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Connexin 43 recruits E-cadherin expression and inhibits the malignant behaviour of lung cancer cells

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Abstract: The interaction of connexin 43 and E-cadherin may play an important role in carcinogenesis and malignant behaviour of tumours. In this study, we examined the relationship between connexin 43 and E-cadherin in human non-small cell lung cancers (NSCLC). Expression levels of connexin 43 and E-cadherin were examined in 107 NSCLC specimens by immunohistochemistry. The connexin 43 gene was transfected into lung cancer LH_7 cells. The protein localizations and levels of connexin 43 and E-cadherin were detected using immunofluorescence staining and western blot. Cell cycle and proliferation of lung cancer cells were examined using flow cytometry and MTT. We found that reduced expression of both connexin 43 and E-cadherin significantly correlated to poor differentiation, advanced TNM stage, and lymph note metastasis of NSCLCs. Connexin 43 and E-cadherin expression significantly correlated with each other. Over-expression of connexin 43 significantly induced E-cadherin expression. Moreover, connexin 43-transfected LH_7 cells showed significantly decreased cell proliferation. The percentage of cells in G1 phase increased, while the number of cells in S and G2 phases significantly decreased. We concluded that concurrent reduction of connexin 43 and E-cadherin may contribute to the development of lung cancer. Connexin 43 may induce E-cadherin expression and inhibit cell proliferation and progression of lung cancer.

Key words: Connexin 43 - E-cadherin - Lung Cancer

Introduction

Both gap junctions and adhesion molecules play a critical role in cell contact inhibition, differentiation, proliferation, and homeostasis of various tissues. Recent evidence suggests that gap junctions are not simply pore-forming proteins, but interact with cell adhesion-associated proteins and participate in signalling events [1-3].

Connexin 43 is one of the most common connexins and the major connexin homolog expressed in lung tissue [4-6]. Connexin 43 and E-cadherin play important roles in carcinogenesis and tumour metastasis [7-9]. They are concurrently expressed in many tumours [10,11]. Hernandez-Blazquez et al. indicated that E-cadherin can regulate connexin 43 expression and function [12]. However, it is still unclear that whether connexin 43 and E-cadherin are concurrently expressed in lung cancers and affect each other or not. In this study, we examined the expression of connexin 43 and E-cadherin in primary lung cancers to clarify the correlation between connexin 43 and E-cadherin, and explore the clinical and pathological implications of expression of these proteins. We also transfected LH_7 cells with connexin 43 gene and investigated the possible effect of connexin 43 on E-cadherin.

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Materials and Methods

Patients. A total of 107 samples and 15 corresponding normal lung tissue samples were selected randomly from patients with squamous cell carcinomas (SCC) or adenocarcinomas who underwent surgery in the First Affiliated Hospital of China Medical University between 2001 and 2003. The study was conducted according to institutional review board regulations at China Medical University. There were 69 males and 38 females in our study, with ages ranging from 33 - 76 years, (mean = 57 years). The tumours were diagnosed as SCC (n = 45) or adenocarcinomas (n = 62) [13]. These tumours showed different degrees of differentiation and were classified as well- (n = 38), moderately- (n = 30), or poorly- (n = 39)

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Table 1. Expression of connexin 43 and E-cadherin and their relation to clinicopathologic characteristics.

Characteristics	n	Connexin 43			E-cadherin		
		Positive	Negative	*P*	Positive	Negative	*P*
E-cadherin							
Positive	72	51	21	<0.001			
Negative	35	3	32				
Differentiation							
Well	38	26	12	0.009	30	8	0.002
Moderate	30	15	15		24	6	
Poor	39	13	26		18	21	
Histological type							
Adenocarcinoma	62	33	29	0.503	42	20	0.907
Squamous cell carcinoma	45	21	24		30	15	
TNM stage							
I	50	33	17	0.006	39	11	0.001
II	30	13	17		23	7	
III-IV	27	8	19		10	17	
Lymph node metastasis							
Yes	57	16	41	<0.001	32	25	0.009
No	50	38	12		40	10	

differentiated [13]. Fifty-seven cases indicated lymphatic metastasis. All the tumours were classified as stages I, II, and III-IV (n = 50, 30, and 27, respectively) [14,15]. All the resected specimens were fixed with 10% neutral-buffered formalin and embedded in paraffin blocks.

