

Impaired gap junctions in human hepatocellular carcinoma limit intrinsic oxaliplatin chemosensitivity: A key role of connexin 26

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Abstract. Hepatocellular carcinoma (HCC) is generally believed to have low sensitivity to chemotherapeutic agents including oxaliplatin (OXA). Studies have demonstrated that gap junctions (GJs) composed of connexin (Cx) proteins have the potential to modulate drug chemosensitivity in multiple tumor cells. In the present study, we investigated the characteristics of Cx and GJs in HCC at both histologic and cytologic levels, and the effects of GJ and its effective components on OXA cytotoxicity in HCC cells *in vitro*. Immunohistochemistry was performed in 76 HCCs and 20 normal liver tissues to detect and locate the expression of Cx26, Cx32 and Cx43. At cytologic levels, the expression and localization of Cxs were evaluated by RT-PCR, western blot and immunofluorescence assay, respectively. The GJ function between adjacent cells was detected using dye transfer assay. The role of GJs in the modulation of OXA toxicity in HCC cells was explored using pharmacologic and molecular biologic methods. We found that Cx expression in HCC tissues was significantly lower than in normal liver tissues, and the 'internalization' from cell membrane to cytoplasm was remarkable. *In vitro* experiments revealed the presence of functional GJs in the SMMC-7721 HCC cells due to a small amount of Cx protein along the plasma membrane at cell-cell contacts. Regulation of this part of GJs positively influenced OXA cytotoxicity. Using RNA interference, only specific inhibition of Cx26 but not Cx32 or Cx43 reduced OXA cytotoxicity. Conversely, Cx26 overexpression by transfection

of Cx26 plasmid DNA enhanced OXA cytotoxicity. This study demonstrated that during hepatocarcinogenesis, the reduced expression and internalization of Cx proteins impaired the GJ function, which further attenuated OXA cytotoxicity. Impaired GJ function may contribute to low intrinsic chemosensitivity of HCC cells to OXA, mediated by Cx26.

Introduction

Hepatocellular carcinoma (HCC) is a significant cause of death worldwide. It has an insidious onset, with a high degree of malignancy and very poor prognosis. Palliative chemotherapy is one of the most important treatments for locally advanced and metastatic HCCs, which are unresectable. In recent years, the third-generation platinum compounds have been proposed as potential active for HCC (1,2). The positive results from the randomized phase III clinical trial EACH (3), further support the role of systemic oxaliplatin (OXA)-based regimen in advanced HCC (4). However, clinical benefits induced by OXA are limited (5), and HCC is traditionally believed to be insensitive to chemotherapy (6). The mechanisms underlying low chemotherapeutic response of HCC are poorly understood.

Gap junction (GJ), a protein channel connecting two adjacent cells, is composed of special proteins called connexins (Cxs). Distinct Cx subtype is named based on its molecular weight. Six Cxs form a hemi-channel (also called connexon) docking with its counterpart in the neighboring cell to form an integral GJ channel. These channels mediate direct intercellular molecular signaling, allowing intercellular exchange of signaling molecules such as inorganic ions, second messengers and other regulatory substances. The intercellular molecular exchange mediates various cellular events, including metabolism, homeostasis, cell proliferation and differentiation, and carcinogenesis (7,8).

Evidence demonstrates that cytotoxicity of multiple drugs is amplified via bystander effect (BE), which is partially mediated by cell-cell communication mediated by GJs (9). It is noteworthy that GJ increases the cytotoxicity of platinum drugs. Jensen *et al* (10) found that cisplatin (CIS)-induced apoptosis produced death signals, which are transmitted to neighboring cells via GJs. CIS toxicity in bladder cancer cells was enhanced

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Abbreviations: 18- α -GA, 18- α -glycyrrhetic acid; ATRA, all-trans retinoid acid; CIS, cisplatin; Cx, connexin; GJ, gap junction; HCC, hepatocellular carcinoma; OXA, oxaliplatin

Key words: hepatocellular carcinoma, oxaliplatin, chemosensitivity, gap junction, connexin 26

after GJ was upregulated by Cx26 transfection (11), and also strengthened in breast cancer cells by quinolines (GJ enhancer) *in vivo* or *in vitro* (12). Supersensitivity of spinal astrocytes to OXA was accompanied by the upregulation of Cx43 and GJ (13). Association of chemo-resistant phenotypes with GJs in a three-dimensional culture model of soft sarcoma has also been documented recently (14).

According to the BE mechanism, the efficacy of platinum drugs positively correlated with GJ level in tumor cells. However, tumor cells (including hepatic) were often accompanied by a decline or loss of GJ (15-17). For instance, normal liver tissue is abundant in GJs, mainly expressing Cx26, Cx32 and Cx43 (18,19). In rat hepatic tumorigenesis induced by oncogenic drugs, GJ and its composed Cx were downregulated (20). The expression of Cx32 and Cx43 in HCC tissues was found to be lower than in normal hepatic tissues (21). Aberrant expression and localization of Cx32 was observed during HCC development and progression (22). Based on these findings, we inferred that when GJ function decreased or was lacking in HCC, cytotoxic signal was not transmitted to adjacent cells, leading to decreased BE, and low chemosensitivity of HCC cells to cytotoxic drugs including OXA. Additionally, the Cx isoform determines the function of GJ channel and also the type of signal transduction (23,24). Therefore, our study systematically investigated the functional status of Cx and GJs in HCC at both histologic and cytologic levels, and further focused on the effect of GJs on OXA cytotoxicity *in vitro*. The effective GJ components participated in the process were also explored. The study may offer new insight into the mechanisms of low response of HCC to chemotherapeutic drugs, and also provide novel potential approaches and strategies to enhance OXA chemosensitivity in liver cancer.

Materials and methods

Materials. SP kits were obtained from Fuzhou Maixin Biotech. Co., Ltd (Fujian, China). OXA, 18- α -glycyrrhetic acid (18- α -GA), all-trans retinoid acid (ATRA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anti-Cx32 and anti-Cx43 mouse IgG were purchased from Sigma (St. Louis, MO, USA). DMEM, fetal bovine serum, TRIzol, Lipofectamine™ 2000, DAPI, DiI-CM, calcein-AM, and anti-Cx26 mouse IgG were procured from Invitrogen (Carlsbad, CA, USA). HPR and FITC-labeled goat anti-mouse secondary antibody were obtained from Amersham Biosciences Corp. (Piscataway, NJ, USA). All other reagents were from Sigma unless stated otherwise.

Cell line and cell culture. Human normal liver cell line LO2 was obtained from KeyGEN BioTECH (Nanjing, China). Human HCC cell line SMMC-7721 was provided by Chinese Type Culture Collection (Shanghai, China). Both cell lines were grown at 37°C in a humidified atmosphere containing 5% (v/v) CO₂ in DMEM supplemented with 10% fetal bovine serum, 100 U/ml streptomycin and 100 mg/ml penicillin. Cells in the exponential phase of growth were selected for experiment.

