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• LIVER CANCER •

Do the expressions of gap junction gene connexin messenger RNA in noncancerous liver remnants of patients with hepatocellular carcinoma correlate with postoperative recurrences?

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Abstract

AIM: To investigate whether the changes of gap junction gene connexin messenger RNA in the noncancerous liver tissue of patients with hepatocellular carcinoma (HCC) could play a significant role in its postresection recurrence.

METHODS: Seventy-nine consecutive patients having undergone curative resection for HCC entered this study. Using a reverse-transcription polymerase chain reaction (RT-PCR)-based assay, connexin (Cx) 26, connexin (Cx) 32 and connexin (Cx) 43 mRNAs were determined prospectively in noncancerous liver tissues from these 79 patients and in the liver tissues from 15 controls. The correlations between connexin mRNA expression and the clinicopathological variables and outcomes (tumor recurrence and recurrence related mortality) were studied.

RESULTS: Compared with liver tissues of control patients, the expression of Cx 32 mRNA in noncancerous liver tissues was significantly lower (mean: 0.715 *vs* control 1.225, $P<0.01$), whereas the decreased Cx 26 mRNA (mean: 0.700 *vs* of control 1.205, $P>0.05$) and increased Cx 43 mRNA (mean: 0.241 *vs* control 0.100, $P>0.05$) had no statistical significance. We defined the value of Cx 32 mRNA or Cx 26 mRNA below 0.800 as a lower value. By multivariate analysis for noncancerous livers, a lower value of Cx 32 mRNA correlated significantly with a risk of HCC recurrence and recurrence-related mortality. The lower value of Cx 26 mRNA did not correlate with recurrence and mortality. The increased value of Cx43 mRNA also did not correlate with postoperative recurrence and recurrence-related mortality. By multivariate analysis, other significant predictors of HCC recurrence included vascular permeation, cellular dedifferentiation, and less encaps-ulation. The other significant parameter of recurrence related mortality was vascular permeation.

CONCLUSION: The decreased expression of Cx 32 mRNA in noncancerous liver tissues plays a significant role in the prediction of postoperative recurrence of HCC.

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Key words: Hepatocellular carcinoma; Gap junctions; Connexins; Local neoplasm recurrences

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors that carries a poor prognosis. Surgery remains the best potentially curative treatment for patients with HCC. The high recurrence rate after resection is one of the main factors that limits the long-term outcome of HCC. However, despite the recent advances in surgery, postoperative recurrence is still common[1-7]. How to predict the prognosis remains a challenging problem to surgeons.

Noncancerous liver tissues from some HCC patients may be at a preneoplastic stage, which may develop postresection recurrence. The association between the shift of γ-glutamyl transpeptidase and the recurrence of HCC has been reported[5].

Gap junctional intercellular communication (GJIC) is performed by intercellular hemichannels, which are formed by six basic protein subunits named connexin (Cx) expressed in neighboring cells[8,9]. GJIC mediated via gap junctions plays important roles in embryonic development, metabolic cooperation, growth control, cell proliferation, cell differentiation, tissue homeostasis, as well as carcinogenesis[10-23]. Uncontrolled tumor cell growth because of the loss of GJIC due to the down-regulated expression of Cx genes appears to be an important event in cell transformation. Transformed cells *in vitro* and *in vivo* having a decreased GJIC capacity among themselves or with surrounding normal cells have been reported[10-13,17].

Connexin 32 (Cx32) and connexin 26 (Cx26) are the major gap junction forming proteins in hepatocytes. Moreover, another gap junction protein, connexin 43 (Cx43) (or α1) is prominent in the liver capsule, and between other liver cell types, including Ito (fat-storing) cells, cholangiocytes, and endothelial cells lining the venules. Some authors reported that Cx32 and Cx26 mRNA and their proteins were significantly decreased in HCC tissues and cell lines whereas expression of Cx43 protein was increased in hepatoma cell line SMMC-7721[8,19,20,24].

To the best of our knowledge, little is known about the prognostic correlation between the changes of connexin mRNA

expression in noncancerous liver tissues and postresection recurrence of HCC. We chose measuring connexin mRNA instead of measuring connexin protein because RT-PCR is thought to provide a more objective quantitification method than immunohistochemistry. We conducted this prospective study to investigate the correlation between connexin mRNA (Cx32, 26, 43 mRNA) expression in noncancerous liver tissues from HCC patients and the development of postoperative recurrence.