Immunohistochemistry. Tissue blocks were cut into 4-μm sections, deparaffinized, rehydrated, and immunostained with monoclonal anti-connexin 43 antibody (1:50; Santa Cruz Biotechnology, USA) and monoclonal anti-E-cadherin antibody (1:100; Santa Cruz Biotechnology, USA). Detection was performed using the streptavidin-peroxidase method. Negative control slides were stained in the absence of primary antibodies.

Based on the scale of Nemeth et al. [16], the immunostaining grade of connexin 43 and E-cadherin was as follows: specimens in which none, ≤ 25%, > 25% - < 75%, and ≥ 75% of tumour cells showed positive staining were defined as (-), (+), (++), and (+++), respectively. Samples scoring (-) - (+) were considered negative, and those that scored (++) - (+++) were considered positive.

Cell culture and transfection. The LH_7 cell line is derived from the PG cell line, established from a human pulmonary giant cell carcinoma. LH_7 cells were a gift from Dr. Jie Zheng, College of Medicine, Beijing University, China. LH_7 cells are highly metastatic and express low levels of connexin 43 [17, 18]. The LH_7 cells were grown in RPMI 1640 medium (GiBCO, USA) with 10% fetal calf serum (GiBCO, USA), at 37°C in a humidified atmosphere (5% CO_2 95% air).

The connexin 43 expression vectors pcDNA3.1(+)-Cx43 were obtained from Professor YW Zhang (Tokyo Medical and Dental University, Japan) as a gift [2]. LH_7 cells were transfected with pcDNA3.1(+)-Cx43 or pcDNA3.1(+) as control using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche, USA). After 36 h, the culture medium was changed and G418 (Roche, USA) was added at a concentration of 350 μg/ml. Culture medium was changed every three days. After four weeks of selection, six neomycin resistant clones were selected and kept in culture with the same concentration of G418 during every following experiment.

Western blot. LH_7 cells transfected with pcDNA3.1(+)-Cx43 (called LH_7-Gja1 cells) or pcDNA3.1(+) [called LH_7(-) cells] were lysed and separated by 10% SDS-PAGE, and were transferred to a polyvinylidene fluoride membrane. The transferred samples were further incubated with anti-connexin 43 antibody (1:300) and anti-E-cadherin antibody (1:300) overnight. The proteins were visualized with an automatic electrophoresis analytic system (ChemiImager 5500, AlPha InnCh, USA). β-actin was used as internal control.

Immunofluorescence staining. Cells were fixed, permeablised and incubated with anti-connexin 43 (1:300) and anti-E-cadherin antibodies (1:300) overnight, and labelled with a FITC-conjugated secondary antibody for 1 h, followed by staining with 0.1% DAPI for 3 min. Cells examined using an immunofluorescence microscope (BX60, OLYMPUS, Japan).

Cell cycle and proliferation. The cells were grown in a 96-well plate at a density of 1.5×10^6 cells/ml for 48 h, and stained with 1 mg/ml propidium iodide (Sigma, USA). The percentages of LH_7-Gja1, LH_7,

