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World J Gastroenterol 2002;8(1):64-68
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

Expression of gap junction genes connexin 32 and connexin 43 mRNAs and proteins, and their role in hepatocarcinogenesis

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Received 2001-02-08 **Accepted** 2001-03-12

Abstract

AIM: **To investigate the relationship between hepatocarcinogenesis and the expression of connexin32 (cx32), connexin43 (cx43) mRNAs and proteins *in vitro*.**

METHODS: **Gap junction genes cx32 and cx43 mRNA in hepatocellular carcinoma cell lines HHCC, SMMC-7721 and normal liver cell line QZG were detected by in situ hybridization (ISH) with digoxin-labeled cx32, and cx43 cDNA probes. Expression of Cx32 and Cx43 proteins in the cell lines was revealed by indirect immuno-fluorescence and flow cytometry (FCM).**

RESULTS: **Blue positive hybridization signals of cx32 and cx43 mRNAs detected by ISH with cx32 and cx43 cDNA probes respectively were located in cytoplasm of cells of HHCC, SMMC-7721 and QZG. No significant difference of either cx32 mRNA or cx43 mRNA was tested among HHCC, SMMC-7721 and QZG (P= 2.673, HHCC *vs* QZG; P = 1.375, SMMC-7721 *vs* QZG). FCM assay showed that the positive rates of Cx32 protein in HHCC, SMMC-7721 and QZG were 0.7%, 1.7% and 99.0%, and the positive rates of Cx43 protein in HHCC, SMMC-7721 and QZG were 7.3%, 26.5% and 99.1% respectively. Significant differences of both Cx32 and Cx43 protein expression existed between hepatocellular carcinoma cell lines and normal liver cell line (P= 0.0069, HHCC *vs* QZG; P= 0.0087, SMMC-7721 *vs* QZG). Moreover, the fluorescent intensities of Cx32 and Cx43 proteins in HHCC, SMMC-7721 were lower than that in QZG.**

CONCLUSION: **Hepatocellular carcinoma cell lines HHCC and SMMC-7721 exhibited lower positive rates and fluorescent intensities of Cx32, Cx43 proteins compared with that of normal liver cell line QZG. It is suggested that lower expression of both Cx32 and Cx43 proteins in hepatocellular carcinoma cells could play pivotal roles in the hepatocarcinogenesis. Besides, genetic defects of cx32 and cx43 in post-translational processing should be considered.**

Ma XD, Ma X, Sui YF, Wang WL. Expression of gap junction genes connexin 32 and connexin 43 mRNAs and proteins, and their role in hepatocarcinogenesis. *World J Gastroenterol* 2002;8(1):64-68

INTRODUCTION

Carcinoma of the liver is one of the most common malignant tumors which seriously threatens to human life[1-14]. About 90% of carcinoma of the liver are hepatocellular carcinoma (HCC)[15-19]. In each year, more than 250 thousands of new cases of HCC were reported around the world, among which 110 000 cases (44.7%) occurred in China[20-24]. Due to the difficulty in early diagnosis and treatment, the tumor molecular mechanisms, early diagnosis and effective methods of clinical treatment for HCC have been a major research project all over the world.

Gap junctions are intercellular channels formed by the interaction of two hemichannels—connexons, one of which is composed of six protein subunits[25,26]. Connexins (Cx) are subunits of gap junctional channels[27,28], by which neighboring cells can exchange low molecular weight ions and molecules, i.e., gap junctional intercellular communication (GJIC)[29,30]. GJIC mediated via gap junctions plays important roles in embryogenesis, cell proliferation, tissue homeostasis and in carcinogenesis[31,32]. Connexins are encoded by a gene family of at least 16 members, which have been divided into two groups based on primary amino acid sequence homology. Connexin32 (Cx32) and connexin43 (Cx43) are the major gap junction forming proteins in liver tissues. Decreased expression of Cx genes and disordered signal transduction pathway of Cx genes contribute to abnormal gap junctional intercellular communication between contacting cells[33]. Uncontrolled tumor cell growth because of the loss of gap junctional intercellular communication due to the down-regulated expression of Cx genes appears to bean important event in cell transformation[34-37]. In this study, the hepatocellular carcinoma cell lines HHCC, SMMC-7721 and normal liver cell line QZG were employed to investigate the relationship between hepatocarcinogenesis and cx32, cx43 mRNA and their protein expression.