Immunohistochemistry

Samples. Without any preoperative treatment, 76 archived HCC paraffin samples were collected from patients following

resection at the First Affiliated Hospital of Bengbu Medical College from January 2008 to December 2013. Twenty normal liver tissues obtained via hepatic resection following hepatic trauma or accidental death (healthy prior to death) served as controls. For the HCC samples, 61 were male and 15 were female, with a median age of 50 years old (range: 22-76 years). The diagnosis was confirmed histologically in all cases. Histology grade was based on the criteria proposed by Edmondson and Steiner. Histological grade I-II accounted for 63% (48 samples), and grade III-IV accounted for 37% (28 samples). The nontumorous liver adjacent to tumor showed cirrhosis or chronic hepatitis in 60 (79%) samples. Staging at the time of diagnosis was based on the tumor-node-metastasis (TNM) classification. Tumors of stage I-II accounted for 71% (54 samples), and tumors of stage III-IV accounted for 29% (22 samples). Tumor size and lymph node status were evaluated separately.

Immunohistochemical staining. Detail experimental procedures were described in our previous studies (25,26). Specific procedures were strictly performed according to the manufacturer's instructions. PBS instead of primary antibody was used as the negative control. Positive staining and cellular localization were observed and photographed with an optical microscope.

Immunohistochemical evaluation. Cx staining was detected on the membrane or in the cytoplasm. Yellow particles in cells were considered positive. We calculated 500 tumor or normal liver cells for every section under high magnification. The distribution of Cx-positive spots was considered as 'internalization' if the spots were removed (or partially removed) from the cell surface and detected in the cytoplasm, and the internalization rate was calculated as follows: (The number of Cx internalization cases/the total number of Cx positive expression cases) x100%.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized by reverse transcription with a random primer. The specific Cx upstream and downstream primers were added to the reverse transcription cocktail to perform PCR. The following primers were used: for human Cx26, 5'-GCT GCAAGAACGTGTGCTAC-3' (upstream), 5'-TGGGTT TTGATCTCCTCGAT-3' (downstream), product size 196 bp; for human Cx32, 5'-TCCCTGCAGCTCATCCTAGT-3' (upstream), 5'-CCCTGAGATGTGGACCTTGT-3' (downstream), product size 156 bp; for human Cx43, 5'-GGTCTGA GTGCCTGAACTTGCCT-3' (upstream), 5'-AGCCACAC CTTCCCTCCAGCA-3' (downstream), product size 184 bp. Human β -actin was used as an internal reference, with the primer sequence 5'-TCCTCCTGAGCGCAAGTACTC-3' (upstream), and 5'-GCATTTGCGGTGGACGAT-3' (downstream), and product size 130 bp. The reaction volume of PCR was 20 μ l. PCR was initiated at 94°C for 3 min followed by 30 cycles consisting of 45 sec at 94°C, 45 sec at 55°C, and 45 sec at 72°C, with the final cycle extended to 10 min at 72°C, followed by termination at 4°C. PCR amplicons (5 μ l) were analyzed on an ethidium bromide-stained 1.5% agarose gel.

Table I. siRNA sequences for human Cx26, Cx32 and Cx43.

Genbank ID	Gene	Sequence	
		Sense (5'-3')	Antisense (5'-3')
NM_004004.5	Human Cx26	GAGGCUCAGAUUGUAAUAUTT	AUAUUACAAUCUGAGCCUCTT
		CCACGUUAAAGGUGAACAUUTT	AUGUUCACCUUUAACGUGGTT
		CCCAGUUGUUAGAUUAAGATT	UCUUAACUAACAACUGGGTT
NM_001097642.2	Human Cx32	GCUCCUGAAAGACAUACUTT	AGUAUGUCUUUCAGGGAGCTT
		GCCGUCUUAUGUAUGUCUTT	AGACAUACAUGAAGACGGCTT
		GCAACACAUAGAGAAGAAATT	UUUCUUCUCUAUGUGUUGCTT
NM_000165.4	Human Cx43	GGCCUUGAAUAUCAUUGAATT	UUCAUGAUUUCAAGGCCTT
		GCCGCAAUUACAACAAGCATT	UGCUUGUUGUAAUUGCGGCTT
		GGAAGCACCAUCUCUAACUTT	AGUUAGAGAUGGUGCUUCCTT

Western blotting. LO2 and SMMC-7721 cells were harvested, centrifuged (12000 x g/min at 4°C for 30 min), and supernatants were obtained. Protein concentration was determined using a DC protein assay kit (Bio-Rad Co., Hercules, CA, USA). Samples (50 µg) from cells were transferred to SDS-PAGE, followed by electrophoresis and blotting. Monoclonal antibodies against Cxs (Cx26-1:1000; Cx32-1:2000; Cx43-1:4000) or β-actin (1:10,000) were used. The immunoreactive bands were visualized using Amersham ECLTM Plus Western Blotting Detection kit (GE Healthcare, Piscataway, NJ, USA).

Immunofluorescence. Cells in exponential growth phase were collected, and fixed with 0.1% Triton X-100-4% paraformaldehyde for 30 min at room temperature. Coverslips were blocked with 2% BSA in PBS and probed with the primary antibodies (Cx26-1:200; Cx32-1:100; Cx43-1:200; diluted in 2% BSA) overnight at 4°C. A secondary antibody FITC anti-mouse IgG (1:200, diluted in 2% BSA) was added, and incubated for 2 h in the dark, at room temperature. Nuclear staining was performed with DAPI at 37°C for 5 min. After rinsing, the coverslips were mounted on slides, and the cells were observed and photographed under a fluorescence microscope (Olympus).

Determination of GJ function: 'Parachute' dye-coupling assay. 'Parachute' dye-coupling protocols were described in our previous studies (27,28). Briefly, LO2 and SMMC-7721 cells were grown to 80%-85% confluence. Donor cells from one well were incubated with a freshly made solution of 5 µM calcein-AM and 2.5 µM CM-DiI in growth medium at 37°C for 30 min. Calcein-AM was converted intracellularly into the small GJ-permeable dye calcein, while CM-DiI was too large to spread to coupled cells through GJs. Unincorporated dye was removed by three gentle washes with PBS. The donor cells were then trypsinized and seeded onto the receiver cells at a density of 500-800 cells/ml, and cultured at 37°C for 4 h. The small molecule calcein (green fluorescence) entered adjacent recipient cells through GJs when stable GJs were formed. The index of GJ function was defined by counting the number of recipient cells containing calcein around one donor cell under fluorescence microscope.

