

# The expression of Cx43, TGF $\beta$ /smads signaling pathways and PCNA in the occurrence and development of gastric carcinoma and the relationship among them

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## ABSTRACT

**Aims and Objectives:** To detect the protein expression level of Cx43, smad4 and PCNA in normal and gastric carcinoma tissue by immuno-histochemistry, western blotting and RT-PCR method. We intend to reveal the relationship between their expression in occurrence and development. Simultaneously through analyzing experimental data we have obtained the correlation among them. **Materials and Methods:** Immuno-histochemistry and western blotting were adopted to detect the expression of Cx43, smad4 and PCNA protein in normal and carcinomatous gastric tissue, expression levels mRNA of Cx43, smad4 and PCNA were detected by RT-PCR in normal and carcinomatous gastric tissue. Experimental data were analyzed by combining clinical pathology data using statistical approach. **Results:** The expression levels of Cx43, and smad4 in carcinomatous gastric tissue were lower than they were in normal gastric tissue. The expression level of PCNA in carcinomatous gastric tissue was higher than that in normal gastric tissue. The expression levels of Cx43, Smad4 and PCNA in groups of age and gender is not significant. The expression of Cx43, Smad4 and PCNA in different clinicopathologic features of carcinomatous gastric tissue including degrees of pathological differentiation, lymph node metastasis condition, depth of tumor invasion and TNM stages is statistically significant. With the progression of gastric carcinoma, the expression levels of Cx43 and Smad4 were decreased, conversely PCNA showed a high expression level. Based on the analysis Cx43 negatively correlated with PCNA, Cx43 positively correlated with Smad4, Smad4 negatively correlated with PCNA. **Conclusions:** The protein and mRNA expressions of Cx43, smad4 and PCNA were significant differences between normal gastric tissue and gastric carcinoma, also were concerned with differentiation degree, lymph node metastasis, TNM stages and depth of invasion, while not significant with age and gender. This suggests that they may play an important role in occurrence and development of gastric carcinoma, but also may have some interaction.

**Key words:** Gastric carcinoma, Cx43, Smad4, PCNA, Signal transduction, Gap junction

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## INTRODUCTION

Gastric cancer is one of the common digestive system malignant tumor, and its morbidity and mortality throughout the world are ranked among the top of various types of tumors. The incidence of male and female ratio is about 2:1 with more than 170000 deaths a year in gastric cancer patients in China harboring about 35% of the cases in the world.<sup>1</sup> Many scholars believe that the occurrence

and development of gastric carcinoma is a multi-factorial, multi-phased and multi-step process which involves the activation of oncogenes and the inactivation of tumor suppressor genes which is the basis of tumor formation and development. Adjacent cells are mediated through gap junction, intercellular communication which is composed of gap junction protein 43, in signal transduction processes of cell metabolism, stable internal environment, the proliferation and differentiation and other physiological

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Cx43 play an important role in regulation. Cx43 sub clusters often appear on the plasma membrane forming gap junction plaque, the number directly affects the signal transmission function between two cells.<sup>2</sup> Smad4 is the important members of the Smads protein family and also the cell TGF- $\beta$ 1/Smad signaling pathways critical components. It can almost combine with all activated R-Smads to form oligomers compounds, then Smad4 translocation to the nucleus to regulate transcription of target genes. Smad4 gene mutation or deletion can lead to its functional inactivation, and thus can not play a role in regulating transcription.<sup>3</sup> PCNA (proliferating cell nuclear antigen) are widely expressed in S phase of cell cycle, as auxiliary of DNA polymerase  $\delta$  it play an important role in DNA replication. It is necessary for eukaryotic cell DNA synthesis of coenzyme, its emergence is closely related to DNA synthesis in the cell cycle. It exists only in proliferating and tumor cells,<sup>4</sup> so the PCNA content is closely related to cell proliferation state and can be used as an indicator to control the degree of tumor cell proliferation and prognosis. Cx43 plays a key role of intercellular signal transduction. Smad4 which is key component of intracellular TGF $\beta$ /smads signaling pathways may be subject to intercellular signal conduction influence, thus affecting PCNA. In development of gastric carcinoma there may be correlations among them and research has not completely unveiled this sequence of events. By comparing the expression levels of Cx43, Smad4 and PCNA in gastric carcinoma and normal gastric mucosa tissues and their relationship with the clinico-pathological characteristics (differentiation, metastasis of lymph nodes, depth of invasion, TNM stages) of gastric carcinoma, they might provide theoretical basis to study the pathogenesis of gastric carcinoma and new approach of clinical treatment.