MATERIALS AND METHODS

Cell culture

Hepatocellular carcinoma cell lines HHCC, SMMC-7721 and normal liver cell line QZG, were kindly provided by Professor Chen in the 863 Research Group. The cells were cultured on slides in RPMI 1640 medium (Gibco BRL, USA), supplemented with 100mL·L^{-1} fetal bovine serum (Gibco BRL, USA), incubated in a humidified atmosphere of 950 mL·L^{-1} air and 50 mL·L^{-1} CO_2 at 37℃. The cells were passaged by trypsinization twice a week.

Probe labeling

pGEM-cx32 plasmid containing cx32 cDNA 1.5kb, pSG5-cx43 plasmid containing cx43 cDNA 1.1kb were gifts from Professor Li in Hunan Medical University. After amplification, isolation and purification, pGEM3-cx32 plasmid was digested by EcoR I (Gibco BRL, USA) and pSG5- cx43 by BamH I (Gibco BRL, USA). The digested plasmids were electrophorated on 7 g·L^{-1} agrose gel with DNA/Hind III + EcoR I Marker (Gibco BRL, USA). cx32, cx43 cDNAs from gel were extracted and purified as the protocol of PCR-pure kit (Clontech, USA), and labeled using Dig

DNA labeling and detection kit (Boehringer Mannheim, Germany).

In situ hybridization

Slides of varied cells were incubated in 0.2mL·L^{-1} DEPC at RT for 10 min , then in 0.2mL·L^{-1} HCl for 10 min, 5mg·L^{-1} PK at 37℃ for 10 min. The digestion reaction was stopped in 0.1mol·L^{-1} glycine and the slides were fixed in 40g·L^{-1} PFA for 10 min, dehydrated in ethanol and air dried in sequence. Prehybridization was performed at 42℃ for 30 min. The labeled cDNA probes were denatured in hybridization buffer at 100℃ for 10 min, then at -20℃ for 3 min, then added on tissues and coverslipped at 42℃ overnight. Washing of sections was done with 2×SSC, 1×SSC, 0.5×SSC and Buffer I. The slides were incubated in NSS at 37℃ for 30 min, and then Dig-Ap (Boehringer Mannheim, Germany, 1:500) for 2h, finally detected with NBT / BCIP of Dig DNA labeling and detection kit (Boehringer Mannheim, Germany). Positive signals were visualized as intensive blue granules in the cytoplasm. Control sections were used. All results were verified by χ^2 test.

Flow cytometry (FCM) analysis

Total 10^6·L^{-1} cells of HHCC, SMMC-7721 and QZG were collected, and blocked with normal serum (Vector, USA) for 30 min at 4℃, then added mouse-anti Cx32 McAb (Zymed, USA, 1:000) and mouse-anti Cx43 McAb (Zymed, USA, 1:1000) respectively for 30 min at 4℃, whereas IgG was added for control. FITC-IgG (Jackson,1:100) was added in the cells for 30 min at 4℃, precipitated and washed by 0.01mol·L^{-1} pH7.5 PBS. Detective rates and the fluorescent intensity of Cx32, Cx43 proteins in the cells were measured by ELITE ESP flow cytometer (Coulter, USA) and phoenix software (Coulter, USA).

RESULTS

Detect of cx32, cx43 mRNA in cell lines by ISH

The cx32, cx43 mRNA in hepatocellular carcinoma cell lines HHCC, SMMC-7721 and normal liver cell line QZG were detected by Dig-labeled cx32, cx43 cDNA probes. After in situ hybridization, blue positive hybridization signals of mRNA were located in cytoplasm of the cells. The results showed that bright blue specific hybridization signals of cx32 mRNA and cx43 mRNA were detected in hepatocellular carcinoma cell lines HHCC, SMMC-7721 and normal liver line QZG. χ^2 tests did not show any significant difference (P> 0.05) between them. The results were showed in Figure 1-3.

