Effects of *Cx43* gene modification on the proliferation and migration of the human lung squamous carcinoma cell line NCI-H226

J.-P. Zang and R. Wei

Department of Respiratory Medicine, People's Hospital of Zhengzhou, Zhengzhou, China

Corresponding author: R. Wei
E-mail: weirandr@163.com

ABSTRACT. In this study, the human lung squamous carcinoma cell line NCI-H226 was transfected with the recombinant plasmid pBudCE4.1_Cx43 to explore the role of the *Cx43* gene in cell growth, cell cycle, and tumor migration. pBudCE4.1-Cx43 was transfected into human lung squamous carcinoma NCI-H226 cells using Lipofectamine TM2000. The mRNA and protein expressions of Cx43 in the transfected cells were detected by reverse transcriptase polymerase chain reaction and western blot analysis. The cell-cell communication was detected using the scratch dye tracer method and the cell cycle was detected by flow cytometry. The CCK-8 proliferation, scratch healing, and cell invasion assays were performed to evaluate the effect of the *Cx43* gene transfection on the proliferation, migration, and invasive abilities of NCI-H226 cells. Cx43 mRNA and protein expressions and the fluorescence intensity in the scratch healing test were significantly higher in the experimental group than those in the control and blank groups ($P < 0.05$ and < 0.01, respectively). The CCK-8 proliferation assay and the scratch healing experiment revealed significantly inhibited NCI-H226 cell proliferation (especially 72 h after incubation) and cell migration, respectively, in the experimental group, compared to the control and

blank groups (P < 0.001 and <0.05, respectively). The transwell chamber test showed a statistically significant decrease in the invasive ability of NCI-H226 cells in the experimental group (P < 0.05). Therefore, Cx43 gene transfection could inhibit the migration of human lung squamous carcinoma cell line NCI-H226, thereby inhibiting tumor cell proliferation.

Key words: Lung squamous carcinoma; Cx43; Transfection; NCI-H226 cells; Proliferation

INTRODUCTION

Lung cancer is a multifactorial and multi-step progressive disease, with high worldwide rates of morbidity and mortality. Lung squamous carcinoma is a histological type of lung cancer, with progressively younger ages of onset; this may be characterized by early lymphatic or blood metastasis, and is a serious threat to human health (Feist et al., 2012). With the continuous development of functional genomics and the completion of the human genome project, scholars have identified a close correlation between tumor cell proliferation and migration and gene regulation (involving the complex activation and deactivation of various genes), which provides a novel and effective way for the treatment of cancer (Bastide et al., 2010; Kunert-Keil et al., 2011). The gap junction is composed of the subunits of integral cell membrane proteins and connexin (Cx); it is the membrane connecting the channel between adjacent cells, including Cx43. The Cx43-mediated gap junction intercellular communication (GJIC) facilitates and maintains the transmission of information and energy between cells, regulation of cell growth, proliferation and differentiation, and homeostasis. Therefore, blockade of the Cx43 expression in tumor cells could inhibit tumor cell proliferation (Wayakanon et al. 2012). Very few studies (at home and abroad) have reported any correlation between Cx43 and the proliferation, invasion, and migration of lung squamous cells. In this study, recombinant plasmid pBudCE4.1_Cx43 was transfected into human lung squamous carcinoma NCI-H226 cells to assess its impact on the proliferation, invasion, and migration of human NCI-H226 cells, in order to provide a theoretical basis for the Cx43 gene modification treatment of lung squamous carcinoma, and to improve the clinical efficacy of squamous carcinoma treatment.

MATERIAL AND METHODS

Materials

Human lung squamous carcinoma NCI-H226 cells were purchased from Shanghai Cell Bank (Chinese Academy of Sciences). Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM were obtained from Gibco (Life Technologies, Carlsbad, CA, USA). Fetal bovine serum was obtained from the Institute of Hematology (Chinese Academy of Medical Sciences); Recombinant eukaryotic expression plasmid pBudCE4.1_Cx43 and empty vector pbudCE4.1 were offered by Professor Liu Fenghua of the Tianjin Cancer Hospital. The liposomal transfection reagent Lipofectamine 2000TM was also obtained from Gibco. The L plasmid extraction kit, RNA extraction reagent TRIzol, G418, and the DNA gel purification and recycling kit was obtained from Nanjing KGI Biotechnology Development Co., Ltd. (Nanjing, China). Lucifer yellow was obtained from Sigma-Aldrich (St. Louis, MO, USA). The DNA marker and RT-PCR two-step kit was obtained from