## MATERIALS AND METHODS

### Tissue samples

We collected 60 cases of gastric carcinoma which were surgically removed and certified by pathology and were not adopted by radiotherapy and chemotherapy in Chengde Medical College affiliated hospital from a period of August 2011 to October 2012. The findings were compared with 25 cases of normal gastric tissue (tissue away the tumor regarding as normal tissue for comparison). Gastric carcinoma tissues according to clinicopathological parameters which included differentiation degree, lymph node metastasis, TNM stages and depth of invasion were classified.

### Immunohistochemistry staining

Sections (5 $\mu$ m) were dewaxed and rehydrated according to pathology standardized procedures, immersed in 3% H<sub>2</sub>O<sub>2</sub> for 15 min at room temperature to quench

endogenous peroxidase activity. After washing twice with phosphate-buffered saline (PBS) for 5 min, Sections were heated in a microwave oven at 600W for 10 minutes in 0.01M citrate buffer (pH-6.0) and unspecified binding was blocked in 5% normal rabbit serum (0.1% BSA in PBS). Sections were incubated at 37°C for 2 h with primary antibody, rabbit anti-rat Cx43, Smad4 and PCNA (Golden Bridge Biotechnology Co Ltd.) 1:100. tissue sections were incubated at 37°C for 30 min with biotin-anti-rabbit IgG. After washing two times in PBS for 5 min, the sections were incubated with streptavidin for 30 min. Then the sections were washed two times in PBS for 5 min, and they were incubated with metal-enhanced 3, 3'-diaminobenzidine solution for 15 min, then they were washed two times in distilled water and counterstained with hematoxylin. In the negative control group, 5% normal rabbit serum was used in place of the primary antibody. The positive staining for Cx43 and Smad4 was expressed as brown-orange granules, which were mainly located in cell cytoplasm. The positive staining for PCNA was expressed as red brown granules, which were mainly located in cell nucleus. At least 5 high-power (x400 field) fields were chosen randomly for cell counting. The ratio of the positive distribution of Cx43, Smad4 and PCNA was calculated by dividing the number of positive cells over the total number of cells, and was expressed as percentage.

### Western blotting detection

A small amount of tissue was cut with clean scissors as possible, plus 400 $\mu$ l single detergent lysis buffer (containing PMSF, RIPA: PMSF volume ratio of 100:1) in the homogenizer. By the BCA method the sample and the standard are added to 96-well plates, protein concentration was determined in a microplate reader and the standard curve. Thirty  $\mu$ g tissue lysates were loaded and separated on polyacrylamide gels and then transferred to positive-charged PVDF membranes according to standard protocol. These blots were blocked for 2 hrs at room temperature in 5% skim milk. The target proteins were probed with primary antibodies and horseradish peroxidase-labeled secondary antibodies (Bioword Technology).  $\beta$ -actin was used as an indicator for equality of lane loading. Antibody positive bands were visualized using ECL Western blotting detection reagents (Solarbio Technology). The x-ray film was scanned and the band density was calculated using the ImagJ software.

### Preparation of RNA samples and RT-PCR

For RT-PCR experiments, 100 mg tissue (stored in liquid nitrogen) were homogenized in ice and were cut using ophthalmic scissors into pieces and immediately joined with precooled 1 mL Trizol reagent. RNA pellet was dried and RNA dissolved in RNase-free water was stored at -80°C for use. RNA was used as a template for amplification. Oligonucleotides as specific primers for Cx43, Smad4 and PCNA were synthesized by a company (Sangon Biotech,

Shanghai, China). As a PCR control reaction,  $\beta$ -actin was also detected in each run. The sequences of primers are as follows: Cx43:

FORWARD PRIMERS 5'-GTTCTATGTGATGCGAAAGGAAG,

REVERSE PRIMERS 5'-AGAGAGGAAACAGTCCACCTGAT;

SMAD4: F 5'-CCATCATAACAGCACTACCACCT,

R 5'-CTTCACTTTACATTCCTCAACTGC; PCNA:

F 5'-AACCTCACCAGTATGTCCAA,

R 5'-ATGTTTCCATTTCCAAGTTCTCC;

B-ACTIN: F 5'-CATGTACGTTGCTATCCAGGC,

R 5'-CTCCTTAATGTCACGCACGAT.