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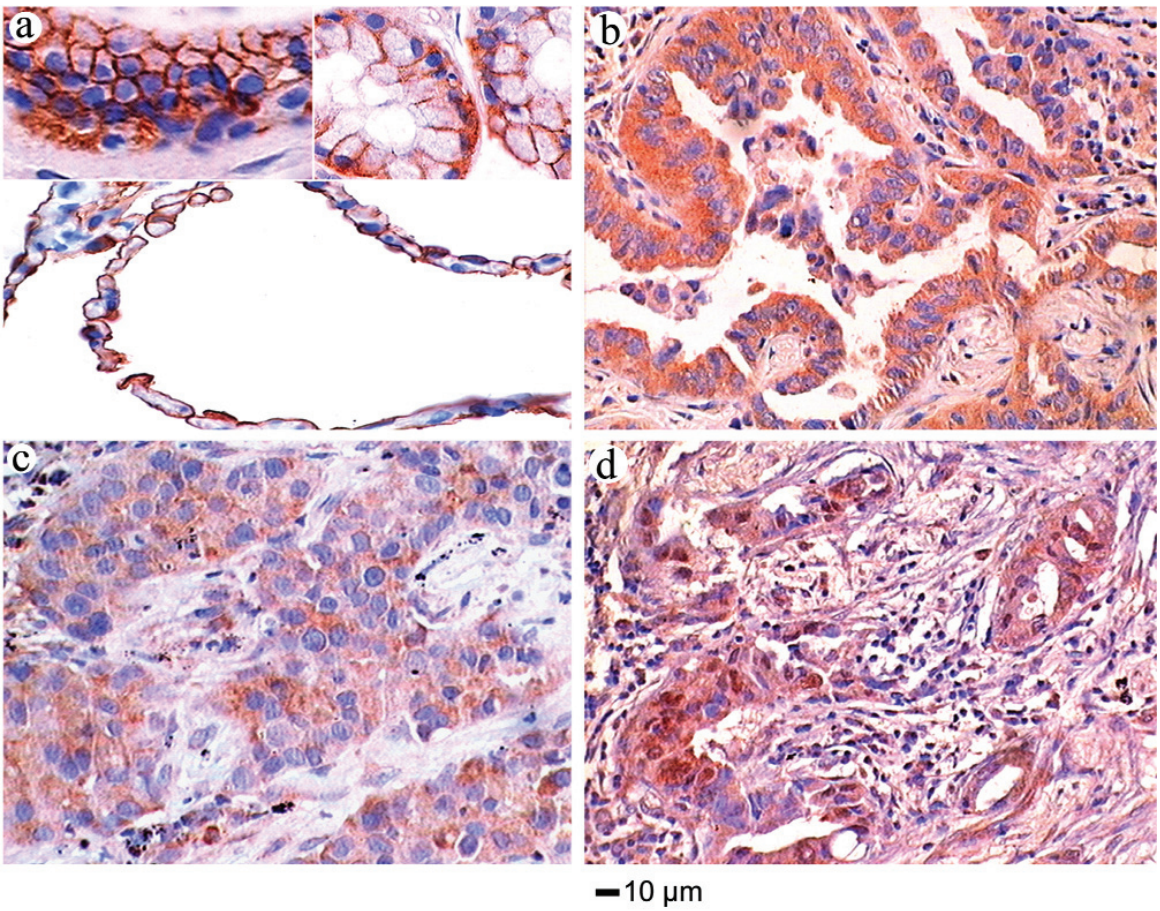

Fig. 1. Expression of connexin 43 and E-cadherin in lung cancers and normal lung tissues. (**a**) upper left: membrane expression of E-cadherin in normal epithelial cells of tunica mucosa bronchiorum. Upper right and below: membrane expression of connexin 43 in normal bronchial submucosal glands and alveolar epithelial cells. (**b**) E-cadherin expressed in cytoplasm of well-differentiated adenocarcinoma cells. (**c**) Connexin 43 expressed in cytoplasm of poorly-differentiated squamous cell carcinoma cells. (**d**) Connexin 43 expressed in cytoplasm and nucleus of moderately-differentiated adenocarcinoma cells.

or LH_7(-) cells in G1 phase, G2 phase and S phase were determined by flow cytometry (FACSCalibur, BD Biosciences, USA).

The CellTiter 96 AQueous cell proliferation assay (Promega, USA) was performed to study alterations in cell proliferation, according to the manufacturer's instructions. The absorbance at 490 nm, which is directly proportional to the number of living cells in culture, was detected in LH_7, LH_7(-) and LH_7-Gja1 cells each day for 5 d after transfection.

Statistical analyses. The χ^2 test was used to clarify the relationships among connexin 43, E-cadherin and clinicopathologic characteristics. The Mann and Whitney U test was used to analyze the results of the western blot, cell growth rate, and cell cycle experiments. P values less than 0.05 were considered statistically significant.