MTT assay. OXA toxicity was assessed by MTT assay. SMMC-7721 cells were seeded at a density of 8x10³/well into 96-well plates for 24 h. OXA of different concentrations were then added for the indicated time periods. MTT (5 mg/ml) was added to each well, and dishes were cultured at 37°C for 4 h. The medium containing MTT was then removed, and 150 µl DMSO was added into each well to fully dissolve formazan crystals in the viable cells. The absorbance at 490 nm of each well was read using a microplate ELISA reader (MRX II; Dynex Technologies, Chantilly, VA, USA). Cell viability was calculated as follows: (OD of experimental group - OD of blank group) / (OD of control group - OD of blank group).

Modulation of GJ function. SMMC-7721 cells were seeded at high (8x10³/well) and low (0.5x10³/well) density into 96-well plates to obtain GJ-formed and non-GJ formed cells, corresponding to conditions in which junctional channel formation was permitted or not, respectively (10). After cells at high density were grown to 80-85% confluence, the cells under both culture conditions were then exposed to a defined dose of OXA for 24 h, to enable the effect of GJ presence on OXA toxicity to be observed. The changes in OXA cytotoxicity were also investigated in high density-cultured cells pretreated with GJ tool drugs, ATRA (10 µM) and 18-α-GA (5 µM), for 24 h and 1 h, respectively.

Specific regulation of Cx expression by RNA interference and Cx26 overexpression. The siRNA fragments of different Cxs were synthesized and provided by Shanghai GenePharma Co., Ltd. (Shanghai, China). Three specific interfering sequences for each Cx gene were synthesized as listed in Table I. Cx26 was overexpressed in SMMC-7721 cells using a pEX-2/hCx26 vector (Shanghai GenePharma Co.). Negative control siRNA (NC in the figures) and empty vector negative control were also maintained. Transfection into SMMC-7721 cells was carried out using Lipofectamine 2000 according to the manufacturer's instructions. Knockdown and upregulation of Cx expression were confirmed by western blotting.

Statistical analysis. Results were analyzed with SPSS version 17.0 software (Chicago, IL, USA). Differences between two

Table II. Expression of Cx26, Cx32 and Cx43 in human normal liver and HCC tissues.

Group	n	Cx26		Cx32		Cx43	
		-	+	-	+	-	+
Normal liver	20	0	20	0	20	2	18
HCC	76	40	36	43	33	30	46
χ^2 -value		18.045		20.497		6.189	
p-value		0.000		0.000		0.015	

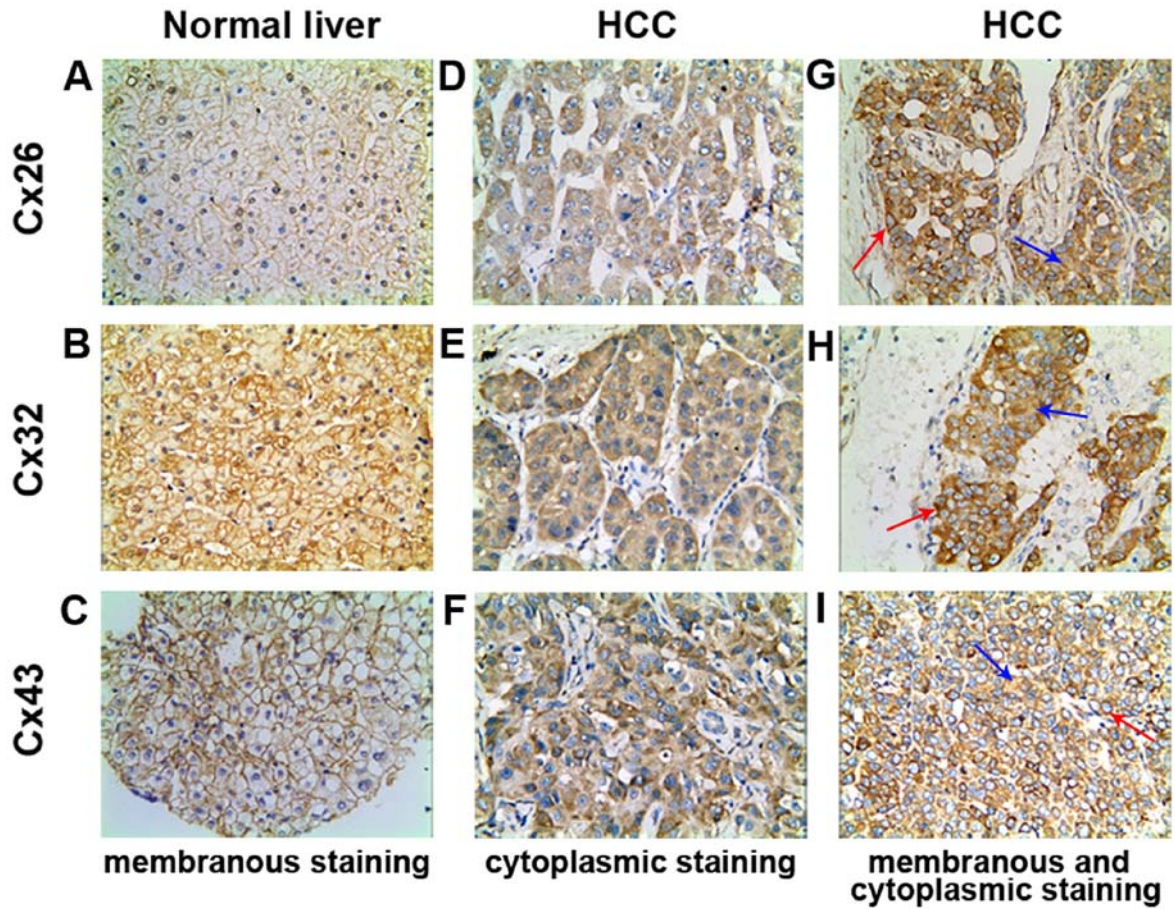


Figure 1. Immunohistochemical analyses of Cx26, Cx32 and Cx43 in sections of human normal liver and HCC tissues. (A-C) Normal liver cells showing extensive membranous staining for the three Cx proteins. (D-F) Cytoplasmic positive staining for Cxs in HCC tissues. (G-I) Both membranous (marked by red arrow) and cytoplasmic (marked by blue arrow) expression of Cxs in certain HCC cases. Representative immunohistochemical examples of staining are shown (original magnification, x400).

groups in immunohistochemistry experiments were evaluated by χ^2 test and Fisher's exact test where appropriate. Numerical data are presented as means \pm SEM and compared with unpaired Student's t test using Sigmaplot 10.0 software (Jandel Scientific, San Rafael, CA, USA). Differences at $p < 0.05$ were considered significant.

Results

Cx expression in human normal and HCC liver tissues. As shown in Table II, Cx26 and Cx32 was 100% and Cx43 showed 90.00% positive expression in normal liver tissues.