The expected sizes of PCR products were 316 bp, 404 bp, 329 bp for Cx43, Smad4 and PCNA respectively. The expected sizes of PCR products were 250 bp for  $\beta$ -actin. Complementary DNA was synthesized using AMV Reverse Transcriptase (Takara Biomedicals). PCR was performed following the procedure: Briefly 300 ng of RNA was used for RT-PCR. For CDNA synthesis 50 microliters system, after adding 1.25  $\mu$ L of a ribonuclease inhibitor (Takara), 10  $\mu$ L of  $MgCl_2$ , 5  $\mu$ L of dNTP (dATP, dCTP, dGTP, dTTP; Takara) and 2.5  $\mu$ L of AMV Reverse Transcriptase (Takara), 5  $\mu$ L of 10 $\times$ PCR buffer, the mixture was incubated at 30 $^{\circ}C$  for 10 min, 42 $^{\circ}C$  for 30 min, 99 $^{\circ}C$  for 5 min and then at 5 $^{\circ}C$  for 5 min as a run. The PCR mixture contained 10  $\mu$ L of cDNA, 10  $\mu$ L of 5 $\times$ PCR buffer, 24.5  $\mu$ L of DEPC-water, 2.5  $\mu$ L of Forward and Reverse PCR primer and 0.5  $\mu$ L of thermostable Taq polymerase (Takara). The amplification was done with a DNA thermal cycler (Research PCR System). After denaturation at 94 $^{\circ}C$  for 2 min, the amplification was done for 35 cycles at 94 $^{\circ}C$  for 30s, at 59 $^{\circ}C$  (Cx43), 58 $^{\circ}C$  (Smad4), 56 $^{\circ}C$  (PCNA) for 45 s, and at 72 $^{\circ}C$  for 60s. This was followed by a final extension for 10 min at 10 $^{\circ}C$ . Five microliter aliquots of the product was analyzed by electrophoresis on a 2% agarose gel and visualized by UV fluorescence after being stained with ethidium bromide.

### Statistical analysis

Using SPSS 17.0 statistical software for statistical analysis, the chi-square test was used to examine positive rates between groups comparison. Measurement data were expressed as mean  $\pm$  SD, Statistical significance was estimated by *t* test. Differences were considered significant when  $P < 0.05$ .

## RESULTS

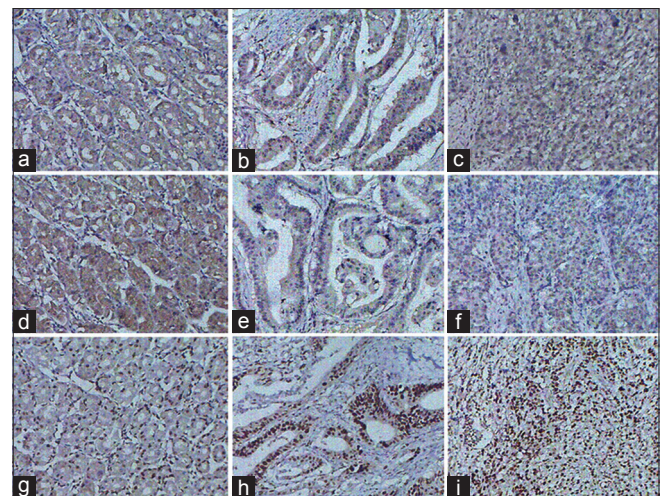
### Expression and localization of Cx43, Smad4 and PCNA by immunohistochemical staining

Normal gastric mucosa adjacent to gastric adenocarcinoma showed strong Cx43 and Smad4 positivity in the glandular compartment of gastric mucosa. Of the 25 cases of normal gastric mucosa, the rates of positive Cx43 and

