Int J Clin Exp Med 2016;9(8):16956-16963
www.ijcem.com /ISSN:1940-5901/IJCEM0016002

Original Article

Effect of electroporation method modified Cx43 gene on human esophageal cancer cell biological behavior

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Received September 11, 2015; Accepted March 15, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: pBudCE4.1_Cx43 was transfected into human esophageal cancer EC9706 cell by electroporation method in order to investigate the effect of Cx43 gene on cell growth, cell cycle and tumor migration and other aspects. Recombinant plasmid pBudCE4.1-Cx43 was transfected into human esophageal cancer EC9706 cells. By RT-PCR, Western blotting method, expression level of Cx43 mRNA and protein after transfection was detected. Scratching dye assay was used to detect intercellular communication function. Flow cytometry was used to detect cell cycle. CCK-8 proliferation assay, scratch healing assay, cell invasion assay were used to measured transfected Cx43 gene on human esophageal cancer cell proliferation, migration, and invasion abilities. The expression level of Cx43 mRNA and protein in experimental group, compared with that in control and blank group, was significantly higher with statistical difference ($P<0.05$). The fluorescence intensity of cell transfection was significantly higher than the control group and blank group with statistical significance ($P<0.01$). Growth rate of the cells slowed down in experimental group. The cell cycle was arrested in G0/G1 phase, and the number of cells in S phase were reduced with statistical difference ($P<0.05$); compared with control and blank group, CCK-8 proliferation experiments showed that in the experimental group, EC9706 cell proliferation was significantly inhibited ($P<0.001$), especially at 72 h. Scratch healing assay showed that in the experimental group migration were significantly decreased with statistical difference ($P<0.05$). Transwell chamber method showed that there was a significant decrease in invasiveness of EC9706 cells in the experimental group with statistical difference ($P<0.05$). The electroporation transfected Cx43 gene may inhibit migration ability and cell proliferation of human esophageal cancer.

Keywords: Esophageal cancer, connexin 43, transfection, EC9706 cell, cell proliferation

Introduction

Esophageal cancer is one of the most common gastrointestinal cancers; the incidence of esophageal cancer in recent years shows a gradually increasing trend; about 30 million patients died of esophageal cancer worldwide each year. An average of about 150,000 patients died of esophageal cancer annually in China; China has become one of a high incidence area of esophageal cancer in the world. Although chemotherapy and neoadjuvant chemotherapy have been widely used in the treatment of esophageal cancer, the prognosis of esophageal cancer is still not very good, especially in patients with clinical metastasis and tumor recurrence [1, 2]. Recent studies have found that reduced connexin (Cx) 43 expressions play a key role in the incidence and development of tumor; the abnormal expression of Cx43 may be associated with the proliferation and metastasis of malignant tumors [3-5]. Electroporation is a kind of efficient, reproducible and non-toxic technology with large samples and easily-controlled experimental parameters [6, 7]. A number of experimental studies have shown that electroporation plays a very good effect in the cells insensitive to traditional transfection, which is considered as the best molecular delivery system [8]. Inhibiting the expression of oncogenes or regulatory molecules by electroporation may be a new treatment strategy. This study used electroporation method to transfect pBudCE4.1-Cx43 into human esophageal cancer EC9706 cells, in order to explore the role of Cx43 gene on cell growth, cell cycle and tumor migration.

Materials and methods

The main materials

Human esophageal cancer EC9706 cells were purchased from Shanghai Institute of Cell Bank;