Promega (Madison, WI, USA), while the cDNA synthesis kit was purchased from Toyobo (Osaka, Japan). The AMV reverse transcription kit, polymerase chain reaction (PCR) primer sequences, CCK-8, and the rabbit anti-human Cx43 polyclonal antibody was purchased from Hangzhou Bioer Technology Company Ltd. (Hangzhou, China), Invitrogen (Life Technologies), Shanghai Yan Bin Chemical Company (Shanghai, China), and Abcam (Cambridge, UK), respectively. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and mouse anti-GAPDH monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-labeled goat anti-rabbit IgG, polyvinylidene difluoride (PVDF) membrane, Matrigel, and the ECL chemiluminescence kit was purchased from Beijing Biosciences (Beijing, China), Bio-Rad (Hercules, CA, USA), BD Biosciences (San Jose, CA, USA), and Thermo Scientific Fisher (Waltham, MA, USA), respectively.

Methods

Cx43 gene modification

NCI-H226 cells were cultured in DMEM supplemented with 10% fetal calf serum, and incubated in a sealed incubator (37°C, 5% CO_2) for culturing and passage. Cells in the logarithmic phase of growth were used for all experiments. NCI-H226 cells were seeded to 6-well plates at a density of 2×10^5 cells/well; upon achieving a fusion rate of 80%, the cells were transfected using Lipofectamine. The transfected cells were divided into three groups: cells transfected with pbudCE4.1-Cx43 were included in the NCI-H226/pbudCE4.1-Cx43 group (experimental group); cells transfected with the empty vector pbudCE4.1 were included in the NCI-H226/control group (control group); while the non-transfected NCI-H226 cells were included in the NCI-H226 group (blank group). The effects of transfection were detected 48 h after transfection.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The cells were rinsed thrice with phosphate buffered saline (PBS) after discarding the culture medium. Total RNA was extracted using TRIzol according to the manufacturer protocols. cDNA was synthesized by reverse transcription, using the commercial cDNA synthesis kit (as per the standard protocols). Cx43 was amplified along with GAPDH internal control. The primer sequences used were as follows: Cx43: upstream, 5'-GTCGACATGGGTGACTGG AGCGCC TT-3' and downstream, 5'-GCGGCCGCCTAGATC T CCAGGTCATCAGG-3' (product: 1149 bp) and GAPDH: upstream, 5'-GA GTCAACGGATTGG TCGT-3' and downstream, 5'-GACAAGCTTCCCGTTCTCAG-3' (product: 185 bp). The PCR conditions were set as follows: pre-denaturation at 95°C for 5 min, and 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR products were separated on a 2% agarose gel via electrophoresis, and the results of the separation were observed under ultraviolet light and photographed using a UVI gel imaging system. The gray values of the bands were analyzed using the Image-Pro Plus 7.0 software (San Francisco, CA, USA). The Cx43/GAPDH value represented the relative expression of *Cx43* mRNA.

Western blot

The cells (1×10^7) were collected and washed thrice with PBS. Total protein was extracted and the protein concentration determined using the bicinchoninic acid (BCA) method. Fifty microgram total protein was dissolved in 20 μL deionized water, and an equal volume of

2X sample buffer was added prior to submitting the samples to denaturation at 99°C for 10 min. The denatured samples were centrifuged (-80°C), and 30 µL of the supernatant were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel. The separated proteins were transferred onto PVDF membranes using semidry electricity, and the samples were sealed using 5% nonfat dry milk at 37°C for 2 h. The membranes were incubated overnight with the Cx43 polyclonal antibody (1:1000) at 4°C. The membranes were washed with Tris-Buffered Saline and Tween 20 (TBST) 5 times (5 min each) The HRP-labeled secondary antibody and GAPDH (1:5000) were added to the samples, which were incubated at 37°C for 2 h. The membranes were subsequently washed with PBS, and the proteins detected using ECL. The X-ray film was exposed in the dark, the film was rinsed, and the membranes photographed using the UVI gel imaging system. Gray values of the bands were analyzed using the Image-Pro Plus 7.0 software, with Cx43/GAPDH representing the relative expression of Cx43.