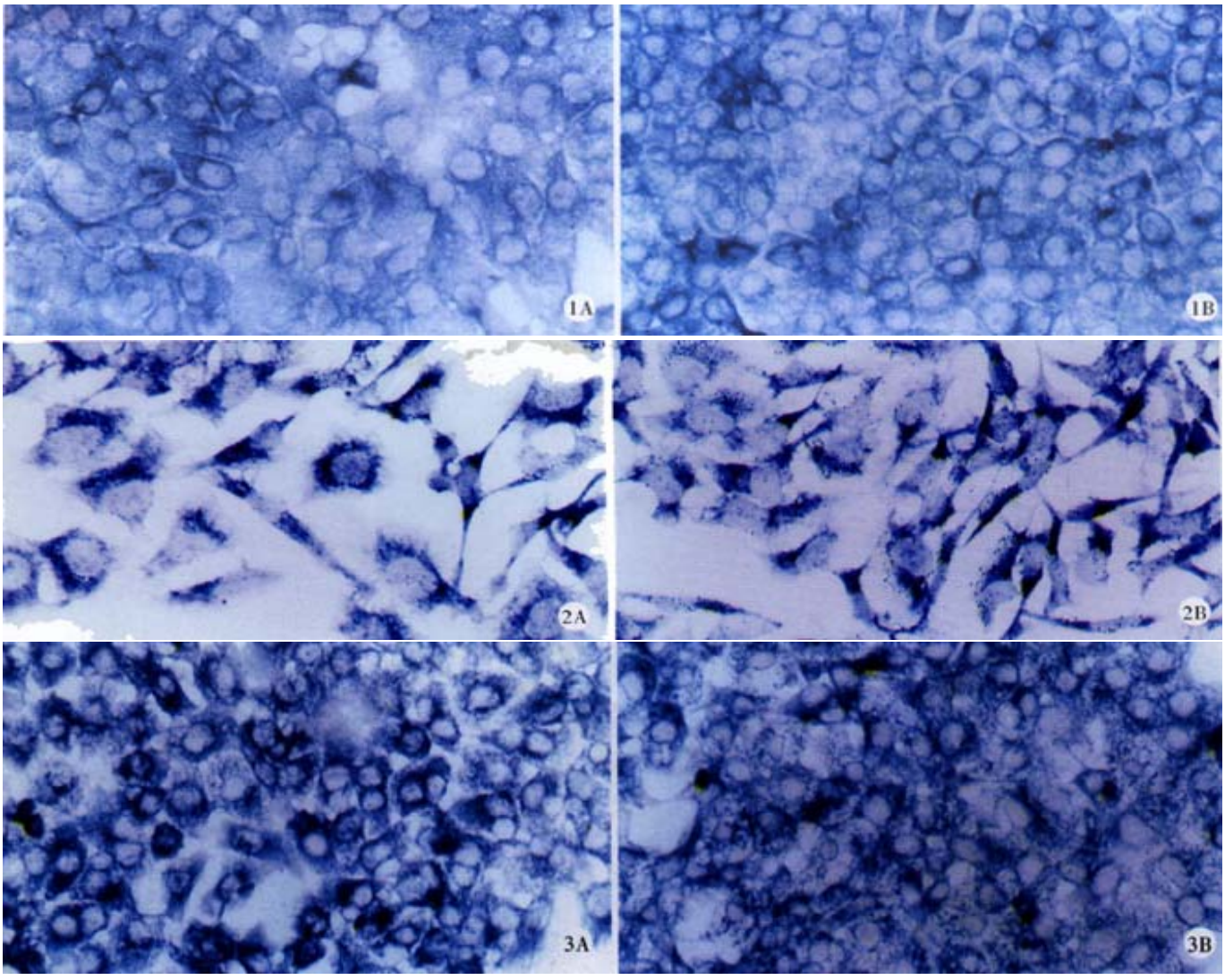

Figure 1A Positive signal of cx32 mRNA in HHCC, ISH×400
Figure 1B Positive signal of cx43 mRNA in HHCC, ISH×400
Figure 2A Positive signal of cx32 mRNA in SMMC-7721, ISH×400
Figure 2B Positive signal of cx43 mRNA in SMMC-7721, ISH×400
Figure 3A Positive signal of cx32 mRNA in QZG, ISH×400
Figure 3B Positive signal of cx43 mRNA in QZG, ISH×400

Expressions of Cx32, Cx43 proteins in hepatocellular carcinoma cell lines by FCM

Expression of Cx32, Cx43 proteins in cultured hepatocellular carcinoma cell lines HHCC, SMMC-7721 and normal liver cell line QZG were detected by FCM after immunoreaction with mouse-anti Cx32 McAb, mouse-anti Cx43 McAb. FCM examined both positive rates of Cx proteins expression and their quantities in each cell line.

FCM assay showed that positive rates of Cx32 protein expression in HHCC , SMMC-7721 and QZG were 0.7%, 1.7% and 99.0%, and those of Cx43 protein were 7.3%, 26.5% and 99.1% respectively. The fluorescent intensity of Cx32 protein and Cx43 protein in HHCC, SMMC-7721 were lower than those in QZG. QZG cells showed both higher positive rates for Cx32, Cx43 proteins and strong fluorescent intensity. The detection rates of Cx32, Cx43 proteins were showed in Figure 4-6 and Table 1.

