located in HCCs<sup>[25]</sup>. Krutovskikh *et al.*<sup>[26]</sup> thought abnormal location of Cx 32 was more important than its translational dysregulation. Omori *et al.*<sup>[17]</sup> drew a similar conclusion regarding Cx 32.

Another mechanism leading to a loss of heterologous host-cancer cell coupling is a change in the expression of connexin species in tumors during differentiation. According to Markert *et al.*<sup>[27]</sup> three important characteristics of cell differentiation were the rate of cell division, the adhesive properties of the cell membrane that determine the capacity of the cell of migrating or metastasizing, and specific patterns of cell metabolism. Connexins are likely related to adhesive properties. An altered pattern of adhesion molecules on the surface of tumorigenic hepatocytes may influence the distribution of gap junctions in neoplastic tissues.

A characteristic feature of a cancerous phenotype is dedifferentiation. In some tissues, cells at different stages of differentiation express different sets of connexin proteins. HCC cells may express the same connexins as surrounding normal hepatocytes and therefore adhere well to each other. However, because of the inability of Cx 32 hemichannels expressed by normal hepatocytes to assemble functional gap junctional channels with Cx 43 hemichannels in neoplastic cells, they failed to communicate with each other<sup>[28,29]</sup>.

The high recurrence rate after resection is the main cause of the poor outcomes of HCC. Variables correlated with tumor recurrence included high serum AFP, hepatitis, vascular permeation, grade of cell differentiation, infiltration or absence of capsule, tumor size, coexisting cirrhosis, presence of daughter nodules, and multiple lesions<sup>[11-7, 30-33]</sup>. According to our study, connexin was significantly correlated with the grade of cell differentiation. In our series, tumors with higher levels of Cx26 and Cx32 mRNAs, that were closer to normal, were more likely to be well-differentiated (Edmondson and Steiner grade I) and less likely to recur than those tumors of grade II to IV. The association between the grade of anaplasia and connexin positivity also varied in other reports. We attribute these differences to the possibility that different histologic grades may coexist within a particular HCC. Additionally, the regulation of connexin may be complex, particularly at different grades of dedifferentiation.

There is also discrepancy in the expression of Cx 43 in human HCCs. Ma *et al.*<sup>[34]</sup> suggested that decreased expression of Cx 32 and Cx 43 might be closely related to liver carcinogenesis.

We found a similar decrease in Cx32 mRNA, but Cx 43 mRNA was increased. The discrepancy might be due to a difference in study samples and examination methods, as well as the background liver diseases. Furthermore, it should be noted that the benign liver tissue surrounding HCC was not normal. Oyamada *et al.*<sup>[25]</sup> studied 6 patients with HCC and found various abnormalities in the surrounding tissues, including cholestasis in 2, fatty liver in 1, chronic persistent hepatitis in 1, cirrhosis in 1, and possible hemochromatosis in 1. In our 25 patients, we found a different distribution of background liver diseases, including cirrhosis in 20 (80%), hepatitis B virus hepatitis in 12 (48%) and hepatitis C virus hepatitis in 10 (40%); 3 patients had both hepatitis B and C hepatitis. We used RT-PCR and Northern and Southern blot analyses. Oyamada *et al.*<sup>[25]</sup> found no decrease in Cx 32 mRNA, while the level of Cx 43 mRNA was higher than that in the surrounding nontumorous tissues but without any amplification of Cx 43 gene. They attributed the increased expression of Cx 43 to a large amount of connective tissues in HCC.

However, another possible explanation for elevated expression of Cx 43 is the stem cell concept of tumor cell origins. This theory proposes that the target cells in carcinogenesis are stem cells present in normal tissues. Assuming that a stem cell that expresses Cx 43 is the target cell in human hepatocarcinogenesis, the resulting neoplasm would be expected to express connexin 43. The mechanism of switching on the Cx 43 gene in human HCC remains to be investigated, which may include DNA methylation, a stabilization of RNA, or positive or negative trans-acting factors<sup>[25]</sup>.

Carcinogenesis is believed to result from block, arrest, or derangement in differentiation. Based on this concept, we could explain the increased expression of Cx 43 by the appearance of isozymes, for example, aldolase and  $\gamma$ -glutamyl transpeptidase, in human HCC.

We proposed that not all HCCs contained connexin mutations, in another word, there might be both connexin-dependent and connexin-independent pathways leading to liver cancer. The selective advantage conferred on liver cells by a mutant connexin gene seems to be significant in later steps of tumorigenesis, after the accumulation of additional genetic changes. The identification of genetic alterations related to connexin changes remains a considerable challenge in the field of liver cancer research.

Surgery remains the best possible therapy for patients with HCC. Examination of connexin mRNA in HCC tissues may provide information about the risk of postoperative recurrences, and our results support this hypothesis. Addition of neoadjuvant therapy after surgery, might be considered for the risk patients. Furthermore, serial measurement of circulating connexin mRNAs after surgery to monitor the effects of therapy or screen for recurrences needs further investigation.

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