MATERIALS AND METHODS

Study population

Seventy-nine patients with HCC who underwent curative hepatectomy at the Department of Surgery, Mackay Memorial Hospital, between January 1997 and December 1998, whose tissue specimens were histopathologically found to have no degeneration or necrosis, were enrolled in this study. The mean age of patients was 56.4±12.6 years (range 16-82 years) with a male to female ratio of 52:27. Clinical details were available from medical records on all patients (Table 1). Surgeries included 73 major resections (38 partial lobectomies,28 lobectomies and 7 extended lobectmies) and 6 minor resections (4 segmentectomies and 2 subsegmentectomy).

Table 1 Characteristics of 79 patients with HCC undergoing curative resection

Variables	No. of patients (%)
Age (mean, yr)	56.4±12.6
Male	52 (65.8)
Cirrhosis	57 (72.2)
Child-Pugh's class A	55 (70.0)
Serum AFP <20 ng/mL	30 (38.0)
20-10^3 ng/mL	29 (36.7)
>10^3 ng/mL	20 (25.3)
HBsAg (+)	60 (57.8)
Anti-HCV (+)	41 (51.9)
Size of HCC <3 cm	24 (30.4)
3-10 cm	25 (31.6)
>10 cm	30 (38.0)
Edmondson-Steiner's grade I	4 (5.1)
grade II	30 (38.0)
grade III	42 (53.2)
grade IV	3 (3.8)
Complete capsule	61 (77.3)
Vascular permeation	56 (70.9)
Daughter nodules	44 (55.7)
Tumor necrosis	55 (70.0)
Tumor hemorrhage	24 (30.4)

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

Both cancerous and noncancerous liver tissues were studied for connexin. A control group including 5 healthy volunteers, 5 individuals with chronic active hepatitis without HCC and 5 individuals with liver cirrhosis without HCC also received liver biopsies for connexin mRNA study during exploratory laparotomy for other reasons. The surgically removed fresh liver samples were immediately transferred to the pathology laboratory, dissected, frozen in liquid nitrogen, and stored at -80 ℃ until RNA extraction. The dissected tumor and surrounding tissues were also studied by pathological examination. No obvious ischemic changes were observed in surrounding liver tissues, suggesting that duration between removal and freezing of samples did not cause problematic artifacts.

Clinicopathological parameters analyzed included sex (male *vs* female), age, presence of liver cirrhosis, hepatitis B virus (HBV) infection (hepatitis B surface antigen), hepatitis C virus (HCV) infection (anti-hepatitis C virus antibody), 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), 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).

After discharge, the patients were assessed regularly to detect HCC 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 other studies suggested possible cancer recurrence. Detection of tumors on any imaging study was defined as recurrence.

Extraction of RNA

We homogenized resected tissues completely in 1 mL of RNA-bee™ (Tel-Test, Protech Technology Enterprises Co., Ltd, Friendswood, TX, USA), added 0.2 mL chloroform, and shook vigorously for 15 to 30 s. We stored the samples on ice for 5 min and then centrifuged them at 12 000 *g* for 15 min. We transferred the supernatant to a new 1.5 mL Eppendorf tube and precipitated the solution with 0.5 mL of isopropanol for 5 min at 4 ℃.We centrifuged the tube at 12 000 *g* for 5 min at 4 ℃ before removing the supernatant and washing the RNA pellet with 1 mL of isopropanol, shaking to dislodge the pellet from the side of the tube. We centrifuged the pellet again at 12 000 *g* for 5 min at 4 ℃, removed the supernatant, and washed the RNA pellet once with 75% ethanol, shaking to dislodge the pellet from the side of the tube. We suspended the pellet in at least 1 mL of 75% ethanol and centrifuged it at 7 500 *g* for 5 min at 4 ℃ before carefully removing the ethanol. The RNA was allowed to air dry and then dissolved in DEPC-H_2O (50 to 100 uL) and stored at -80 ℃.