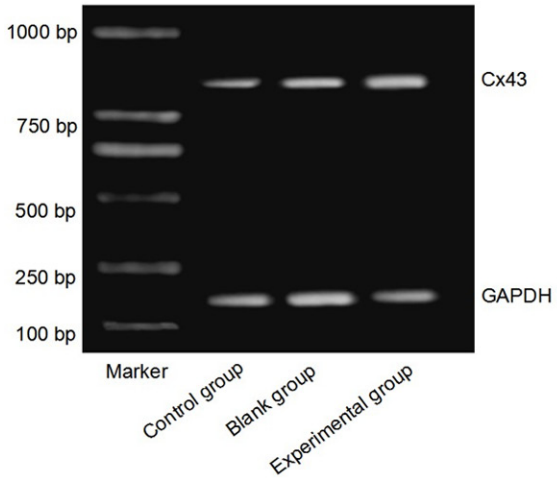
The following were other materials: DMEM medium, Opti-MEM (GIBCO BRL, USA), fetal bovine serum (Institute of Hematology, Chinese Academy of Medical Sciences); L plasmid extraction kit, RNA extraction reagent Trizol, G418, DNA gel purification extraction kit (Nanjing KGI Biotechnology Development Co., Ltd.) eukaryotic expression vector and empty vector pBudCE4.1_Cx43 pbudCE4.1, liposomal transfection reagent Lipofectamine 2000TM (Gibco US company); CR primer sequences (chemically synthesized by Invitrogen); C; Roche yellow liquid (lucifer yellow, sigma); DNA marker, RT-PCR two-step kit (US Promcga company); cDNA synthesis kit (Japan TOYOBO Company); AMV reverse transcription kit (Hangzhou Bioer); PCK-8 (Shanghai Yan Bin Chemical Company); Cx43 polyclonal rabbit anti-human antibody (Abcam US companies); mouse anti-GAPDH monoclonal antibody (Santa Cruz, USA); HRP labeled goat anti-mouse IgG (Santa Cruz, USA); HRP-labeled goat anti-rabbit IgG (Beijing Biosynthesis company); Matrigel gel (US BD Biosciences); PVDF membrane (Bio-Rad US companies); ECL chemiluminescence kit (Thermo American company).

Methods

Electric transfected Cx43 gene modification: Esophageal carcinoma cell line EC9706 were cultured in DMEM medium containing 10% fetal bovine serum at 37°C 5% CO_2 incubation chamber. Take the cell when they were in logarithmic growth phase to make them into a single cell suspension. They were washed with PBS, trypsin digested and centrifuged. Electroporation solution was added and they were resuspended. The cells were centrifuged at 1000 r/min for 5 min. The cells were washed for 3 times and resuspended. After the pellet was resuspended in electroporation solution, and the suspension was transferred to the cuvette, and added 10 μg plasmids. They were mixed and placed on ice for 0.5 h. The electroporation was performed, and the condition were set to voltage 450 V/cm, capacitor 25 μF, time 0.9 ms. At room temperature they were set for 0.5 h, then they were transferred to the dish with DMEM medium (containing 10% calf serum, 1% dual antibody). Finally they were placed in 37°C 5% CO_2 incubator and closed the box to culture. While electrical transfected with empty vector was served as the control. While transfecting, the cells were divided into three groups: transfected pbudCE4.1-Cx43 carrier was the EC9706/pbudCE4.1-Cx43 group (experimental group), transfected with empty vector pbudCE4.1 was the EC9706/control group (control group). untransfected HCC was EC9706 EC9706 group (control group). RNA interference was detected at 48 h after transfection.

RT-PCR detection: After discarding the culture medium, the cells were washed for three times with PBS. Total RNA was extracted using TRIzol method. By cDNA synthesis kit the cDNA was synthesized by reverse transcriptase. Cx43 and internal reference GAPDH were amplified. Cx43 upstream primer was: 5'-GTCGACATGGGTGACTGG AGCGCCTT-3 and downstream primer was: 5'-AATACCAGTGGATGTGATGCGG-3'. The amplification product was 1149 bp; GAPDH upstream primer was: 5'-GA GTCAACGGATTGG TCGT-3', and downstream primer was: 5'-GACAAGCTTCCCGTTCTCAG-3'. The amplified product was 185 bp. RT-PCR reaction conditions were as follows: 95°C pre-denaturation for 5 min, 95°C denaturation for 30 s, 60°C annealing for 30 s, 72°C extension for 60 s, with a total of 40 cycles. Through 1.2% agarose gel electrophoresis, PCR products were observed by gel imager. UVI gel imaging systems was used to photo. Image-Pro Plus 7.0 software was used to analysis strips gray value. The Cx43/GAPDH ratio represented Cx43 mRNA relative expression.