Scratch loading dye transfer test

The cells in all three groups were seeded onto culture dishes. Upon reaching 100% confluence, the medium was discarded, and cells were washed thrice with PBS. Lucifer yellow solution (0.05%) was added to these cells. Light crosses (scratches) were made to the slides using scalpel blades. The cells were then soaked for 3 min to remove the dye. The cells were washed thrice with PBS and subsequently wetted with PBS. The cells were then observed under a fluorescence microscope and photographed. Ten regions were randomly selected for photography, and observed using an optical microscope (Olympus,Osaka, Japan). The fluorescence intensity was analyzed using the Image Pro 5.1 image analyzer; the average was calculated as the relative intensity of fluorescence.

Flow cytometry

Cells from each group were collected and washed twice with cold PBS. These cells were then precipitated and mixed with 70% cold ethanol (4°C) for further experiments. The cells were washed and the cell concentration adjusted to 1×10^6 cells/L with PBS. The cells were then incubated for 30 min with Tris-HCL buffer (pH 7.4) containing 50 µg/mL RNAse. One microgram per milliliter of 100 µg/mL propidium iodide (PI) was to stain DNA, and placed in a dark room for 1 h. The cell cycle was detected by flow cytometry. This experiment was repeated thrice.

CCK-8 assay

Cells in the exponential phase of growth were seeded in 96-well plates at a density of 2×10^3 cells/well, and cultured in DMEM (200 µL) supplemented with 10% FBS (6 wells per group) for 24 h. CCK-8 (10 µL) was added to each well and incubated for 4 h (blank wells, without cells, were used as controls). Absorbance (A_{450}) was detected using a microplate reader (Beijing, China). The growth curve was extrapolated using the average A values as the vertical axis and the time points (at 48, 72, 96, and 120 h) as the horizontal axis.

Scratch healing assay

Cells in the logarithmic phase of growth in all three groups were seeded to 6-well plates at

a density of 5×10^3 cells/well. Cells were cultured until the formation of a monolayer; the monolayers were subjected to "-" scratches using 10 L pipette tips. After gentle PBS washing, 2 mL serum-free DMEM was added to each well for continuous culture. Cell migration was observed after 48 h under a 100-fold inverted microscope (Olympus,Osaka, Japan).

Chamber invasion assay

Polycarbonate membrane filters were capped with Matrigel (50 µg/hole). Fetal bovine serum (10%) was added as conditioned medium to the polymerized lower chamber. One hundred microliter of the NCI-H226 cell suspension (3×10^5 cells/ L) was added to the upper chamber. This set-up was incubated for 24 h, and the tumor cells on the filter surface that did not pass through the chamber were carefully scraped off with a swab, fixed in 95% ethanol for 5 min, gently rinsed thrice with PBS, and stained with hematoxylin (10 min). The chamber was washed with PBS and dried naturally. The upper polycarbonate membrane was then carefully removed with a scalpel slice along the edge, fixed on glass slides with resin glue (inner side facing up), and sheet-sealed. The cells were dried, and the invasive cells in the upper, lower, left, right, and middle fields were counted under a 200X light microscope (calculating the average). Three parallel chambers were set up in each group, and the experiment was repeated thrice.

Statistical analysis

The SPSS v.16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to analyze the obtained results. The measured data was presented as the mean ± standard deviation (mean ± s). Differences among the groups were compared using one-way ANOVA and the q-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Cx43 mRNA expression

RT-PCR detection of the *Cx43* mRNA expression showed that the bands in the NCI-H226/pbudCE4.1-Cx43 (experimental) group were significantly wider than those in the NCI-H226/control (control) and NCI-H226/pbudCE4.1 (blank) groups. Statistically significant differences were observed between the experimental and control groups, and the experimental and blank groups ($P < 0.05$ each; Figure 1).
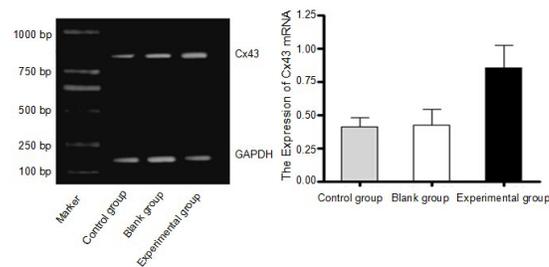
Figure 1. Detection of Cx43 mRNA expression in NCI-H226 cells by reverse transcriptase polymerase chain reaction (RT-PCR).