However, the positive rate for the three Cxs was significantly reduced to 47.37, 43.42 and 60.53% in HCC tissues, respectively ($\chi^2=18.045$, $p=0.000$; $\chi^2=20.497$, $p=0.000$; $\chi^2=6.189$, $p=0.015$). In Cx localization, while the positive particles were distributed mainly linearly on membrane in normal liver tissues (Fig. 1A-C), in HCC tissues Cxs stained positive mostly in the cytoplasm (Fig. 1D-F), and occasionally on the membrane of cancer cells, concurrently in certain cases (Fig. 1G-I). The phenomenon of Cx removal from membrane to cytoplasm is known as 'internalization'. The internalization rates of the three Cx proteins in HCC group were 100% and statistically significant compared with the normal liver tissue

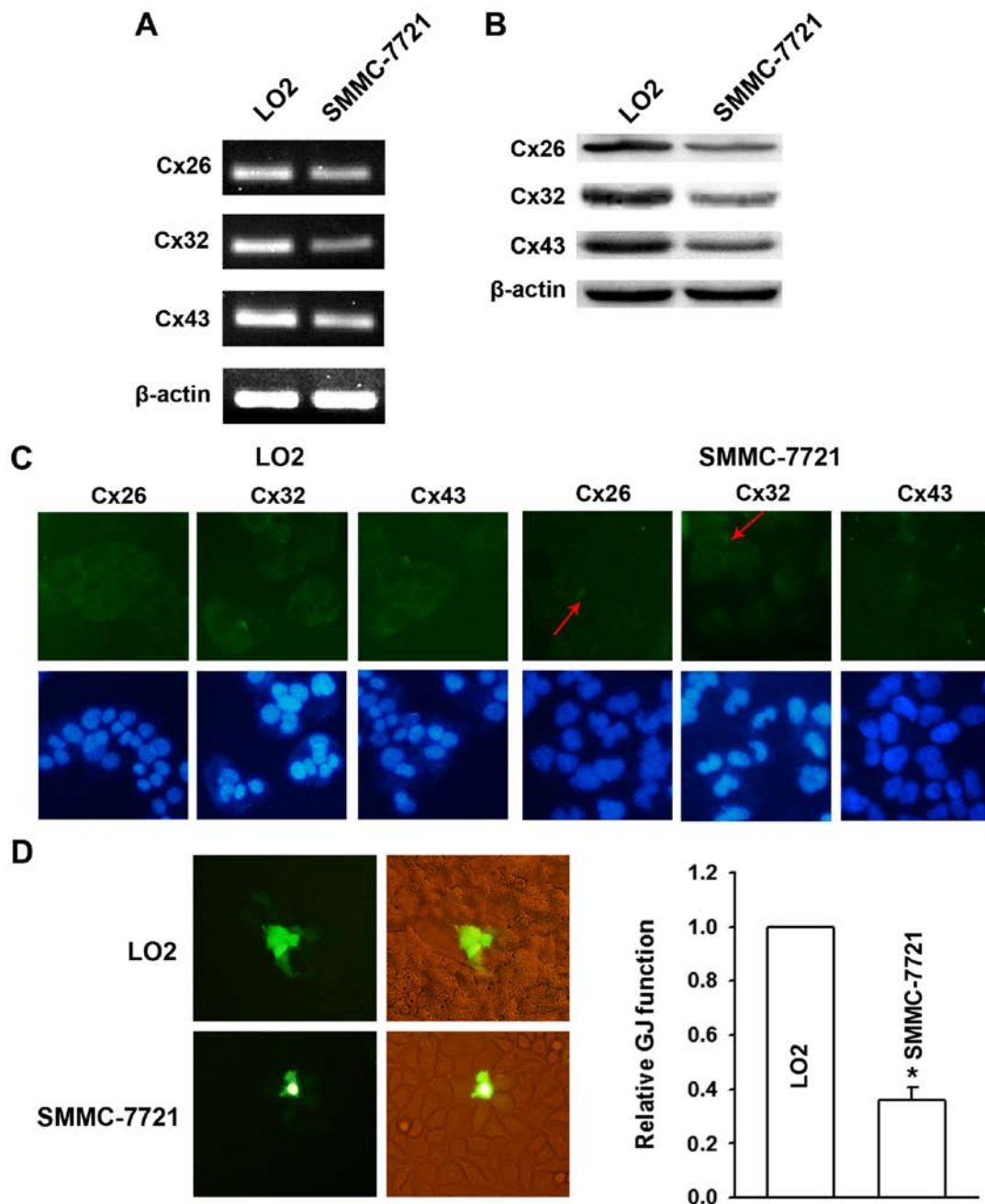


Figure 2. Detection of Cx26, Cx32 and Cx43 expression and GJ status in human normal liver cell line LO2 and hepatoma cell line SMMC-7721. (A and B) Decreased mRNA and protein expression of Cx26, Cx32 and Cx43 in SMMC-7721 cells compared with those in LO2 cells, as shown by agarose gel electrophoresis of RT-PCR products and western blotting, respectively. (C) Immunofluorescent staining of Cx26, Cx32 and Cx43 proteins *in vitro*. LO2 cells showed mainly membranous staining for Cxs, while diffuse cytoplasmic staining for Cxs in SMMC-7721 cells was observed. Only a partial Cx26 and Cx32 expression on SMMC-7721 cell membrane was concurrently detected (marked by red arrow), and Cx43 was only observed in the cytoplasm of SMMC-7721 cells (x200). (D) GJ activity of SMMC-7721 cells was markedly reduced compared with that of LO2 cells, as evidenced by parachute dye coupling assay (x200). Data represent the mean \pm SEM of three independent experiments. * $p < 0.05$ vs. LO2 group.

group ($\chi^2=43.938$, $p=0.000$; $\chi^2=31.392$, $p=0.000$; $\chi^2=41.358$, $p=0.000$, Table III).

Cx expression and GJ function in human normal liver LO2 and hepatoma SMMC-7721 cells. The reduced levels and cytoplasmic localization of Cx protein suggested a decreased GJ function in HCC carcinogenesis. To further validate the histologic results of the Asian population, two Asian-derived human cell lines including the normal liver cell line LO2 and HCC cell line SMMC-7721 were used *in vitro*. The RT-PCR

and western blot results revealed a positive expression of Cx26, Cx32 and Cx43 at both mRNA and protein levels. Compared with LO2 cells, the expression of Cxs was markedly reduced in SMMC-7721 cells (Fig. 2A and B). Immunofluorescence analysis revealed a clear membranous localization of the three Cxs in LO2 cells. While in SMMC-7721 cells, Cx26 and Cx32 were detected mainly in the cytoplasm and only a partial expression on membrane; and for Cx43 only cytoplasmic staining was observed (Fig. 2C). Thus cytologic and histologic results were highly consistent. Subsequent 'parachute' dye-coupling assay