Smad4 expression were 92% and 84% respectively. While 60 cases of gastric adenocarcinoma, the rates of positive for Cx43 and Smad4 were 65% and 58%, the findings were statistically significant for Cx43 and Smad4 between normal gastric mucosa and gastric adenocarcinoma. The rates of positive PCNA expression were 48% (gastric adenocarcinoma) and 82% (normal gastric mucosa), there was statistical significance (Table 1). Expression of Cx43 protein showed more actively patterns in well-moderately differentiation (23cases/27cases, 85%), without lymph node metastasis (20/25, 80%), invasion superficial muscular and submucosa (16/18, 89%), TNM stages I+II (23/28, 82%) gastric adenocarcinoma than poorly differentiation (16cases/33 cases, 48%), lymph node metastasis (19/35, 54%), invasion deep muscularis and serosa (23/42, 55%), TNM stages III+IV (16/32, 50%). The expressions of Smad4 in each group were very similar to Cx43 expression. On the contrary, higher PCNA protein expression levels in poorly differentiation (30cases/33 cases, 91%), lymph node metastasis (33/35, 94%), invasion deep muscularis and serosa (38/42, 90%), TNM stages III +IV (30/32, 94%) were noted. Expression levels of Cx43, Smad4 and PCNA protein in gastric adenocarcinoma were not statistically associated with age or gender (Table 2, Figure 1).

### Cx43, Smad4 and PCNA protein expression in gastric adenocarcinoma by Western blotting

To further verify the role of Cx43, Smad4 and PCNA, western blotting was employed to confirm the expression



**Figure 1:** a/b/c: Immunohistochemical stain for Cx43 protein( $\times 200$ ), a: strongly positive expression in normal gastric mucosa, b: positive expression in Well-moderately differentiated gastric adenocarcinoma, c: negative expression in poorly differentiated; d/e/f: Smad4 protein staining( $\times 200$ ), d: strongly positive expression in normal gastric mucosa, e: positive expression in Well-moderately differentiated gastric adenocarcinoma, f: negative expression in poorly differentiated; g/h/i: PCNA protein staining( $\times 200$ ), g: negative expression in normal gastric mucosa, h: positive expression in Well-moderately differentiated gastric adenocarcinoma, i: strongly positive expression in poorly differentiated

**Table 1: The expression of Cx43 in normal and carcinomatous gastric tissue**

Group	Cases no.	Cx43		Smad4		PCNA		P
		Preserved	Reduced	Preserved	Reduced	Preserved	Reduced	
Normal mucosa	25	23	2 <sup>Δ</sup>	21	4 <sup>Δ</sup>	12	13 <sup>Δ</sup>	P<0.05
Gastric Carcinoma	60	39	21 <sup>#</sup>	35	25 <sup>#</sup>	49	11 <sup>#</sup>	

<sup>Δ</sup> VS <sup>#</sup>: P<0.05

**Table 2: The expression of Cx43, Smad4 and PCNA in different clinicopathologic features of carcinomatous gastric tissue**

Group	Cases no.	Cx43		Smad4		PCNA		P
		Preserved	Reduced	Preserved	Reduced	Preserved	Reduced	
Gender								P>0.05
Male	38	24	14	19	19	31	7	
Female	22	15	7	16	6	18	4	
Age								P>0.05
>60	29	21	8	18	11	24	5	
≤60	31	18	13	17	14	25	6	
Differentiation								P<0.05
Well-moderately	27	23	4	20	7	19	8	
Poorly	33	16	17	15	18	30	3	
L/N mets								P<0.05
+	35	19	16	15	20	33	2	
-	25	20	5	20	5	16	9	
Depth of invasion								P<0.05
Superficial muscular and submucosa	18	16	2	15	3	11	7	
Deep muscularis and serosa	42	23	19	20	22	38	4	
TNM stages								P<0.05
I+II	28	23	5	21	7	19	9	
III+IV	32	16	16	14	18	30	2	