Cx43 protein expression

The western blot analysis revealed significantly wider bands in the experimental NCI-H226/pbudCE4.1-Cx43 group compared to the NCI-H226/control (control) and the NCI-H226/pbudCE4.1 (blank) groups. Statistically significant differences were observed among the gray values ($P < 0.05$; Figure 2).
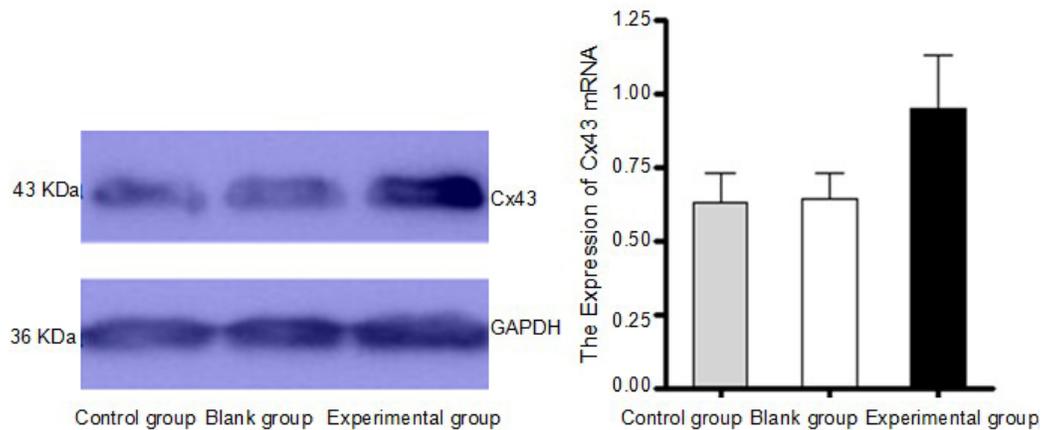
Figure 2. Detection of Cx43 protein expression in NCI-H226 cells by western blot analysis.

Scratch loading dye transfer test

Changes in the gap junctional intercellular communication (GJIC) were detected by the scratch loading dye transfer test. The results showed missing cell communication in the NCI-H226/control (control) and NCI-H226/pbudCE4.1 (blank) groups. However, the communication of pbudCE4.1-Cx43-transfected cells was significantly recovered in the NCI-H226/pbudCE4.1-Cx43 (experimental) group, with the fluorescent dye being propagated to the subsequent 3–4 rows of cells (Figure 3).
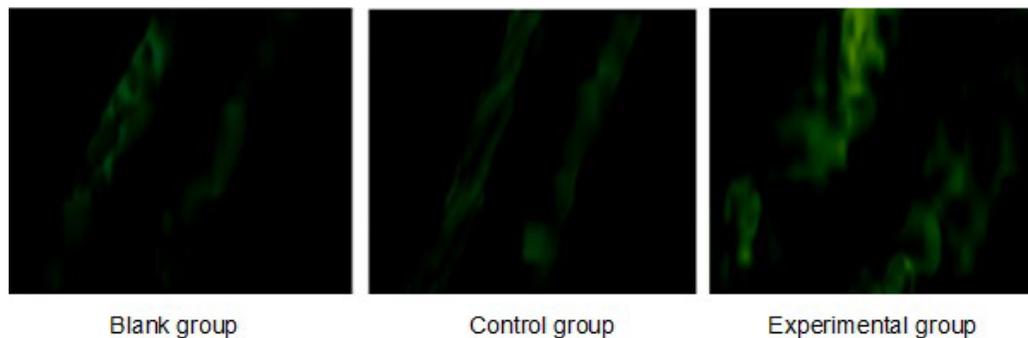
Figure 3. The communication of pbudCE4.1-Cx43-transfected NCI-H226 cells was significantly recovered in the NCI-H226/pbudCE4.1-Cx43 (experimental) group, compared to the control and blank groups; the fluorescent dye was passed on to the next 3-4 rows of cells.