Table 1 Positive expression rates of Cx32 and Cx43 proteins in various cell lines

Cell line	Positive rates %	
	Cx32	Cx43
HHCC	0.7[b]	7.3[b]
SMMC-7721	1.7[b]	26.5[b]
QZG	99.0	99.1

[b]$P<0.01$, *vs* QZG.

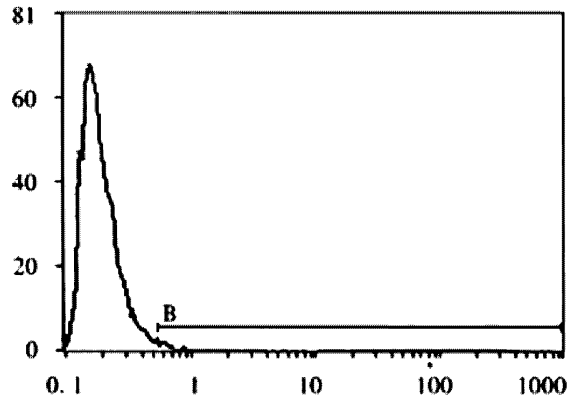

Figure 4A Positive expression rate of Cx32 protein in HHCC, FCM
(X: FL1; Y: cell count)

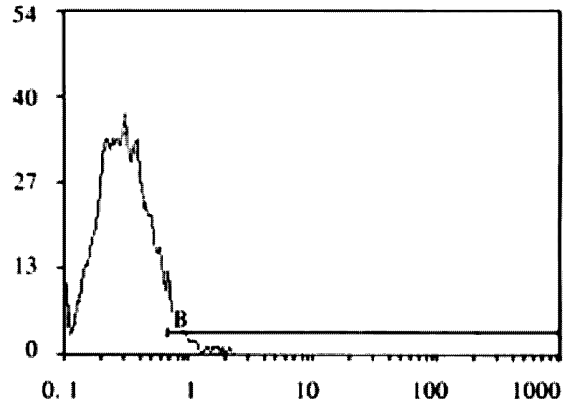

Figure 4B Positive expression rate of Cx43 protein in SMMC-7721, FCM
(X: FL1; Y: cell count)

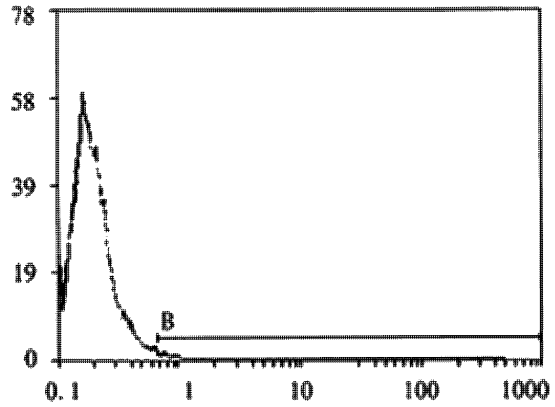

Figure 5A Positive expression rate of Cx43 (X: FL1; Y: cell count)
protein in HHCC, FCM

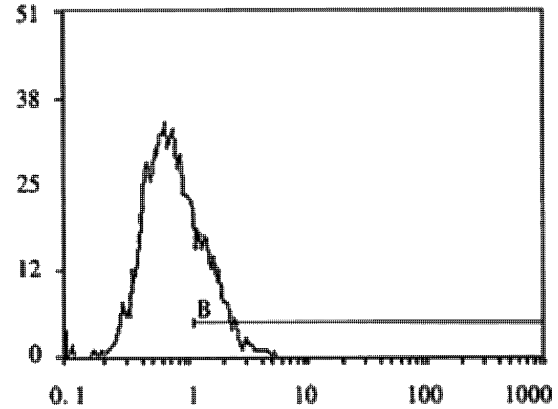

Figure 5B Positive expression rate of Cx32 protein in QZG, FCM
(X: FL1; Y: cell count)

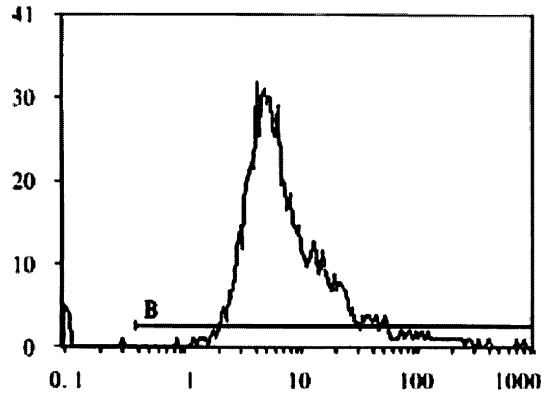

Figure 6A Positive expression rate of Cx32 protein in SMMC-7721, FCM
(X: FL1; Y: cell count)