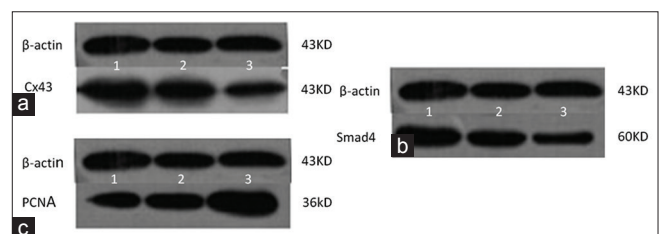
levels of these proteins. Expression was measured by the Optical density (OD). Of the 25 cases of normal gastric mucosa Cx43 protein mean OD value was 0.80±0.06, Of 60 cases of gastric adenocarcinoma the value was 0.37±0.06, there was statistical significance for Cx43 protein between normal gastric mucosa and gastric adenocarcinoma (Table 3). Specific expression of each group is as follows: Well-moderately differentiation (0.50±0.04), Poorly differentiation (0.23±0.03); Lymph node metastasis (0.21±0.03), without lymph node metastasis (0.59±0.04); Invasion deep muscularis and serosa (0.29±0.02), invasion superficial muscular and submucosa (0.55±0.03); TNM stages I +II (0.51±0.03), TNM stages III+IV (0.25±0.03), these expression differences between the groups were statistically significant (p<0.05). The expression of Cx43 in groups of age and gender were not significant. Expression of Smad4 protein in each group was very similar to Cx43 expression. Expression of PCNA is contrary to Cx43 and Smad4 expression form. Groups of poorly differentiation, lymph node metastasis, invasion deep muscularis and serosa and TNM stages III+IV have a higher protein OD value (Table 4, Figure 2).

**Expression of Cx43 mRNA, Smad4 mRNA and PCNA mRNA**

The expression of Cx43 mRNA and Smad4 mRNA in carcinomatous gastric tissue were lower than that of in

**Table 3: The expression of Cx43, Smad4 and PCNA Protein in normal and carcinomatous gastric tissue (x̄±S)**

Group	Cases no.	Protein OD			P
		Cx43	Smad4	PCNA	
Normal mucosa	25	0.80±0.06	0.86±0.05	0.32±0.03	P<0.05
Gastric Carcinoma	60	0.37±0.06	0.36±0.07	0.76±0.06	



**Figure 2:** 1. normal gastric mucosa, 2. Well-moderately Differentiated gastric adenocarcinoma; 3. Poorly differentiated gastric adenocarcinoma. a: Western blotting for Cx43 protein, b: Western blotting for Smad4 protein, c: Western blotting for PCNA protein

normal gastric tissue (p<0.05). The expression of Cx43 mRNA and Smad4 mRNA in gastric carcinoma which were well-moderately differentiated were higher than poorly differentiated gastric carcinoma (p<0.05) (Table 5). The expression of Cx43 mRNA and Smad4 mRNA in gastric carcinoma without lymph node metastasis were higher than

**Table 4: The expression of Cx43, Smad4 and PCNA Protein in different clinicopathologic features of carcinomatous gastric tissue ( $\bar{x}\pm S$ )**

Group	Cases no.	Protein OD			P
		Cx43	Smad4	PCNA	
Gender					
Male	38	0.35±0.02	0.33±0.02	0.74±0.05	P>0.05
Female	22	0.39±0.03	0.41±0.03	0.79±0.04	
Age					
>60	29	0.38±0.03	0.35±0.02	0.77±0.04	P>0.05
≤60	31	0.36±0.03	0.37±0.03	0.75±0.05	
Differentiation					
Well-moderately	27	0.50±0.04	0.50±0.04	0.59±0.05	P<0.05
Poorly	33	0.23±0.03	0.25±0.03	0.90±0.08	
Lymph node metastasis					
Yes	35	0.21±0.03	0.24±0.03	0.92±0.07	P<0.05
No	25	0.59±0.04	0.53±0.04	0.54±0.04	
Invasion depth					
Superficial muscular and submucosa	18	0.55±0.03	0.66±0.05	0.51±0.05	P<0.05
Deep muscularis and serosa	42	0.29±0.02	0.23±0.02	0.87±0.07	
TNM stages					
I+II	28	0.51±0.03	0.49±0.04	0.62±0.04	P<0.05
III+IV	32	0.25±0.03	0.25±0.03	0.88±0.06	