Cell cycle detection

Flow cytometry results revealed a slight increase and decrease in the number of cells in the G0/G1 and S phases of growth, respectively, in the experimental NCi-H226/pbedCE6.1-Cx43 group, compared to the C-7721/control (control) and NCI-H226/pbudCE4.1 (blank) groups. The differences were statistically significant ($P < 0.05$). No significant changes were observed in the M phase, and the difference was not statistically significant ($P > 0.05$), indicating that the cell cycle was arrested in the G0/G1 phase by the modification of the *Cx43* gene (Table 1).

Table 1. Effect of the Cx43 gene modification on the NCI-H226 cell cycle (mean ± s; N = 3).

Group	G0/G1	S	G2/M
Blank	56.5 ± 3.4	35.1 ± 2.3	8.9 ± 0.8
Control	55.7 ± 3.2	36.3 ± 2.7	9.2 ± 1.0
Experimental	67.0 ± 3.6*	25.4 ± 1.4*	8.3 ± 1.2

*P < 0.05, compared to the blank and control groups.

Effects of cell proliferation

Based on the results of the CCK-8 test, the growth curve of the NCI-H226/pbudCE4.1-Cx43 (experimental) cells was significantly lower than that of the C-7721/control (control) and the NCI-H226/pbudCE4.1 (blank) groups (Figure 4). These differences were statistically significant ($P < 0.05$; Table 2).
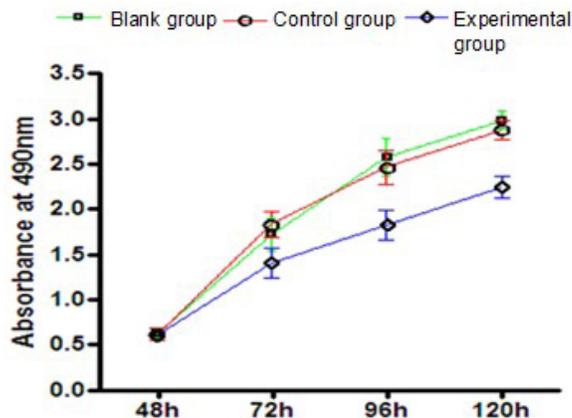

Figure 4. Growth curves of the experimental, control, and blank groups.

Table 2. Absorbance at 450 nm (mean ± s; N = 5).

| Group	48 h	72 h	96 h	120 h
Blank	0.60 ± 0.10	1.73 ± 0.15	2.43 ± 0.19	2.91 ± 0.20
Control	0.61 ± 0.09	1.73 ± 0.14	2.35 ± 0.14	2.82 ± 0.21
Experimental	0.60 ± 0.10*	1.30 ± 0.11	1.73 ± 0.14	2.01 ± 0.10

Scratch healing assay

The scratches in the experimental NCI-H226/pbudCE4.1-Cx43 group were observed to heal slowly after 48 h, while those in the NCI-H226/control (control) and NCI-H226/pbudCE4.1 (blank) groups were almost covered (Figure 5).
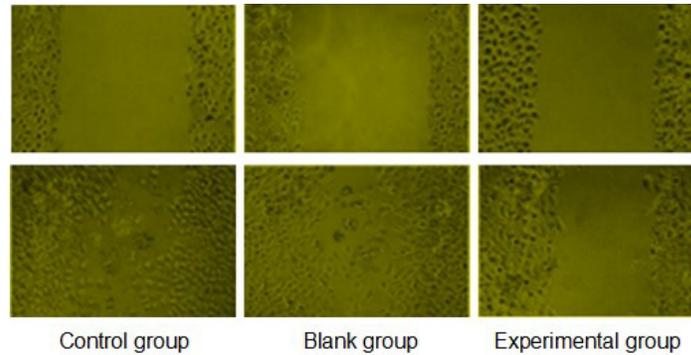
Figure 5. Results of the scratch healing assay. After 48 h, the scratches in the NCI-H226/pbudCE4.1-Cx43 (experimental) group were observed to heal slowly, while the scratches in the NCI-H226/control (control) and NCI-H226/pbudCE4.1 (blank) groups had basically recovered, indicating a significant decrease in the migration of the experimental group.