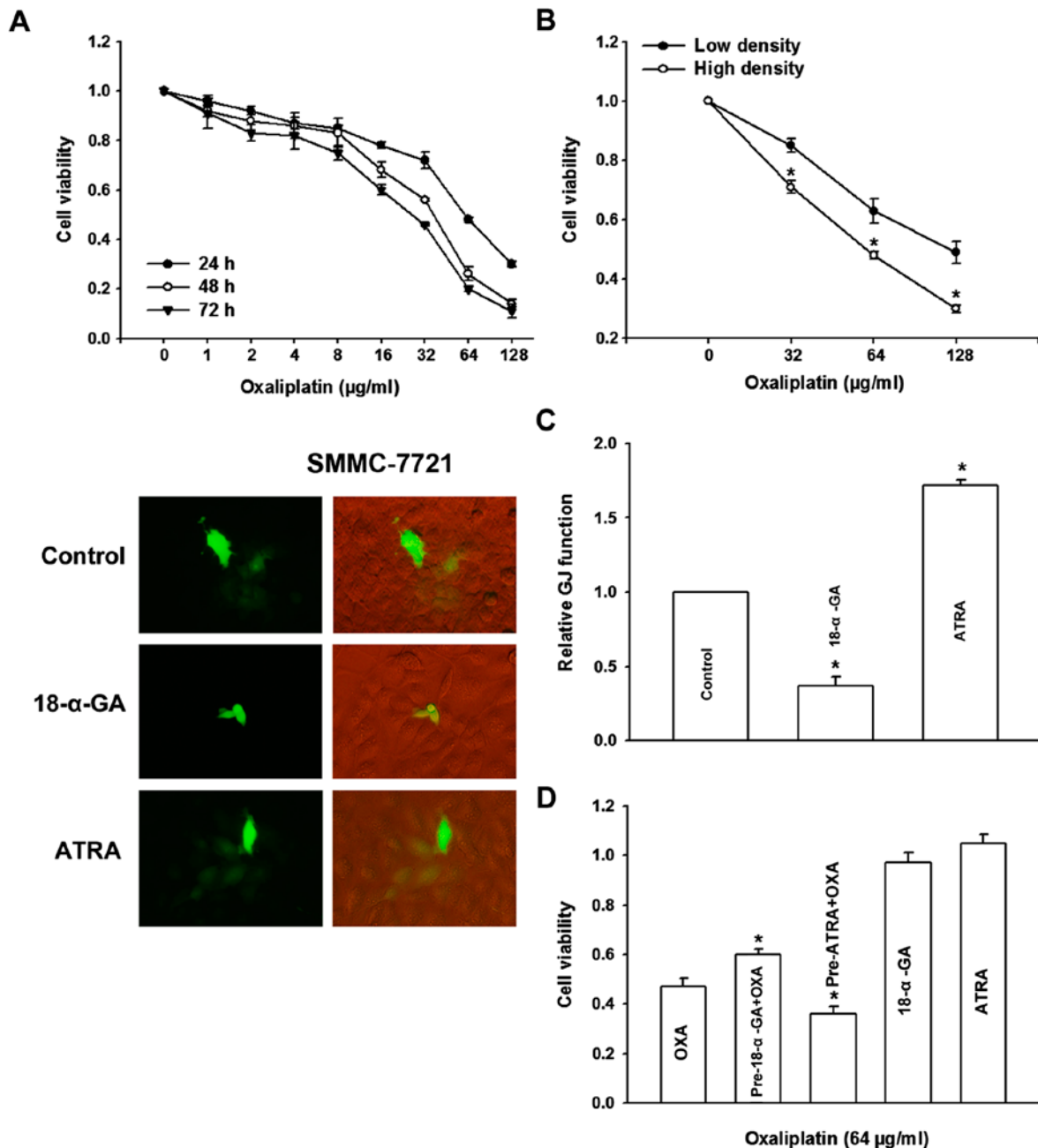


Figure 3. GJ modulates OXA cytotoxicity in SMMC-7721 cells. (A) Growth inhibition of SMMC-7721 cells by a range of OXA concentrations for the indicated time periods. (B) Cell viability of SMMC-7721 cells incubated for 24 h with increasing doses of OXA at low and high cell density. Culture at high density enhanced OXA toxicity compared with that at low density. (C) Fluorescence images showed the degree of dye coupling by the parachute assay. 18- α -GA treatment (5 μ M) for 1 h decreased GJ function while 10 μ M ATRA treatment for 24 h increased GJ function in SMMC-7721 cells. (D) Cell viability of SMMC-7721 cells incubated with 64 μ g/ml OXA for 24 h with or without 18- α -GA or ATRA pretreatment at high cell density. Data represent the mean \pm SEM of 3 to 6 independent experiments. * p <0.05 vs. low cell density group (B); * p <0.05 vs. control (C); * p <0.05 vs. OXA treatment alone (D).

in vitro revealed that GJ was abundant in LO2 cells, and was attenuated to ~36% in SMMC-7721 cells (Fig. 2D).

GJ modulates OXA cytotoxicity in SMMC-7721 cells. As shown in Fig. 3A, results of MTT indicated that cell viability of SMMC-7721 cells decreased with increase in OXA concentration and treatment time. The effects of 32, 64 and 128 μ g/ml dose groups were significant, and the inhibition rates for 24 h were 28, 52 and 70%, respectively. In the experiments involving high-density and low-density cell seeding, we found that OXA cytotoxicity of high-density cultures was

substantially greater than that of low-density cultures (p <0.05, Fig. 3B). The formation of GJs is just one of the several potential differences between low and high density cultures, and therefore, GJ coupling was further manipulated in the cultures by chemical drugs.

18- α -GA, recognized as a GJ channel inhibitor (29,30), and ATRA as an enhancer of reinforced Cx expression in various tumor cells (31,32), have been widely used in the GJ analysis. We then observed the alteration of GJ function in SMMC-7721 cells by these two drugs. As shown in Fig. 3C, 18- α -GA (5 μ M) treatment for 1 h significantly inhibited GJ