Western blotting detection: 1×10^7 cells were collected from each group. They were washed by PBS for three times. The total protein was extracted and protein concentration was determined by BCA method. Take 50 μg total proteins and add deionized water to 20 μl. Add 2 × the same volume of the above buffer, and denaturized at 99°C for 10 min. After centrifugation, draw 30 μl samples in the above. After separated by 10% SDS-PAGE, semidry method was used to electric transfected them onto PVDF membrane. They were closed at 37°C for 2 h with 5% nonfat dry milk. 1:1000 dilution of Cx43 polyclonal anti was added at 4°C overnight. TBST was used to wash membrane five times with each time for 5 min; 1:5000 HRP-labeled secondary antibody and GAPDH were added, and they were incubated at 37°C for 2 h. Then they were washed by PBS. ECL was



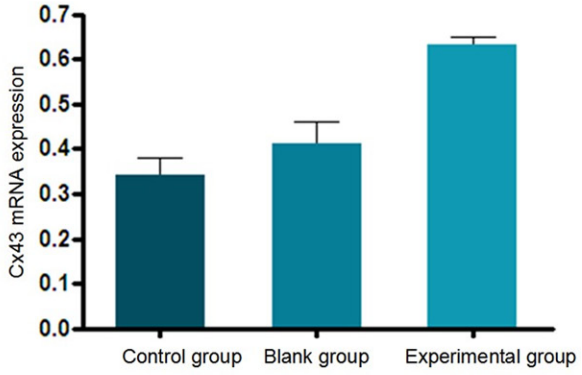
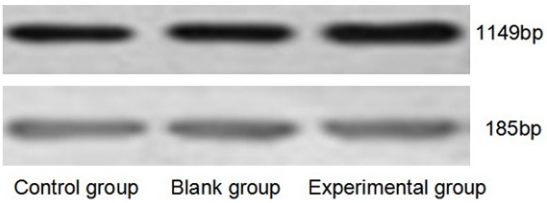


Figure 1. Detection of Cx43 mRNA expression in EC9706 cells by RT-PCR.



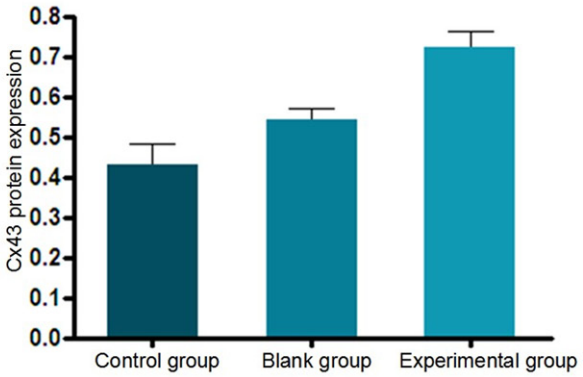


Figure 2. Detection of Cx43 protein expression in EC9706 cells by Western blot.

used for detection. X-rays was used for tabletting, developing and fixing in the dark room. UVI gel imaging systems was used to photo. Image-Pro Plus 7.0 software was used to analysis strips gray value. The Cx43/GAPDH ratio represented Cx43 protein relative expression.

Scratching loaded dye transfer test: Three groups of cells were seeded in culture dishes until the cells reached 100% confluence. The medium was discarded, and they were washed with PBS 3 times. 0.05% Roche yellow liquid (lucifer yellow, sigma) was added. Blades were crossed over the slide slightly several times, and then the cells were soaked for 3 min to remove the dye. PBS was used to wash three times, and then add a small amount of PBS to wet the cell. Fluorescence microscope was used to observe and photo. Randomly selected 10 regions from the beginning to the end and then observe with an OLMPUS optical microscope. Imagepr05.1 image analyzer was used to analysis fluorescence intensity. The average was calculated and it was served as the relative intensity of the fluorescence.

Flow cytometry detection: Collect three groups of cells and then washed by cold PBS twice. The cells were precipitated with 70% 4°C ice ethanol and mixed. Then wash the cells and adjust cell density to 1×10^6/L. They were incubated for 30 min with Tris-HCl buffer (pH 7.4) which containing 50 μg/ml RNA enzyme. After the DNA was stained by 1 $\mu g.ml^{-1}$ propidium iodide, they were stored in the dark at room temperature for 30 min. Flow cytometry was used to detect cell cycle. The experiment was repeated three times.