Changes in NCI-H226 invasiveness *in vitro*

Higher numbers of invasive cells were observed in the NCI-H226/control (control) group and NCI-H226/pbudCE4.1 (blank) groups (64.34 ± 3.65 and 65.21 ± 4.70, respectively). Significantly reduced numbers of invasive cells were observed in the NCI-H226/pbudCE4.1-Cx43 (experimental) group (27.48 ± 4.54). This difference was statistically significant ($P < 0.05$). The results revealed that modifications in the *Cx43* gene can effectively reduce the invasiveness of NCI-H226 cells (Figure 6).
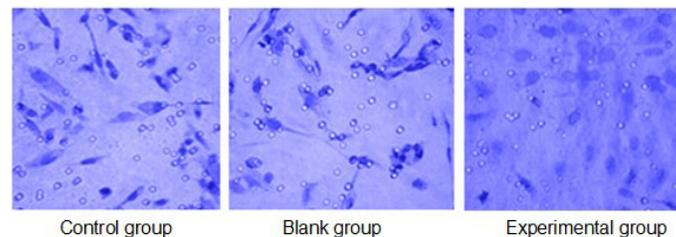
Figure 6. Results of the chamber invasion assay. After 48 h, the number of invasive cells in the experimental group was significantly reduced (27.48 ± 4.54) compared to that in the control and blank groups; this difference was statistically significant ($P < 0.05$).

DISCUSSION

Lung cancer is a systemic disease, with increasing incidence and a gradual younger age of onset. Lund cancer may result in early lymph or blood metastasis, seriously affecting human health (Feist et al. 2012). Currently, clinicians adopt surgery, chemotherapy, radiotherapy,

hormonal therapy, biological molecular targeted therapy, and other comprehensive programs for the treatment of lung cancer patients. However, its local recurrence and distant metastasis are complex biological processes and are major problems plaguing the clinicians (Ho et al. 2013). Lung squamous carcinoma is a type of histological lung cancer. Cancer gene therapy has attracted the attention of medical practitioners worldwide with the development of functional genomics and the completion of the human genome project (Bastide et al. 2010). Its proliferation, invasion, and migration are related to the abnormalities in gene function and structure. Therefore, the identification of key genes involved in the incidence of lung squamous carcinoma is significant for the treatment of lung squamous carcinoma (Chen and Zhang, 2006).

Cell connections mainly include the gap, tight, and desmosome junctions (Ruttenstock et al. 2012). Gap junctions are involved in energy, material, and information transfer between normal cells, the regulation of cell proliferation and differentiation, and the maintenance of cellular homeostasis and metabolism. Cx43 is the major protein subunit of gap junctions in the cell membrane (Ton and Kathryn Iovine, 2012). Tumor incidence and metastasis are related to uncontrolled proliferation and the abnormal differentiation of tumor cells (Teixeira et al. 2007). Studies have revealed the positive, but significantly reduced, expression of Cx43 in tumor cells (Moore et al. 2008; Solan and Lampe, 2014), resulting in the disappearance of the most important contact inhibition in tumor cells, and a decline in tumor cell binding capacity. This suggested that the down-regulation of the *Cx43* gene could cause GJIC dysfunction or disappearance, allowing for the escape of normal cells from growth control and immune surveillance, leading to greater growth and easy proliferation and metastasis of tumor cells. It is involved in the pathogenesis and metastasis of tumors, a novel tumor suppression gene discovered in recent years. The development of RNA interference technology has led to its becoming a hot spot in cancer gene therapy. Transcribed silencing genes mediated by dsRNA molecules have been discovered to efficiently and specifically inhibit the expression of various genes associated with cancer incidence, in order to effectively treat the tumors (Neuhaus et al. 2009). In this study, recombinant plasmid pBudCE4.1-Cx43 was transfected into human lung squamous carcinoma NCI-H226 cells using Lipofectamine TM2000. The Cx43 mRNA and protein expressions in the transfected cells were detected by RT-PCR and western blot, respectively. The scratch dye tracer method was used to detect cell-cell communication, and the cell cycle detected by flow cytometry. The CCK-8 proliferation assay, scratch healing assay, and cell invasion assay was performed to evaluate the effects of transfection of the *Cx43* gene on the proliferation, migration, and invasion of NCI-H226 cells. The results revealed a statistically significant increase in the Cx43 mRNA and protein expressions in the experimental group, compared to the control and blank groups. The fluorescence intensity in the experimental group was also (statistically) significantly higher than that in the control and blank groups. On the other hand, the growth rate of NCI-H226 cells was (statistically) significantly reduced in the experimental group; that is, the cell cycle was arrested in the G0/G1 phase and a reduced number of cells entered the S phase. The CCK-8 proliferation assay showed a significant inhibition in the proliferation of NCI-H226 cells in the experimental group, compared to the control and blank groups, especially at 72 h. The scratch healing assay showed a statistically significant decrease in the NCI-H226 cell migration in the experimental group compared to the control and blank groups. The transwell chamber test showed a statistically significant decrease in the invasive ability of NCI-H226 cells in the experimental group, compared to the control and blank groups.