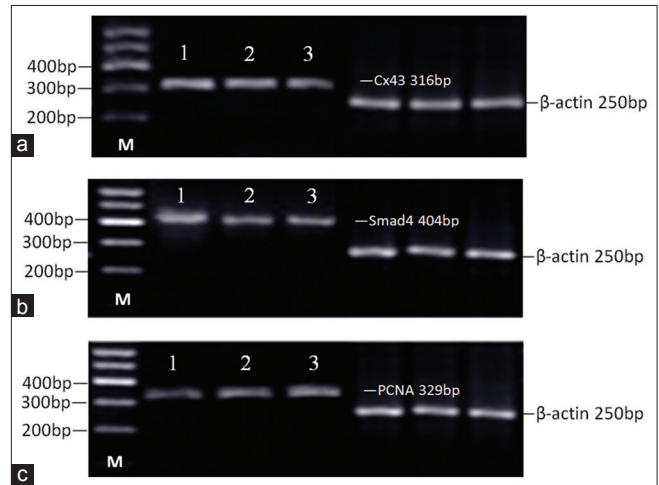
**Table 5: The expression of Cx43, Smad4 and PCNA mRNA in normal and carcinomatous gastric tissue ( $\bar{x}\pm S$ )**

Group	Cases no.	mRNA OD			P
		Cx43	Smad4	PCNA	
Normal mucosa	25	0.75±0.05	0.76±0.04	0.31±0.03	P<0.05
Gastric Carcinoma	60	0.37±0.07	0.30±0.07	0.69±0.08	

that of lymph node metastasis ( $p<0.05$ ). The expression of Cx43 mRNA and Smad4 mRNA in gastric carcinoma with TNM I+II were higher than that of TNM III+IV ( $p<0.05$ ). The expression of Cx43 mRNA and Smad4 mRNA invasion deep muscularis and serosa of gastric carcinoma were higher than that of invasion superficial muscular and submucosa gastric carcinoma ( $p<0.05$ ). PCNA mRNA expression for Cx43 mRNA and Smad4 mRNA has the opposite trend. Groups with poorly differentiation, lymph node metastasis, invasion deep muscularis and serosa and TNM stages III+IV had a higher mRNA OD value. The expression of Cx43 mRNA, Smad4 mRNA and PCNA mRNA in groups of age and gender were not statistically significant (Table 6, Figure 3).

**Correlation research among Cx43 and Smad4 and PCNA in the occurrence and development of gastric carcinoma**

To research the correlation among Cx43, smad4 and PCNA in development of gastric carcinoma through analyzing experimental data among them. In immunohistochemistry, the correlation coefficient Cx43 of and Smad4 protein is:  $r=0.53(P<0.01)$ ; the correlation coefficient of Smad4 and PCNA protein is:  $r=-0.49(P<0.01)$ ; the correlation coefficient Cx43 of PCNA protein is:  $r=-0.55(P<0.01)$ . By



**Figure 3:** M. 100 bp DNA Marker, 1. normal gastric mucosa, 2. Well-moderately Differentiated gastric adenocarcinoma, 3. Poorly differentiated gastric adenocarcinoma. a: RT-PCR for Cx43 mRNA, b: RT-PCR for Smad4 mRNA, c: RT-PCR for PCNA mRNA

Western blotting detection the correlation coefficient Cx43 of and Smad4 protein is:  $r=0.88(P<0.01)$ ; the correlation coefficient of Smad4 and PCNA protein is:  $r=-0.79(P<0.01)$ ; the correlation coefficient Cx43 of PCNA protein is:  $r=-0.75(P<0.01)$ . The correlation coefficient of Cx43 mRNA and Smad4 mRNA is:  $r=0.83(P<0.01)$ ; the correlation coefficient of Smad4 mRNA and PCNA mRNA is:  $r=-0.81(P<0.01)$ ; the correlation coefficient of Cx43 mRNA and PCNA mRNA is:  $r=-0.76(P<0.01)$ .