CCK-8 detection: Take the cells when they were in logarithmic growth phase, and adjust the cell density to 2×10^3 for each hole. 10% fetal calf serum was added to 200 μl DMEM medium, and each group were six wells. After cultured for 24 h, 10 μL CCK-8 were added to each hole. After incubated for 4 h in the incubator, the blank wells were served as the control. The

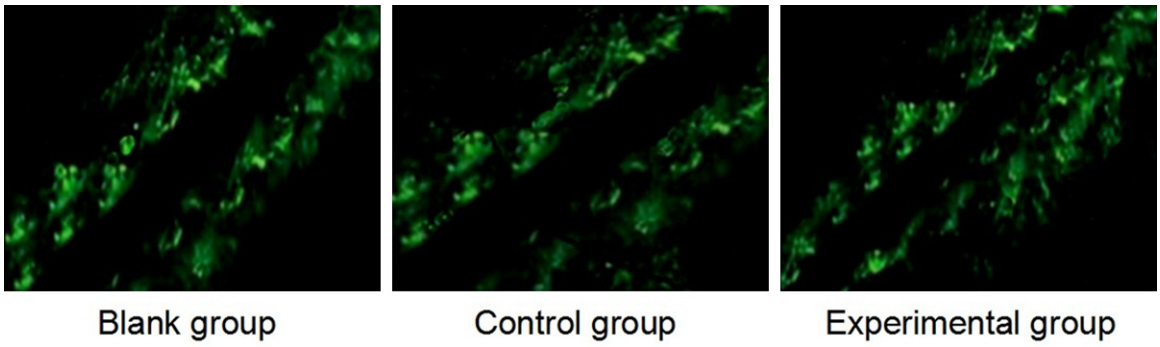


Figure 3. After transfected by pbudCE4. 1-Cx43, EC9706 cell communication was significantly restored compared with the control group and blank group; The intracellular fluorescent dye can be passed to the next 3-4 rows of cells.

Table 1. Effect of Cx43 gene modification on EC9706 cell cycle ($\bar{x}$±s, n=3)

Groups	G0/G1	S	G2/M
Blank	56.6±3.4	34.9±3.1	8.6±1.9
Control	55.8±4.1	35.2±2.9	9.1±1.3
Experimental	67.8±3.6*	25.2±1.8*	8.4±1.0

Compared with the blank a control groups, *P<0.05.

micro plate reader detected absorbance values (A) at 450 nm and cell growth curve was drawn. The mean absorbance value of the cell was the vertical axis, while time 48, 72, 96 and 120 h were the horizontal axis and the growth curve was drawn.

Cell scratching impairment experiment: Take the three kinds of cells when they were in logarithmic growth phase, and they were seeded in 6-well plates with density 5 × 10^3 cells/well. When the cells grew to monolayer cells in each group, 10 μl pipette tip was used to scratch in the monolayer with the style "-". PBS was used to wash slightly and 2 ml cell culture medium without Serum was added for culture. After 48 h, cell migration was observed under an inverted microscope with 100 times amplification.

Chamber invasion assay: The Polycarbonate microporous membrane was capped with Matrigel (50 μg/hole); in the lower room of the well-polymerized chamber, 10% fetal bovine serum was added as the conditioned medium; in the upper chamber, 100 μl suspended solutions of treated EC9706 cells of above three groups were added (total number of cells was 3 × 10^5/L); after incubated in an incubator for 24 hours, the tumor cells which did not pass through membrane were carefully scraped off with a cotton swab; the chamber was fixed in 95% ethanol for 5 minutes, gently rinsed with PBS for three times, stained with hematoxylin for 10 min, rinsed with BS, and dried naturally. The polycarbonate membrane of the upper chamber was carefully removed with a scalpel blade along the edge, secured on the slides with a resin glue (with the inner surface side up), and mounted; after drying, under a 200 × optical microscope, invasion cells in five horizons (upper, lower, left, right, middle) in each film were counted respectively, and the average was calculated. In each group, three chambers were arranged in parallel; the experiment was repeated three times.

Statistical analysis

SPSS16.0 statistical software was used; measurement data were expressed as $\bar{x}$±s; differences among many groups were compared using One-way ANOVA and q test; P<0.05 or P<0.01 indicated a statistically significant difference.

Results

Cx43 mRNA expression

RT-PCR to detect the expression of Cx43 mRNA showed that the bands of EC9706/pbudCE4. 1-Cx43 group (experimental group) was significantly wider compared with EC9706/control group (control group) and EC9706/pbudCE4. 1 group (blank group); Differences were statistically significant between the experimental group and control group, between the experimental group and blank group (P<0.05), shown in **Figure 1**.