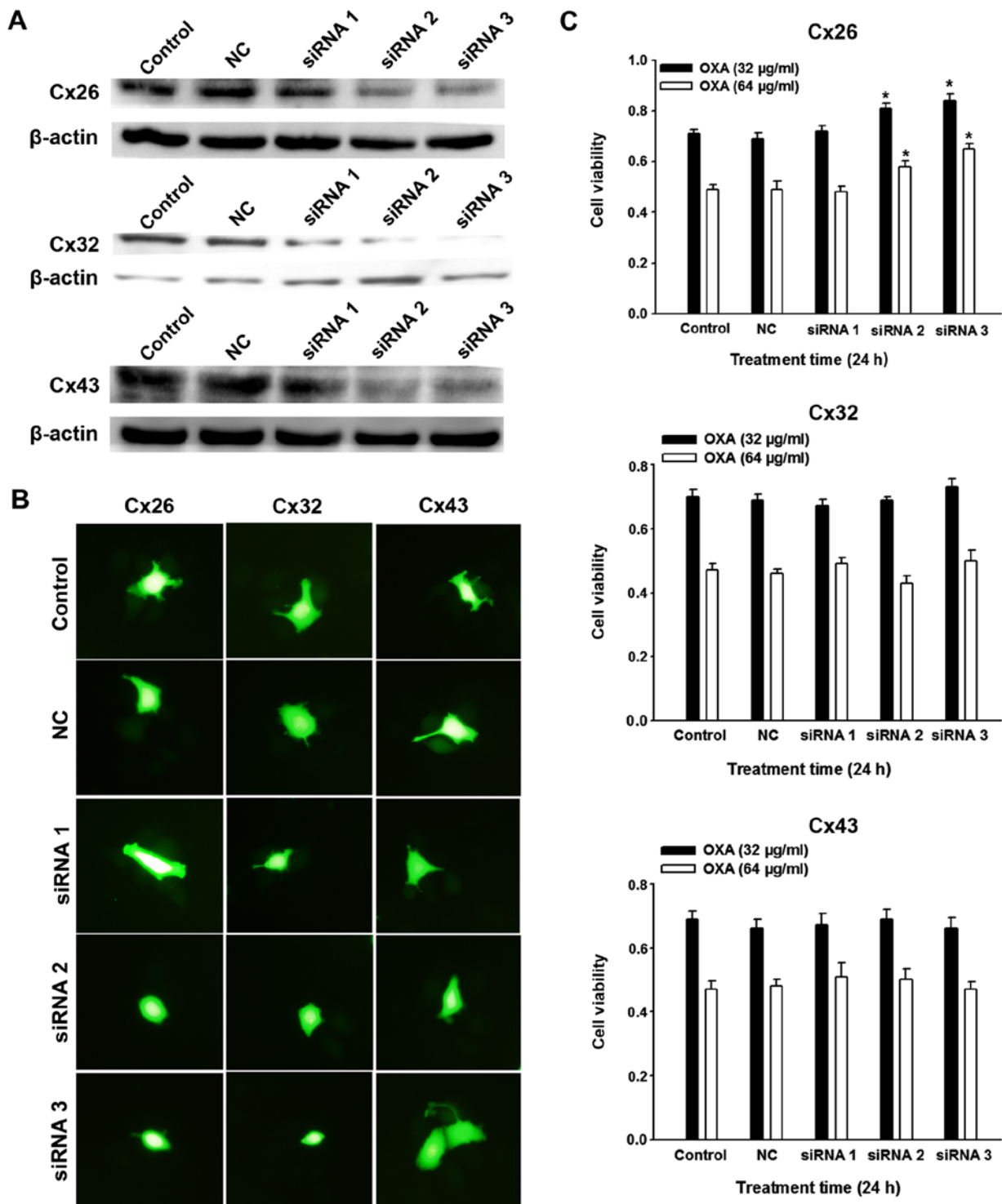


Figure 4. Effect of siRNA-mediated knockdown on Cx expression, GJ function and OXA cytotoxicity in SMMC-7721 cells. (A) Western blot of siRNA-mediated knockdown of Cx26, Cx32 and Cx43 expression. (B) Fluorescence images showed the degree of dye coupling by the parachute assay. Cells in which Cx expression was knocked down by siRNA targeting Cx26 or Cx32 showed lower dye transfer than control cells (no siRNA) and cells transfected with negative control siRNA (NC group). Negative control, siRNA: cells transiently transfected with synthetic negative control siRNA or siRNA targeting Cx26, Cx32 and Cx43, respectively. Representative images are shown (original magnification x200). (C) High-density cultured cell viability after 24 h incubation with both 32 µg/ml and 64 µg/ml OXA of control, negative control (NC) and Cx knockdown. Data represent the mean \pm SEM of four independent experiments. *p < 0.05 vs. control.

function by about 63%; conversely, ATRA (10 µM) treatment for 24 h markedly strengthened GJ function by about 72%. Subsequent studies revealed that 18-α-GA (5 µM) and ATRA (10 µM) alone had no obvious effect on SMMC-7721 cell growth. However, upon pretreatment with 18-α-GA for

1 h to inhibit GJ function, OXA toxicity was reduced. On the contrary, upon pretreatment with ATRA for 24 h to increase GJ function, OXA toxicity was increased (p < 0.05, Fig. 3D). These results suggested a potential role of GJ in modulating OXA cytotoxicity in SMMC-7721 cells.