Results

Reduced expression of both connexin 43 and E-cadherin is associated with poor differentiation, advanced TNM stages and lymph node metastasis in lung cancers

Connexin 43 and E-cadherin were typically expressed at the cell membrane in all 15 normal lung tissues (Fig. 1a). However, in lung cancer tissues, expression of connexin 43 was positive in only 50.5% (54/107) of samples. E-cadherin expression was positive in only 67.3% (72/107) of lung cancer samples. Expression of connexin 43 and E-cadherin in the samples was primarily cytoplasmic, and membrane expression was dramatically reduced (Figs. 1b and 1c). Several samples showed simultaneous nuclear and cytoplasmic expression (Fig. 1d). Correlations between the clinicopathologic characteristics of the samples and expression patterns of connexin 43 and E-cadherin are summarized in Table 1. Significant reduction of connexin 43 and E-cadherin expression was associated with patient samples that are poorly-differentiated, had advanced TNM stages and lymph node metastases. Reduced connexin 43 and E-cadherin levels were independent of tumour histological types. Interestingly, expression of connexin 43 and E-cadherin were significantly correlated with each other ($P < 0.001$, Contingency coefficient = 0.504; Table 1).

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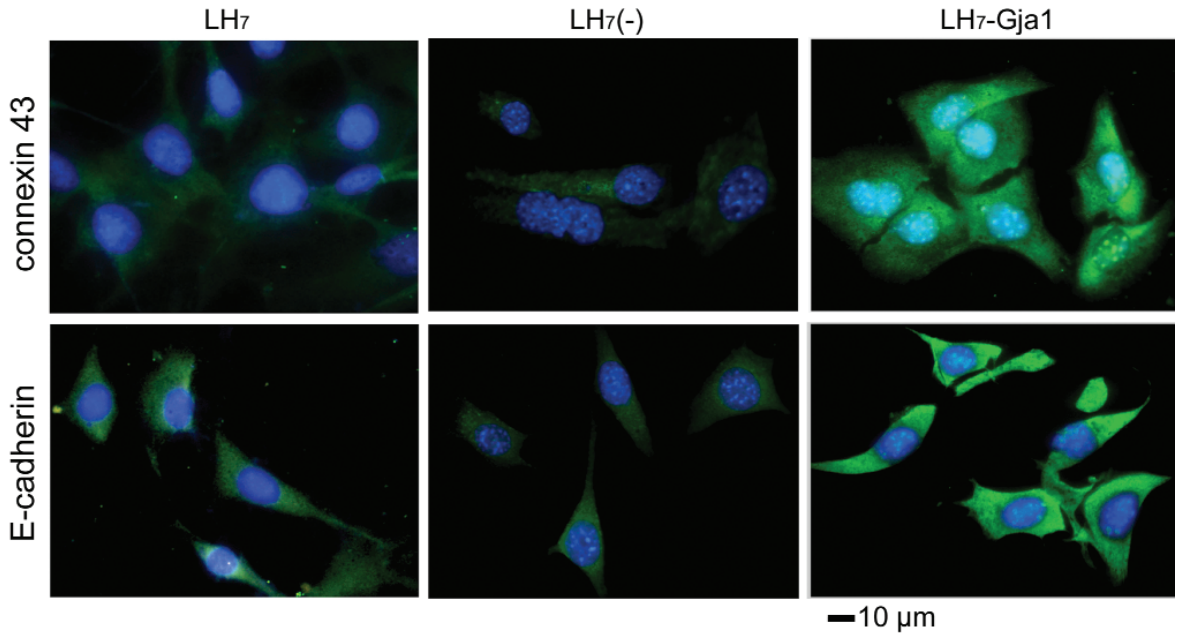

Fig. 2. Immunofluorescence of connexin 43 and E-cadherin in LH_7, LH_7(-) and LH_7-Gja1 cells. The expression of connexin 43 and E-cadherin in LH_7 or LH_7(-) cells is very low. After transfection, the expression of connexin 43 and E-cadherin is enhanced in LH_7-Gja1 cells.