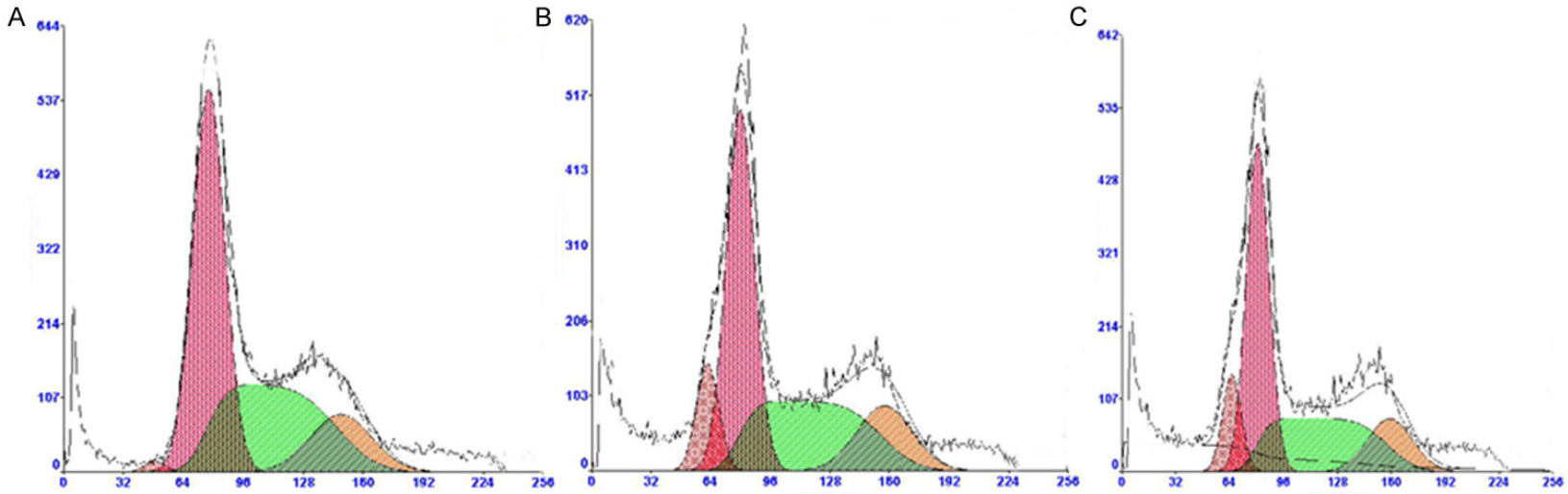


Figure 4. The flow cytometry picture for all groups. A: Blank group; B: Control group; C: Experimental group.

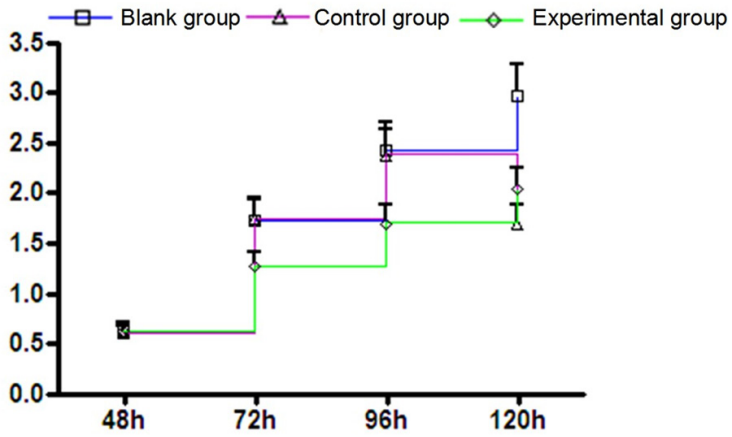


Figure 5. Growth curve of three groups.

Table 2. Absorbance at 450 nm ($\bar{x}$±s, n=5)

Groups	48 h	72 h	96 h	120 h
Blank	0.63±0.08	1.73±0.22	2.43±0.27	2.97±0.31
Control	0.61±0.07	1.74±0.19	2.38±0.25	2.91±0.24
Experimental	0.62±0.09*	1.27±0.15	1.70±0.18	2.04±0.21

Cx43 protein expression

Detection of Cx43 protein expression by Western blot showed that the bands of EC9706/pbudCE4. 1-Cx43 group (experimental group) was significantly wider compared with EC9706/control group (control group) and EC9706/pbudCE4. 1 group (blank group); There were statistically significant differences in the gray value (P<0.05), shown in **Figure 2**.