Reverse transcription

We heated the RNA sample at 55 ℃ for 10 min, chilled it on ice, and then added the following reagents: 4 μL 5× RT buffer containing Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, and 10 mmol/L DTT (dithiothreitol); 3 μL 10 mmol/L dNTP (deoxyribonucleoside triphosphate); 1.6 μL Oligo-d $(T)_{18}$ and 0.4 μL random hexamers (N) 6 (1 ug/uL); 0.5 μL RNase inhibitor (40 units/μL); 3 μL 25 mmol/L $MnCl_2$; 6 μL RNA in DEPC-H_2O; and 0.5 μL DEPC-H_2O. We incubated the mixture at 70 ℃ for 2 min and then chilled it to 23 ℃ to anneal the primer to the RNA. We added 1 μL of Moloney murine leukemia virus reverse transcriptase (M-MLV RTase), 200 units/μL, (Promega) and incubated it for 10 min at 23 ℃ followed by 60 min at 40 ℃. We then heated it at 94 ℃ for 5 min, chilled it on ice, and stored the cDNA at -20 ℃.

Amplification of connexins26, 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 20-μL final volume containing 2 μg of random hexamer (Gene Teks 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 ℃ 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 ℃ followed by 5 min at 94 ℃. 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 of both sense and antisense oligonucleotide primers according to Cx 32, Cx 26 and Cx 43 to be detected, 2.5 mmol/L MgCl2, 2.5 U 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], 1% Triton-X-100). PCR primer sequences of the sense and antisense oligonucleotides for Cx 32, Cx 26 and Cx 43 as well as the direction, size and reaction conditions are shown in Table 2. For example, Cx 32 sense oligonucleotide (5'-CTGCTCTACCCG GGCTATGC-3') and its anti-sense sequence (5'-CAGGCTGAG CATCGGTCGCTCTT-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 a fraction of their intensity compared to GAPDH mRNA. To eliminate any possibility of genomic DNA contamination, PCR amplification reaction was carried out on each sample of 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.

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

Type of connexin mRNA	Primers Probes	Nucleotide sequences
Cx32	Sense	5 CTGCTCTACCCGGGCTATGC
	Antisense	5 CAGGCTGAGCATCGGTCGCTCTT
Cx26	Sense	5 CCGAAGTTCATGAAGGGAGAGAT
	Antisense	5 GGTCTTTTGGACTTCCCTGAGCA
Cx43	Sense	5 TACCATGCGACCAGTGGTGCGCT
	Antisense	5 GAATTCTGGTTATCATCGTCGGGGAA

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 (SPSS for Windows, version 8.0, Chicago, IL) was employed, Student's *t*-test was used to analyze continuous variables and a chi-square test or Fisher's exact test for categorical variables. A Cox proportional hazard model was used for multivariate stepwise analysis to identify significant factors for predicting recurrence and mortality. *P* value <0.05 was considcred statistically significant.

RESULTS

RT-PCR analysis of connexin transcript in liver and HCC tissues

Compared with the mean values of mRNAs of Cx 32, 26 and 43 in livers obtained from 15 controls (1.225, 1.205, 0.100, respectively), the values of Cx 32 mRNA of noncancerous liver tissues from HCC patients (mean: 0.715) were significantly lower ($P<0.01$), whereas lower Cx 26 mRNA (mean: 0.700) and higher Cx 43 mRNA (mean: 0.241) were not found ($P>0.05$).

Correlation between clinical and histopathological features and different connexin mRNA expressions in liver remnants

We defined the value of Cx 32 mRNA or Cx 26 mRNA below 0.800 as a lower value. According to the value of Cx 32 mRNA, we categorized the 79 study patients into group A (lower than 0.800) and group B (higher than 0.800). When the clinicopathological characteristics of primary HCC were compared between the two groups, the difference was statistically significant in poor cellular differentiation ($P = 0.0203$), less encapsulation ($P = 0.0088$), and vascular permeation ($P = 0.0107$) respectively by univariate analysis. The presence of daughter nodules achieved a borderline significance ($P = 0.0527$,Table 3).

Whereas, no significant difference was noted between the two groups in age, gender, tumor size, coexisting cirrhosis, Child-Pugh's class, chronic HBV or HCV carriage, serum AFP level, tumor necrosis, or tumor hemorrhage ($P>0.05$, Table 3).