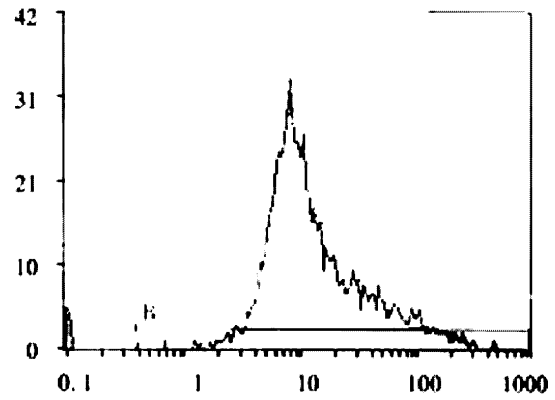

Figure 6B Positive expression rate of Cx43 protein in QZG, FCM
(X: FL1; Y: cell count)

DISCUSSION

Gap junction intercellular communication (GJIC) mediated by gap junction channels[38,39] has been postulated to be an important tool for the maintenance of tissues homeostasis, metabolism, control growth and differentiation[40,41]. Carcinogenesis is one of the pathological processes in which disorders of GJIC may play an important role. The inhibited GJIC in many kinds of tumor cells has been found, which could make important contributions to neoplastic progression by allowing tumor cells to escape by either systemic or local control mechanisms[42-44].

Cx32, Cx43 are widely expressed in many tissues, especially in normal liver. This study revealed that Cx32 and Cx43 proteins expressed at a high level in normal liver cell line but at low level in hepatocellular carcinoma cell lines[45-47].

Hepatocarcinogenesis is dramatically enhanced in liver neoplasm tissues lacking of Cx32 and Cx43 proteins, as we shown previously[48]. The results showed that cx32 and cx43 mRNAs and their proteins were highly expressed in normal liver tissues and cell lines, and had significantly decreased in hepatocellular carcinoma tissues and cell lines except expression of Cx43 protein in hepatoma cell line SMMC-7721. cx32 is the specific expression gene in human normal liver tissues and cell line whereas cx43 is a kind of variable expressing gene in either normal liver or hepatoma cell lines.Decrease of Cx proteins related with abnormal function of GJIC between hepatocellular carcinoma cells and surrounding normal cells finally results in hepatocarcinogenesis. Aberrant localization of Cx32 and Cx43 may be not only essential for the reduced GJIC in HCC[49], but also disturb the mechanism of a bystander effect[50]. Expression of the gap junctional proteins is often decreased in tumor tissues, but recruited expression could suppress malignant phenotypes of the tumor cells. The mechanism is that enhanced GJIC on basis of normal gap junctional protein expression regulates homologous and heterologous communication between tumor cells, surrounding normal hepatocytes and other cells[51].

Many observations demonstrate that the lower expression of Cx32 and Cx43 may be involved in the development of hepatocellular carcinoma[52]. It is interesting, moreover, that our results have showed even lower expression of Cx32, Cx43 proteins in hepatocellular carcinoma cell lines HHCC, SMMC-7721 than that in normal liver cell line QZG, but ISH results have shown no decreases of cx32 and cx43 mRNA in hepatocellular carcinoma compared with normal liver cell line. Therefore, it appears that cx32 and cx43 genes transcription is not responsible for aberrant expression of Cx32 and Cx43 proteins during human liver tumorigenesis. Besides, the results have indicated that cx32 and cx43 genes, the specific genes expressing in normal liver tissues, are expected to be the potential unmutated tumor suppressor genes.

Some abnormal regulatory events promote carcinogenesis[55,56], through multiple mechanisms, including post translation process and other potential mechanisms[53-56]. The carcinogenesis and development of hepatocellular carcinoma are related with the abnormal expression of cx genes, signal transduction disorders[57, 58], such as reduce of $[Ca^{2+}]$[59,60] and post-translational phosphorylation on tyrosine of Cx proteins, which are associated with dramatic changes in gap junctional intercellular communication and carcinogenesis[61]. The possibility is that defects in post-translational processing of Cx32 and Cx43 proteins may be obstacle for their transportation to cell membranes[62]. Furthermore, phosphorylation on tyrosine of Cx protein can affect the structure of Cx proteins, which is related to channel properties[63]. Post-translational phosphorylation on tyrosine of Cx32 and Cx43 may be important factors controlling the GJIC in hepatocellular carcinoma and substantially responsible for the assembly and function of these proteins as well. Further investigation is expected to understand the mechanism in detail.

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Edited by Zhang JZ