**DISCUSSION**

Gap junction which is used for the exchange of intercellular information is a special membrane structures. Gap junction

**Table 6: The expression of Cx43, Smad4 and PCNA mRNA in different clinicopathologic features of carcinomatous gastric tissue ( $\bar{x}\pm S$ )**

Group	Cases no.	mRNA OD			P
		Cx43	Smad4	PCNA	
Gender					
Male	38	0.35 $\pm$ 0.04	0.28 $\pm$ 0.03	0.68 $\pm$ 0.04	P>0.05
Female	22	0.40 $\pm$ 0.04	0.33 $\pm$ 0.04	0.70 $\pm$ 0.05	
Age					
>60	29	0.38 $\pm$ 0.05	0.31 $\pm$ 0.05	0.70 $\pm$ 0.05	P>0.05
$\leq$ 60	31	0.36 $\pm$ 0.04	0.29 $\pm$ 0.04	0.67 $\pm$ 0.04	
Differentiation					
Well-moderately	27	0.50 $\pm$ 0.04	0.41 $\pm$ 0.05	0.56 $\pm$ 0.04	P<0.05
Poorly	33	0.27 $\pm$ 0.02	0.21 $\pm$ 0.03	0.79 $\pm$ 0.07	
Lymph node metastasis					
Yes	35	0.26 $\pm$ 0.03	0.20 $\pm$ 0.03	0.83 $\pm$ 0.06	P<0.05
No	25	0.53 $\pm$ 0.05	0.44 $\pm$ 0.05	0.49 $\pm$ 0.04	
Invasion depth					
Superficial muscular and submucosa	18	0.53 $\pm$ 0.05	0.42 $\pm$ 0.06	0.42 $\pm$ 0.03	P<0.05
Deep muscularis and serosa	42	0.30 $\pm$ 0.03	0.25 $\pm$ 0.03	0.80 $\pm$ 0.06	
TNM stages					
I+II	28	0.55 $\pm$ 0.04	0.40 $\pm$ 0.04	0.56 $\pm$ 0.04	P<0.05
III+IV	32	0.21 $\pm$ 0.02	0.21 $\pm$ 0.02	0.80 $\pm$ 0.07	

is composed of the connexin subunits (connexin, Cx) across the membrane. Tumor characteristics embodied in the uncontrolled proliferation and differentiation abnormalities, there exists abnormal expression of Cx in tumors. Cx43 gap junction protein as a major participation in a variety of diseases and tumors may also have a great relationship with gastric cancer.<sup>5</sup> Cx43 is part of gap junction channel, in exchange of information between cells, and play the role of cell-cell contact inhibition. Normal cells and adjacent cells were positive expression, but contact inhibition role in tumor cells often disappear.<sup>6</sup> The most important features in cancer cells is loss of contact inhibition, resulting to significantly decreased Cx43 in cancer tissues, suggestive of Cx43 downregulation leading to abnormal or disappearance of cell gap junctional intercellular communication (gap junction intercellular communication, GJIC) which might be involved in gastric carcinogenesis process. As a new target in gastric cancer therapy remains to be our further research.

Our results from immunohistochemistry, Western blotting and RT-PCR technology confirms high expression of Cx43 in normal gastric mucosa and significantly reduced expression in gastric cancer tissue. The expression of Cx43 and clinical pathology of gastric cancer indicators, such as differentiation, lymph node metastasis, depth of invasion, TNM stage and so on have a great relationship with gastric cancer progression, metastasis and invasion, Cx43 expression gradually weakened, suggesting the absence of Cx43 may contribute to the progress of gastric cancer, which consistent with the findings reported by Nishitani et al.<sup>7</sup> In Western blotting Cx43 expression, the study conducted by Carystinos et al<sup>8</sup> found that the expression of

Cx43 in gastric mucosa tissue compared with normal gastric mucosa has been falling, Mine et al<sup>9</sup> study also confirmed the expression of Cx43 in normal mucosa was significantly higher than gastric cancer tissue, and were fully expressed in the normal gastric mucosa. Since very few research based on Cx43 expression in relation to gastric cancer prognosis. Another study conducted by Carystinos et al<sup>10</sup> found that the connection proteins involved in tumor metastasis, and the results also show that Cx43 may be involved in the process of tumor metastasis. So whether Cx43 is involved in gastric cancer lymph node metastasis, and may be an indicator of prognosis of gastric cancer, as new targets for gastric cancer treatment needs further research. Smad4 was first discovered Smads family abnormal gene, most of the pancreatic cancer exists in the gene absence or mutation.<sup>11</sup> In the TGF- $\beta$ 1 signaling transduction pathway, when the receptor-binding Smads (R-Smads) and T $\beta$ RI phosphorylation after separation, Smad4 then combined with the R-Smads to form heterologous oligomers, Smad4 translocation to the nucleus regulate transcription of target genes. Because it can almost combine with all activated R-Smads to form oligomers compounds, so in the TGF- $\beta$ 1 signal transduction it plays a central role. Smads molecular abnormalities, usually characterized by pathway regulating disorder, and transcription regulation would be unbalanced.<sup>12</sup> Smad4 tumor inhibition may be that it mediated TGF- $\beta$ 1 growth inhibition, including cut c-myc proto-oncogene expression and rise in CDK inhibitors P15 and P21 expression, thus played a entrainment effect of TGF- $\beta$ 1.<sup>13</sup> The majority of pancreatic cancer has been found to exist in absence of Smad4 gene, or mutation, and Smad4 inactivation also be found in gastric