Table 3 Comparison of characteristics of primary HCC between different levels of connexin 32 mRNA in noncancerous liver tissues

Characteristics	Group A (n = 64,%)	Group B (n = 15,%)	*P*
Age (mean, yr)	52.3	48.8	NS
Male	65.6	66.7	NS
Liver cirrhosis	68.8	73.3	NS
Child- Pugh's class A	76.6	53.3	NS
Tumor size <3 cm	35.9	40.0	
>10 cm	34.4	13.3	NS
HBsAg (+)	53.1	46.6	NS
Anti-HCV (+)	78.1	66.7	NS
Serum AFP <20 ng/mL	21.9	40.0	
>1 000 ng/mL	37.5	40.0	NS
Tumor necrosis	76.6	53.3	NS
Tumor hemorrhage	35.9	40.0	NS
Edmondson-Steiner grade I[a]	1.6	20	0.0203
Capsule incomplete or absent[b]	78.1	40	0.0088
Daughter nodules	60.9	33.3	0.0527
Vascular permeation[c]	76.6	40	0.0107

Low Cx 32 mRNA: <0.800 (group A), high Cx 32 mRNA: ⩾0.800 (group B). *P*: The *P* value of univariate analysis. a, b, and c: the significant variables in multivariate analysis with *P* values of 0.0120, 0.0420 and 0.019 respectively. AFP: alpha-fetoprotein, NS: no statistical significance.

Correlation of connexin mRNA expression and tumor recurrence and recurrence related death

Fifty-five patients (69.6%) had clinically detectable recurrence during the follow-up period (median 58 mo; range 38 to 72 mo), of whom 26 died. A lower Cx 32 mRNA in liver remnant correlated significantly with tumor recurrence both univariately ($P = 0.0107$) (Table 4) and multivariately ($P = 0.0203$) (Table 5). The lower level of Cx 32 mRNA in noncancerous liver tissues significantly correlated with death from recurrence both univariately ($P = 0.0002$) (Table 4) and multivariately ($P = 0.0333$) (Table 5). However, the differences of the recurrence-free interval and the duration of survival between group A and group B did achieve borderline sgnificance ($P = 0.0595$, $P = 0.0620$), respectively (Table 4). The lower Cx 26 mRNA and the increased Cx 43 mRNA had no significant correlation either with recurrence ($P = 0.0880$, $P = 0.0710$, respectively) or mortality ($P = 0.1240$, $P = 0.0866$, respectively) (Table 5).

Table 4 Comparison of recurrence, death, recurrence, free interval and survival between different levels of Cx 32 mRNA in noncancerous liver tissues

Outcome	Group A (n = 64)	Group B (n = 15)	P
Recurrence (number) (%)	49 (76.6)	6 (40.0)	0.0107
Death[1] (number) (%)[2]	25 (39.0)	1 (6.7)	0.0002
Recurrence free interval (median, mo)	8.5	43.0	0.0595
Duration of survival (median, mo)	11.5	41.5	0.0620

Note: Low Cx 32 mRNA: <0.800 (group A), high Cx 32 mRNA: ≥0.800 (group B), Death[1] : patients died of HCC recurrence. 2: percent of recurrence patients.

By multivariate analysis, other significant predictors of recurrence included vascular permeation (P = 0.0002), poor cellular differentiation (P = 0.0203), and less encapsulation (P = 0.0160) (Table 5). Whereas, by multivariate analysis, only vascular permeation significantly correlated with mortality (P<0.0001) (Table 5).

Table 5 Factors influencing tumor recurrence and death of patients in multivariate analysis

Variables	P	OR
Recurrence		
Vascular permeation	0.0002	5.36
Cellular dedifferentiation	0.0203	4.18
Incomplete or absent capsule	0.0160	3.10
Lower Cx 32 mRNA in liver remnant	0.0203	4.18
Lower Cx 26 mRNA in liver remnant	0.0880	2.29
Higher Cx 43 mRNA in liver remnant	0.0710	2.38
Death		
Vascular permeation	<0.0001	8.35
Lower Cx 32 mRNA in liver remnant	0.0333	3.80
Lower Cx 26 mRNA in liver remnant	0.1240	2.10
Higher Cx 43 mRNA in liver remnant	0.0866	2.40

OR: odds ratio; Lower Cx 32 mRNA: value <0.800.

DISCUSSION

Our study showed that compared with control group liver tissues, a lower value of Cx 32 mRNA in the noncancerous liver remnant tissues was significantly associated with an increased risk of postoperative recurrence and disease mortality. The lower value of Cx 26 mRNA and the increased value of Cx 43 mRNA were not significantly predictive of outcomes. The reasons remain unknown.