In summary, down-regulated Cx43 expression was correlated with tumor incidence and progression. The results of this study revealed that the *Cx43* gene modification inhibits the proliferation and migration of human lung squamous carcinoma NCI-H226 cells. This lays an experimental foundation for lung squamous carcinoma gene therapy.

Conflicts of interest

The authors declare no conflict of interest

REFERENCES

Bastide K, Ugolin N, Levalois C, Bernaudin JF, et al. (2010). Are adenosquamous lung carcinomas a simple mix of adenocarcinomas and squamous cell carcinomas, or more complex at the molecular level? *Lung Cancer* 68: 1-9.

Chen X and Zhang Y (2006). Myocardial Cx43 expression in the cases of sudden death due to dilated cardiomyopathy. *Forensic Sci. Int.* 162: 170-173.

Feist A, Lee R, Osborne S, Lane J, et al. (2012). Increased incidence of cutaneous squamous cell carcinoma in lung transplant recipients taking long-term voriconazole. *J. Heart. Lung. Transplant.* 31: 1177-1181.

Ho CF, Chan KW, Yeh HI, Quo J, et al. (2013). Ketone bodies upregulate endothelial connexin 43 (Cx43) gap junctions. *Vet. J.* 198: 696-701.

Kunert-Keil C, Steinmüller F, Jeschke U, Gredes T, et al. (2011). Immunolocalization of glycodelin in human adenocarcinoma of the lung, squamous cell carcinoma of the lung and lung metastases of colonic adenocarcinoma. *Acta Histochem.* 113: 798-802.

Moore JC, Tsang SY, Rushing SN, Lin D, et al. (2008). Functional consequences of overexpressing the gap junction Cx43 in the cardiogenic potential of pluripotent human embryonic stem cells. *Biochem. Biophys. Res. Commun.* 377: 46-51.

Neuhaus J, Heinrich M, Schwalenberg T and Stolzenburg JU (2009). TGF-beta1 inhibits Cx43 expression and formation of functional syncytia in cultured smooth muscle cells from human detrusor. *Eur. Urol.* 55: 491-498.

Ruttenstock EM, Doi T, Dingemann J and Puri P (2012). Prenatal retinoic acid upregulates connexin 43 (Cx43) gene expression in pulmonary hypoplasia in the nitrofen-induced congenital diaphragmatic hernia rat model. *J. Pediatr. Surg.* 47: 336-340.

Solan JL and Lampe PD (2014). Specific Cx43 phosphorylation events regulate gap junction turnover *in vivo*. *FEBS Lett.* 588: 1423-1429.

Ton QV and Kathryn Iovine M (2012). Semaphorin3d mediates Cx43-dependent phenotypes during fin regeneration. *Dev. Biol.* 366: 195-203.

Teixeira TF, da Silva TC, Fukumasu H, de Lima CE, et al. (2007). Histological alterations in the livers of Cx43-deficient mice submitted to a cholestasis model. *Life Sci.* 81: 380-384.

Wayakanon P, Bhattacharjee R, Nakahama K and Morita I (2012). The role of the Cx43 C-terminus in GJ plaque formation and internalization. *Biochem. Biophys. Res. Commun.* 420: 456-461.