Scrape loading dye transfer test

GJIC changes detected by scrape loading dye transfer test: Fluorescent dye transfer showed that, cellular communication was missing in EC9706/control group (control group) and EC9706/pbudCE4.1 group (blank group); cellular communication in EC9706/pbudCE4.1-Cx43 group (experimental group) was significantly restored after pbudCE4.1-Cx43 transfection: the intracellular fluorescent dye can be passed to the next 3-4 rows of cells (**Figure 3**).

Detection of cell cycle

Flow cytometric to detect cell cycle showed that, cells in the experimental group (EC9706/pbudCE4. 1-Cx43 group) increased slightly in the G0/G1 phase compared with the control group and the blank group, which showed a slight decrease in S phase. Between the experimental group and control group or blank group, there were significant differences (P<0.05); M phase cells had no significant changes; the difference was not statistically significant (P>0.05), indicating significant G0/G1 phase arrest (**Table 1**; **Figure 4**).

Effect on cell proliferation

The growth curve drawn by CCK-8 test results showed that, the curve of EC9706/pbudCE4. 1-Cx43 group (experimental group) was significantly lower than that of C-7721/control group and EC9706/pbudCE4. 1; The difference was statistically significant (P<0.05, **Figure 5**; **Table 2**).

Cell scratch wound experiment

48 hours later, scratches of EC9706/pbudCE4. 1-Cx43 group were slowly healed, while scratches in M EC9706/control group and EC9706/pbudCE4. 1 group had been basically covered, shown in **Figure 6**.

Vitro invasiveness of EC9706 cells

As shown in **Figure 7**: the number of cells across the membrane in M EC9706/control group and EC9706/pbudCE4. 1 group were higher (65.48±5.72) and (63.26±6.84); Penetrating cells in EC9706/pbudCE4. 1-Cx43 group were significantly reduced (35.44±4.27); The difference was statistically significant (P<0.05). Results showed that Cx43 gene modification can effectively reduce the invasiveness of EC9706 cells.

Discussion

Cx-mediated gap junction intercellular communication (GJIC) plays an important role in cell growth and differentiation. Cell gap junction gene is a class of non-mutated tumor suppressor gene discovered in recent years; the connexin encoded by the gene family (Cx) is the structural and functional unit of gap junctions. The defects in gap junction caused by the decrease of Cx expression are related with

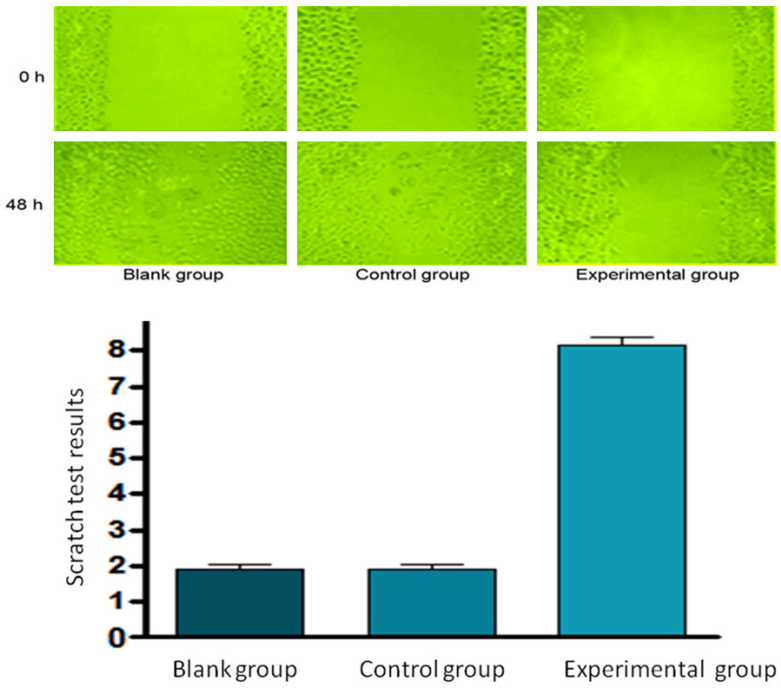


Figure 6. Cell scratch wound experiment results: 48 hours later, scratches of EC9706/pbudCE4. 1-Cx43 group were slowly healed, while scratches in M EC9706/control group and EC9706/pbudCE4. 1 group had been basically covered, indicating that migration of the experimental group was significantly decreased (× 100).