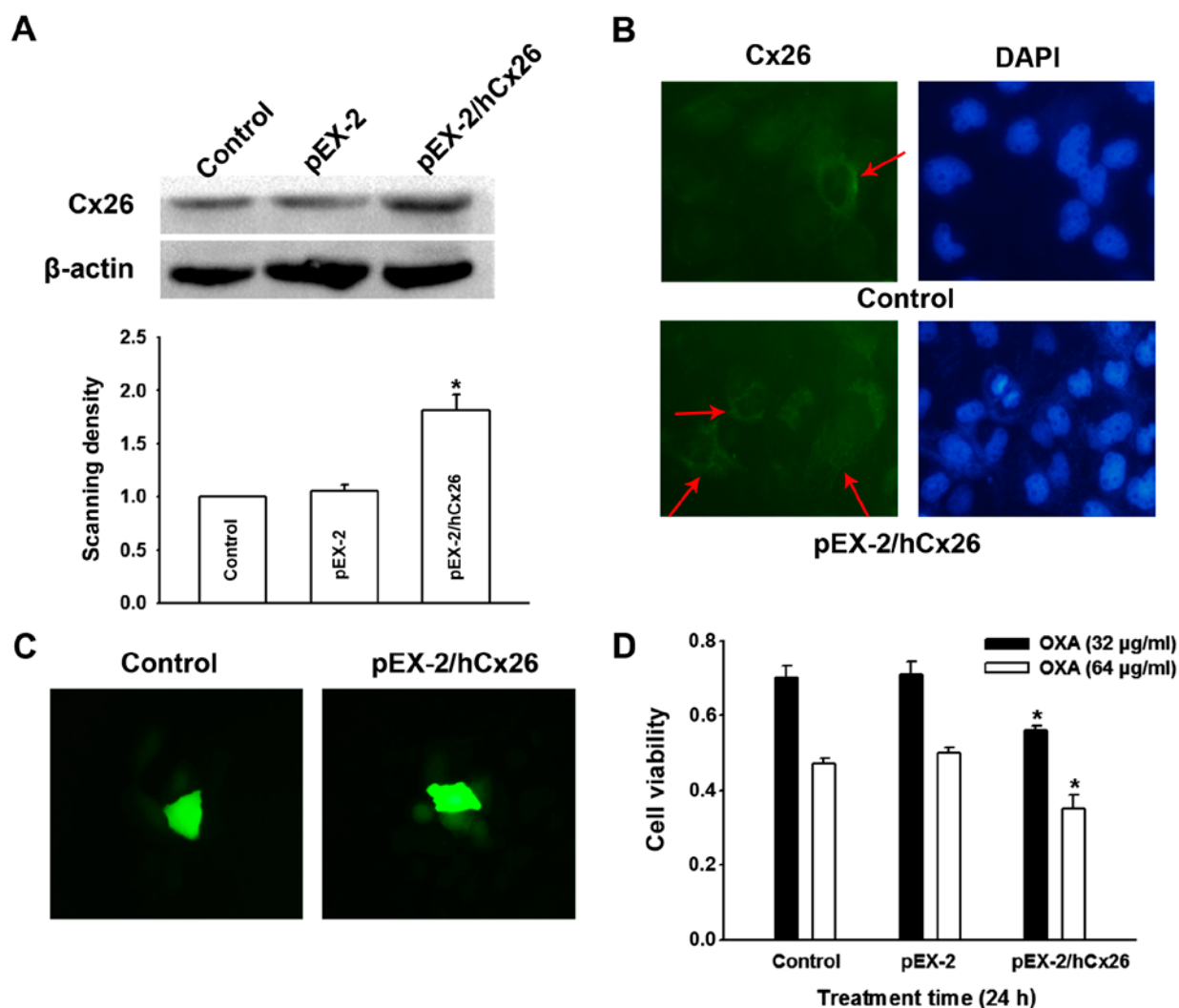


Figure 5. Effect of Cx26 overexpression on OXA cytotoxicity in SMMC-7721 cells. (A) Western blot analyses confirmed overexpression of Cx26 in SMMC-7721 cells following transfection by pEX-2/hCx26. (B) More membranous localization of Cx26 was observed in SMMC-7721 cells by Cx26 transfection (marked by red arrow). (C) Fluorescence images showed an enhanced dye coupling between adjacent SMMC-7721 cells by the parachute assay. (D) OXA toxicity in SMMC-7721 cells was increased by Cx26 transfection. Data represent the mean \pm SEM of four independent experiments. * $p < 0.05$ vs. control.

Table III. Internalization of Cx expression in human normal liver and HCC tissues.

Group	n	Cytoplasmic Cx26		n	Cytoplasmic Cx32		n	Cytoplasmic Cx43	
		-	+		-	+		-	+
Normal liver	20	17	3	20	14	6	18	14	4
HCC	36	0	36	33	0	33	46	0	46
χ^2 -value		43.938			31.392			41.358	
p-value		0.000			0.000			0.000	

Effect of specific regulation of SMMC-7721 Cx expression on OXA cytotoxicity. To verify that the effects of cell density and the drugs on OXA cytotoxicity were due to GJ-mediated cell-cell communication, RNA interference of the dominant Cxs was conducted in SMMC-7721 cells. After inhibition of Cx26, Cx32 and Cx43 expression was confirmed by western blot analysis (Fig. 4A), 'parachute' dye transfer assay demonstrated

that Cx26 and Cx32 downregulation led to decreased GJs, but not Cx43 (Fig. 4B). These observations are important as they indicate the effective component of GJs in SMMC-7721 cells. Further studies showed that only the knockdown of Cx26, but not Cx32 or Cx43 expression depressed OXA toxicity (Fig. 4C). We further explored the effect of overexpression of Cx26 by transfection of pEX-2/hCx26. Western blot confirmed that

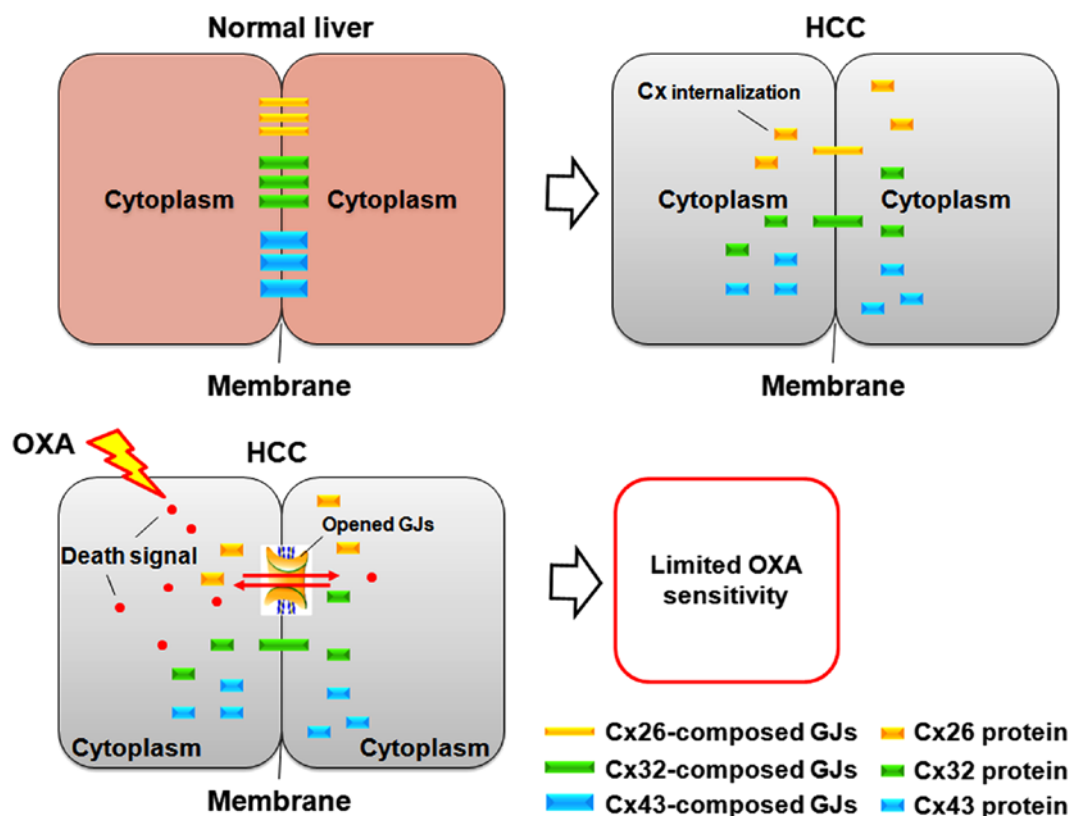


Figure 6. A proposed model for an inherent defective GJs to limit OXA-induced toxicity in HCC cells. HCC cells orchestrate impaired GJs due to the reduced expression and internalization of dominant Cx proteins, leading to low intrinsic OXA chemosensitivity. The effective component responsible for this effect is attributed to Cx26, not Cx32 or Cx43.

the expression of Cx26 was markedly enhanced relative to its untreated counterparts and pEX-2 negative controls (Fig. 5A). Accompanied by Cx26 upregulation, more Cx26 particles were located on SMMC-7721 cell membrane (Fig. 5B), and 'parachute' dye-coupling assay confirmed an increased GJ function (Fig. 5C). Expectedly, SMMC-7721 OXA cytotoxicity was finally strengthened by the upregulation of Cx26 expression (Fig. 5D). These results together demonstrate that the Cx26 protein component was specifically responsible for the role of SMMC-7721 GJs in OXA toxicity.