cancer, colorectal cancer, breast cancer, lung cancer.<sup>14</sup> Our experimental results show that Smad4 in normal gastric mucosa tissues increased even fully expressed which means that the normal cell Smad4 in the regulation role of TGF-β1/Smads signaling pathway is crucial. Yet another study by Rodeck<sup>15</sup> findings on the role of Smad4 is consistent to the findings of the current study. In gastric clinical pathology such as the poorly differentiated, lymph node metastasis, invasion of deep myometrial even serosa and TNM stage into III, IV stage Smad4 expression levels were significantly decreased. Aiminleng et al<sup>16</sup> reported that Smad4 protein in gastric carcinoma was significantly lower than the adjacent tissues and Smad4 protein expression associated with the degree of differentiation of gastric carcinoma, poorly differentiated gastric carcinoma tissues Smad4 protein expression levels was significantly lower than well and moderate differentiation of gastric carcinoma, which is consistent with our findings.

In the process of the occurrence of gastric cancer, abnormal cell cycle regulation and uncontrolled cell proliferation are considered to be an important mechanism for gastric cancer. PCNA mainly exist in the G1 phase and S phase of cell cycle, as one of the important biological indicators which reflect the cell proliferation activity, and PCNA is closely related with the biological behavior of malignant tumors, including metastasis, invasion and prognosis.<sup>17</sup> PCNA expression in normal gastric mucosa weak and regular, limited to the gastric gland part zone, it may have to do with the metabolism of cells, new cells to replace aging nearly apoptosis of the cells. PCNA in poorly differentiated gastric carcinoma, lymph node metastasis, deep myometrial even serosa invasion and TNM stage into III, IV stage, PCNA expression showing a higher level, the results were consistent with Maga et al<sup>18</sup> findings, indicating that PCNA have a great relationship with the degree of invasion and prognosis. This study found that high expression of Cx43 and low expression of PCNA in normal gastric mucosa, low expression of Cx43 and high expression of PCNA in gastric carcinoma tissue. Wang Chunhong et al<sup>19</sup> pointed out the level of Cx43 expression and tumor cell proliferation ability was negatively correlated, which provide a reliable basis for our findings. Cx43 further decreased expression induced gap junction intercellular communication dysfunction, malignant proliferation of gastric epithelial cells, and the Cx gene may have a negative regulation role on cell proliferation. Our experiments found Smad4 expression at high levels in normal gastric mucosa, PCNA almost no expression, Smad4 expression in gastric cancer was significantly reduced while PCNA significantly higher PCNA expression. When Smad4 reduced expression will affect the TGF-β1 signaling pathway in normal transfer, resulting in gastric epithelial cell hyperplasia, which may

be a reflection of PCNA overexpression. Our experiments show that in normal gastric mucosa Cx43 and Smad4 keep a state of high expression and are downregulated in gastric carcinoma. Ping Dai et al<sup>20</sup> research have shown that TGF-β and Smad signaling pathway were through the medium of Cx43, found the TGF-β function relation with the Cx43 activities by Smad2/3, microtubule and Cx43 interactions.

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**Authors Contribution:**

**CL** – Contributed to the original idea, designed the study. **LP, HL and RS** – Conceived hypothesis, designed study, preparing of manuscript and reviewing the manuscript. **XZ** – Contributed to the study design, data analysis. **YC** – Contributed to patient enrolment, data analysis.

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