E-cadherin expression is recruited by connexin 43 transfection

After 4 weeks of G418 selection, stable LH_7-Gja1 cell clones were obtained. These clones were named LH_7Gja1#1, LH_7Gja1#2, LH_7Gja1#4, and LH_7Gja1#6. As shown in Fig. 2, the immunofluorescence detection of connexin 43 and E-cadherin expression in the LH_7-Gja#1 clone is obviously stronger than in LH_7 or LH_7(-) cells. However, localization of both proteins was cytoplasmic rather than at the cell surface. The western blot results show that E-cadherin expression is significantly recruited in LH_7-Gja1 cells than in LH_7 or LH_7(-) cells ($P < 0.001$, Fig. 3).

Overexpression of connexin 43 inhibits cell cycle transition and proliferation of LH_7-Gja1 cells

The percentage of G1 phase cells in all the LH_7-Gja1 cell clones was significantly higher than that of LH_7 or LH_7(-) cells, while the percentages of LH_7-Gja1 cells

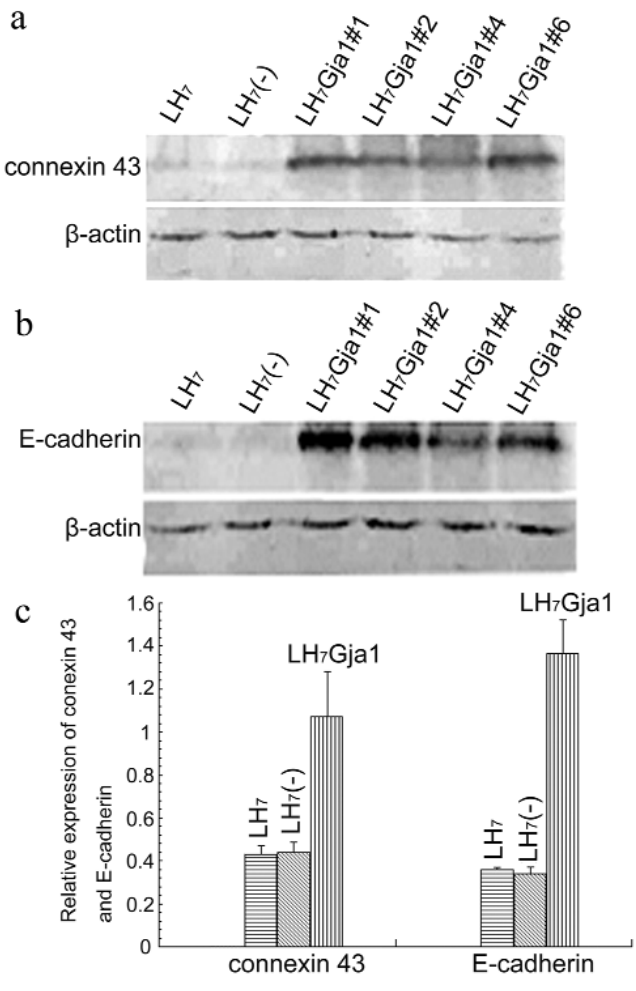

Fig. 3. Connexion 43 and E-cadherin expression in LH_7, LH_7(-) and LH_7-Gja1 cell clones. (**a**) After connexion 43 transfection, expression was enhanced in LH_7-Gja1 cell clones, LH_7Gja1#1, LH_7Gja1#2, LH_7Gja1#4, and LH_7Gja1#6, compared with LH_7 and LH_7(-) cells. Expression of E-cadherin (**b**) was greatly enhanced in these cell clones, but not in LH_7 or LH_7(-) cells. β-actin served as internal control. (**c**) The relative expression was quantified by the ratios of connexion 43 or E-cadherin and β-actin. Expression of connexin 43 and E-cadherin was significantly higher in LH_7-Gja1 cells than in LH_7 or LH_7(-) cells ($P < 0.001$). The results are the average of three independent experiments.