Discussion

Studies have demonstrated that injury or death signals induced by cytotoxic drugs such as platinum-based agents have the potential to be transferred between adjacent tumor cells via GJs, leading to the cytotoxicity amplified (10-12). Therefore, the final anti-tumor effect of chemotherapeutic drugs depends largely on the tumor GJ levels. However, Cx and its composed GJs are frequently reduced or absent in cancer cells compared with the original normal tissue (33,34). GJ abnormalities compromise the regulatory framework of the organism to certain initial transformed cells, resulting in malignant tumors followed by uncontrolled excessive proliferation. Based on these findings, it is plausible that GJ decline limits the transmission of toxic signal induced by chemotherapeutic drugs between adjacent cells, resulting in restricted drug sensitivity or even drug resistance in its targeted cells (Fig. 6). It may be a mechanism underlying the poor chemotherapeutic response

in tumors such as HCC. To verify the hypothesis, we first investigated the differences in Cx expression between HCC and normal liver tissues in histological specimens using a relatively large number of samples. Human normal liver cell line and HCC cell line were then used *in vitro* to confirm the Cx expression profile and its GJ status, and the role of GJs in mediating OXA cytotoxicity was also explored.

The present study showed that expressions of Cx26, Cx32 and Cx43 decreased significantly in human HCC tissues compared with normal liver tissues, which was in accordance with the results of liver cancer animal model (20). Further, we noted marked localization changes for the three Cxs: in normal liver tissues they were located mainly on the intercellular membranes of hepatocytes. In HCC tissues, they were located in the cytoplasm mainly, due to 'internalization', which was discovered in a prior study focused on the role of Cx32 in HCC development (22). Considering the dominant liver Cx proteins exhibit a similar pattern, Cx internalization may be a significant marker of hepatocarcinogenesis. GJs channels are formed by the end-to-end docking of two hemichannels in adjacent cells, and functional GJs occur only when Cxs are located on the membrane. Thus, an internalization of Cx proteins may lead to depressed or deficient GJs. Note is that Cx membranous localization is still retained in a few HCC cases, suggesting a theoretical possibility of GJ formation. We then indeed confirmed in subsequent *in vitro* experiments that functional GJs were reduced in human HCC cell line SMMC-7721 compared with the normal liver cell line LO2, due to reduced Cx expression and aberrant localization of the dominant Cx

proteins. From the localization of Cx by immunofluorescence assay, we inferred Cx43 was not the main effective component of SMMC-7721 GJs.

Human liver cancer tissues or cells retain partial GJs enabling observation of the influence of GJ regulation on OXA cytotoxicity. GJ is the direct channel communicating with the adjacent cytoplasm, suggesting that the growth and confluence were proportional to GJ formation in cells with Cx expression. We found that OXA cytotoxicity in high density cultures (with GJ formation) was substantially greater than that of low density cultures (without GJ formation), showing that cell density affected OXA cytotoxicity. In addition to promoting GJ formation, the intimate contact between cells occurred via other mechanisms such as increased adherent proteins or vascular endothelial growth factor (VEGF) affecting cell growth and drug action (35,36). Subsequent experiments of pharmacologic modulation of GJs by chemical agents elucidated an additional positive relationship between GJs and OXA toxicity. To more directly assess the role of GJs in cell density-dependent OXA sensitivity, and also determine its effective component, we explored the effects of specific alteration of SMMC-7721 Cx expression using knockdown of Cxs expression with RNA interference and Cx overexpression by gene transfection. Data showed that knockdown of Cx26 expression depressed OXA toxicity. Conversely, upregulation of Cx26 expression enhanced OXA toxicity. The differences of OXA cytotoxicity mediated by Cx and cell density were similar, suggesting that OXA cytotoxicity was mediated by Cx26-composed GJs.

The role of GJs composed of different Cx subtypes in regulating OXA cytotoxicity was distinct. We found that specifically the inhibition of Cx26, not Cx32 or Cx43 expression, decreased OXA cytotoxicity. Additionally, upregulation of Cx26 expression enhanced GJ formation and ultimately OXA toxicity. It is not surprising that Cx43 expression knockdown did not affect the anti-tumor effect of OXA, as it may not be the main Cx isoform for GJs in SMMC-7721 cells. Its deficiency in modulating OXA toxicity, together with the results from initial cytologic study, strongly suggested a non-GJ-dependent Cx43 function in SMMC-7721 cells. Cx26 and Cx32 both have the potential to form liver GJs based on histologic and cytologic results, however, Cx32 did not exert any effect on OXA toxicity. The reason may be that the GJ channels, composed of different Cxs, mediate varying signal transduction (23,24). For example, Cx expression is cell- and tissue-specific, with multiple Cxs associated with unique functions (7). The transfection of Cx26 gene into HepG2 hepatoma cells localized to the membrane, inducing the recovery of GJ function, and reduced the malignant phenotype. However, Cx32 did not exhibit this function (37). The result was similar to our present study, confirming the idea that different Cx proteins perform different physiological and pathological functions (38). Similarly, the Cx component determines the selectivity of GJ channels for signaling ligands, and may thus result in the differential permeability to death or injury signal molecules induced by OXA.

In the present study, the toxic signals induced by OXA were propagated by Cx26 channels more easily or were specifically permeable to this channel, which may explain the discrepancy of OXA toxicity following siRNA knockdown of

distinct Cx in targeted SMMC-7721 cells (Fig. 6). We believe that these signals include the toxic OXA itself and its metabolites, or the molecules mediating the cellular death pathway. OXA and its cytoplasmic species have a molecular mass of about 400 Da, lower than the upper limit of GJ permeability. Alternatively, following OXA treatment of SMMC-7721 cells for 24 h, the induction and transfer of relevant proteins such as cyclins, apoptosis-related proteins and DNA injury-linked proteins cannot be ruled out (39,40). However, further studies are needed to explore the explicit signal molecules mediating the process.

In conclusion, histologic and cytologic data consistently demonstrate reduced expression and aberrant localization of the three dominant Cx proteins including Cx26, Cx32 and Cx43, abrogating the function of GJs in HCC tissues and cells significantly. Downregulated GJs comprising Cx26 specifically, but not Cx32 or Cx43 limited OXA cytotoxicity. Targeting Cx26 and even transiently increasing its expression, or reversing its internalization may represent an effective therapeutic strategy in liver cancer treatment.

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