To explain this discrepancy, we propose the following five possible reasons.

The first is that among the three connexins, Cx 32 gene may be more predominant in tumor suppression. Some studies suggested that Cx32 and Cx26 genes, the specific genes expressing in normal liver tissues, be the potential unmutated tumor suppressor genes though some authors have suggested Cx32 and Cx43 genes[24-26]. When the liver remnants in some patients develop a preneoplastic transformation, a reduction between either homologous or heterologous GJIC, which has been demonstrated in many tumors, may contribute to neoplastic progression by allowing tumor cells to escape from intercellular signals involving regulations of proliferation, differentiation and apoptosis[10-26]. Ma *et al.* found that HCC cells often expressed less connexin, but the mechanisms are unknown[12]. Eghbali *et al*. found that transfection of tumor cells with connexin 32 cDNA could retard tumor growth *in vivo*[25]. Oyamada *et al*. suggested that the molecular mechanism might be different from those in rat hepatocarcinogenesis[19]. However, in rat multistage hepatocarcinogenesis studies, several authors found that a significant decrease in connexin 32 expression at the mRNA or protein level occurred in preneoplastic nodules and HCCs induced by chemicals[24,27].

The second is that though both Cx 32 and Cx 26 are the main connexins in hepatocytes, they have differences. According to Plante *et al*.[28] Cx 32 is about 10 times more abundant than Cx 26 in the liver, the importance of both connexins in promoter-induced tumors may therefore be different. Animal experiments found that Cx32 was involved in tumor promotion by chemicals[29].

The third is that the detailed mechanisms of the discrepancy of their decrease in HCC recurrence is unknown. We attribute it to that the pathways for Cx 32 and Cx 26 in liver may be different. There was a dramatic increase in the presence of Golgi in the case of Cx 32[30]. Sixty percent of Cx 26 present in the gap junction plaques support the possible existence of an alternative trafficking pathway for Cx 26 in liver. The alternative pathway proposed may not contain conventional signal peptide sequences and does not require endoplasmic reticulum (ER)/Golgi postranstational modification. Connexins lack a signal peptide sequence and Cx 26 is not phosphorylated.

The fourth is that Cx 32 may be more important in the shift of host-cancer cell coupling, which may be important in developing recurrence. Krutovskikh V highlighted that the direct intercellular host-tumor interactions may play a role in natural host resistance against neoplastic growth, and emphasized on the underlying connexin function impairment[31]. Among the 3 connexins, the change of Cx 32 may be earlier and more evident.

The fifth is that some authors have previously found that after partial hepatectomy, there was a reciprocal correlation between the expression of connexin and the mitotic activity of hepatocytes during liver regeneration[32]. The significant reduction of Cx32 expression in S-phase cells may play an important role in the control of proliferation in liver. More Cx 32 in the liver[28] may contribute to this significant reduction. Timmen *et al*. found that the partial loss of gap junctions provided a selective advantage for the preneoplastic liver cells to rapidly proliferate into carcinoma cells[20].

In our study, the decreased connexin 32 mRNA in liver remnants significantly correlated with some tumor invasiveness variables[33,34], including the grade of cellular differentiation, less encapsulation, and vascular permeation, which correlate with recurrence. The more invasive the HCC is, the earlier the recurrence in the remnant liver may develop.

Three characteristics during cellular differentiation have been emphasized, namely the rate of cell division, the adhesive properties of cell membranes that influence cells to migrate or metastasize, and specific patterns of cellular metabolism[35]. Among them, connexin may be important in the adhesive properties. An altered pattern of adhesion molecules on the surface of tumorigenic hepatocyte may influence the distribution of gap junctions in preneoplastic tissues.

Among patients with recurrence, 10.9% (6 among 55) patients had no decrease in Cx 32 mRNA. Not all patients revealed connexin mutations, suggesting that there may be both connexin-dependent and connexin-independent pathways leading to liver cancer. The identification of contributing genetic alterations related to connexin changes remains a considerable challenge in the field of liver cancer research.

In conclusion, decreased expression of Cx 32 mRNA in noncancerous liver tissues plays a significant role in the prediction of postoperative recurrence of HCC. Such patients may be the candidates for neoadjuvant therapy after surgery.

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