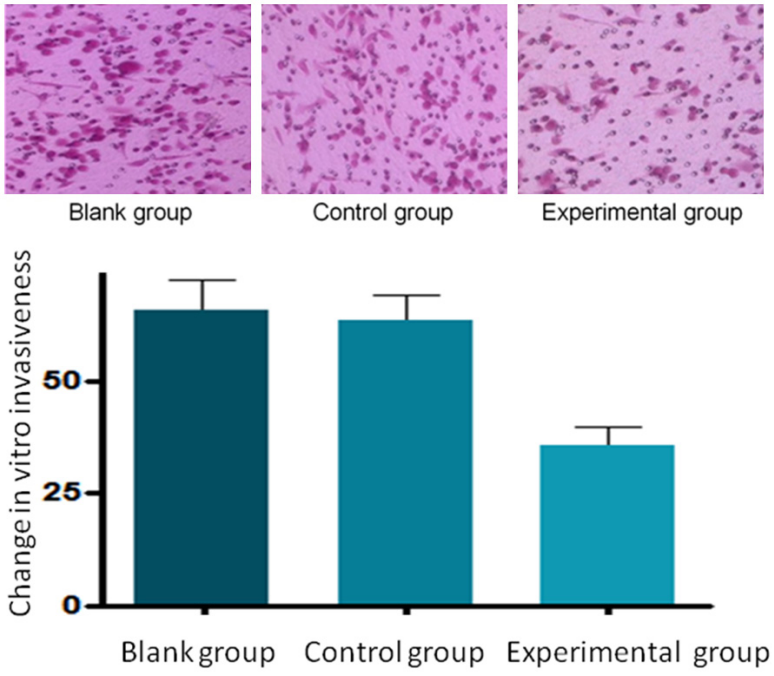


Figure 7. Chamber invasion assay results: As it can be seen from **Figure 4** 48 hours later, the number of cells across the membrane in the experimental group was significantly reduced (35.44±4.27) compared with the control group (65.48±5.72) and the blank group (63.26±6.84); the differences were statistically significant (P<0.05).

malignant proliferation of a variety of tumors, which may be an important part of tumorigenesis [9]. The study shows that decreased or absent connexin 43 (Cx43) gene expression exists in a variety of tumors, with gap junctional intercellular communication interruption [10]. Studies have reported [11] that in esophageal squamous cell carcinoma, the relative expression of Cx43 mRNA and protein expression rate were lower than those in normal esophageal mucosa and adjacent atypical hyperplasia tissues. In esophageal squamous cell carcinoma, the relative expression levels of Cx43 mRNA decreased with the degree of differentiation decreased. It showed a positive correlation between the expression of Cx43 mRNA and protein. Positive rate of Cx43 protein and m RNA was significantly reduced with the depth of cancer invasion deepened; in cancerous tissues with lymph node metastasis, it was significantly lower than that in the cancerous tissues without lymph node metastasis. Cx43 gene can inhibit the occurrence, development, invasion and metastasis of ESCC, which is an effective indicator to early predict ESCC invasion and metastasis. Decrease in Cx43 gene expression may lead to the weakening or disappearance of GJIC, causing loss of normal growth control, which is closely related to the formation of esophageal squamous cell carcinoma and is one of the important mechanisms of the formation of esophageal squamous cell carcinoma [12].

Presently the mechanisms of Cx43 gene inhibiting cell proliferation is not very clear. Most scholars believe that upregulated Cx43 gene expression in tumors can promote the recovery of GJIC to play a role in the inhibition of tumor cell growth [13]. In this study, after recombinant plasmid pBudCE4.1_Cx43 was transfected into human esophageal cancer EC9706 cells, Cx43 mRNA and protein expression was significantly increased compared with the control group and the blank group; scratches loading dye transfer test showed that intercellular communication had been effectively recovered; after up-regulating Cx43 gene to recover GJIC of tumor cells, the cell cycle was arrested in G0/G1 phase; MMC-7721 cell proliferation was inhibited significantly, especially at 72 h. Scratch healing assay showed that cell migration was significantly decreased; Transwell chamber assay showed a significant decrease in MMC-7721 invasiveness in the experimental group, indicating that Cx43 gene transfection could inhibit the migration of human esophageal cancer EC9706 cells and inhibit tumor cell proliferation.

In short, the dysfunction in GJIC caused by reduced expression of Cx43 not only is closely related with tumor cell migration and invasion, but also plays an important role in promoting tumor cell proliferation. It showed good efficacies in inhibiting tumor cell growth, inducing the apoptosis of tumor cells, preventing cancer invasion and metastasis, and anti-tumor angiogenesis. Therefore, transfecting recombinant plasmid pBudCE4.1_Cx43 into human esophageal cancer EC9706 cells may provide new ideas for the treatment of human esophageal cancer and other malignant tumors.

Disclosure of conflict of interest

None.

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References

[1] Wei WQ, Yang J, Zhang SW, Chen WQ and Qiao YL. Analysis of the esophageal cancer mortality in 2004-2005 and its trends during last 30 years in China. Zhonghua Yu Fang Yi Xue Za Zhi 2010; 44: 398-402.

[2] Hou L, Gong HJ, Li J and Zhang XZ. Effects of radiation on the expression of cyclooxygenase-2 in Eca-109 cell line and it's radiosensitization value. Journal of Modern Oncology 2012; 20: 35-38.

[3] Shimizu M, Zaninotto G, Nagata K, Graham DY and Lauwers GY. Esophageal squamous cell carcinoma with special reference to its early stage. Best Pract Res Clin Gastroenterol 2013; 27: 171-186.

[4] Fokas E, Weiss C and Rödel C. The role of radiotherapy in the multimoda management of esophageal cancer. Dig Dis 2013; 31: 30-37.

[5] Zeng Q and Xia XB. Comparison of electroporation and lipofection efficiency in retinal Müller cells. International Eye Science 2010; 2: 247-249.

[6] Wei F, Wang F, Liu XJ and Li HM, Tian SH, Huang Q. Inhibition of choroidal melanoma cell growth by electroporation-mediated transfer of immunologic cytokines or anti-angiogenesis genes. Chinese Journal of Ocular Fundus Diseases 2011; 27: 33-36.

[7] Lassmann S, Schuster I, Walch A, Göbel H, Jütting U, Makowiec F, Hopt U and Werner M. STAT3 mRNA and protein expression in colorectal cancer: effects on STAT3-inducible targets linked to cell survival and proliferation. J Clin Pathol 2007; 60: 173-179.

[8] Chen JX, Xia J, Liu JW and Cui XY. Experimental Study on Apoptosis Induced by Ginsenoside Rg3 in Human MCF-7 Breast Cancer Cell Line. Carcinogenesis, Teratogenesis & Mutagenesis 2005; 17: 213-216.

[9] Bodenstine TM, Vaidya KS, Ismail A, Beck BH, Diers AR, Edmonds MD, Kirsammer GT, Landar A, Welch DR. Subsets of ATP-sensitive potassium channel (KATP) inhibitors increase gap junctional intercellular communication in metastatic cancer cell lines independent of SUR expression. FEBS Lett 2012; 586: 27-31.

[10] Li YY, Zhang B, Han F, Liu ZY and Jin FG. Progress on Connexin43. Progress in Modern Biomedicine 2012; 12: 3731-3733.

[11] Dong ZW, Qiao YL, Wang GQ, Zhang SZ, Qian GS, Tang BJ, You WC, Zhou Q, Zhang LW, Wan DS, Chen JG, Liu Q, Yuan Y, Li JB, Zhao FH, Wei WQ, Meng XZ, Zhen S, Wang GQ, Li GL, Lei ZL and Kong LZ. The Exploration of Evaluating Indicators for Early Detection and Treatment of Cancers in China 2010; 19: 633-638.

[12] Lin HW, Bai H, Su M, Xiao P, Chen KS and Zhang HX. Expression of gap junction protein Cx26 and Cx43 and its relationship with the invasion and metastasis of esophageal squamous cell carcinoma. Cancer Research on Prevention and Treatment 2011; 38: 809-813.

[13] Schlemmer SR and Kaufman DG. Kaufman. Re-establishment of gap junctional intercellular communication (GJIC) between human endometrial carcinomas by prostaglandin E2. Exp Mol Pathol 2012; 93: 441-448.