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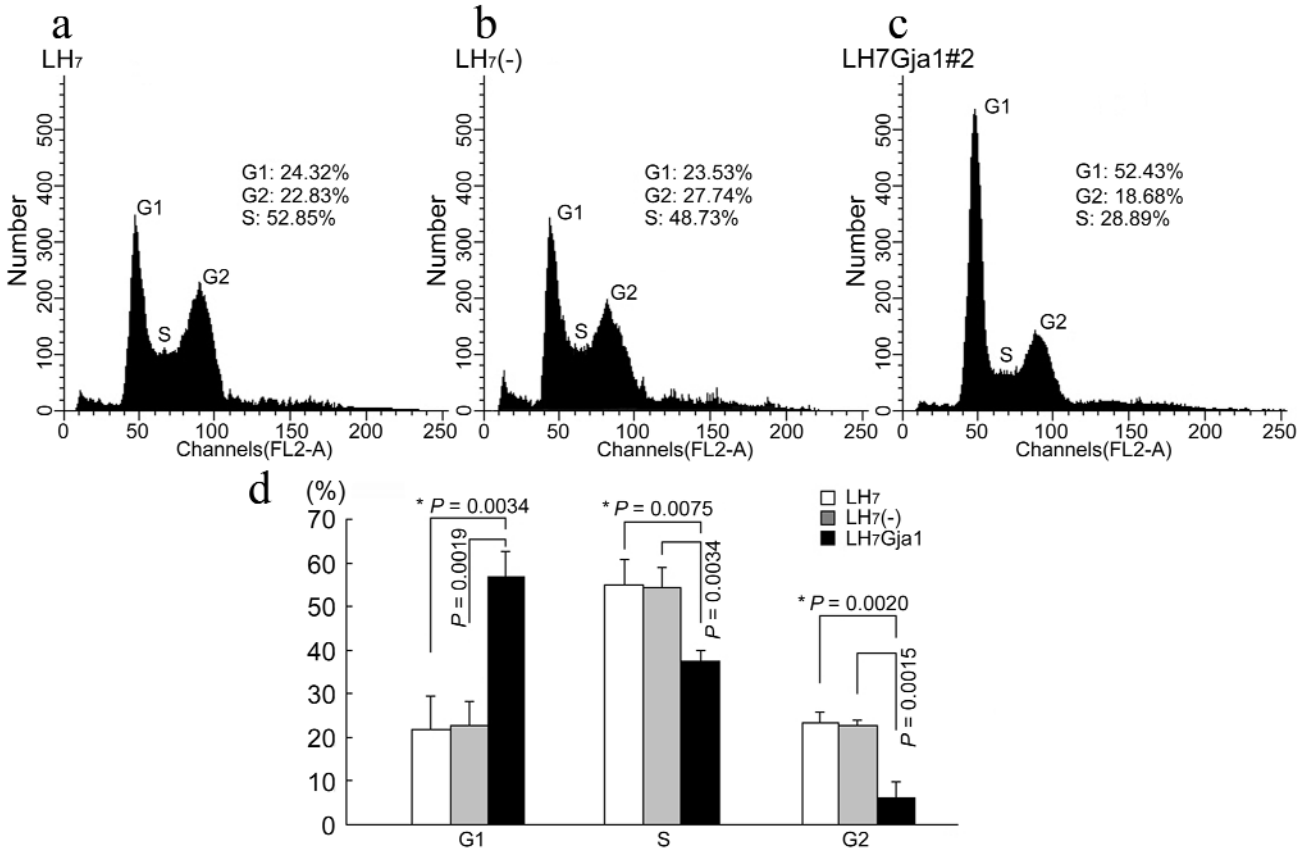

Fig. 4. (**a-c**) Cell cycle distribution of LH_7, LH_7(-) and LH_7-Gja1 clone (LH_7Gja1#2) assayed by flow cytometry after PI staining. (**d**) Histogram of LH_7, LH_7(-) and LH_7-Gja1 cells. The bars represent the percentages of G1, S or G2 phase cells in LH_7, LH_7(-) or LH_7-Gja1 cells. The results are the average of three independent experiments.

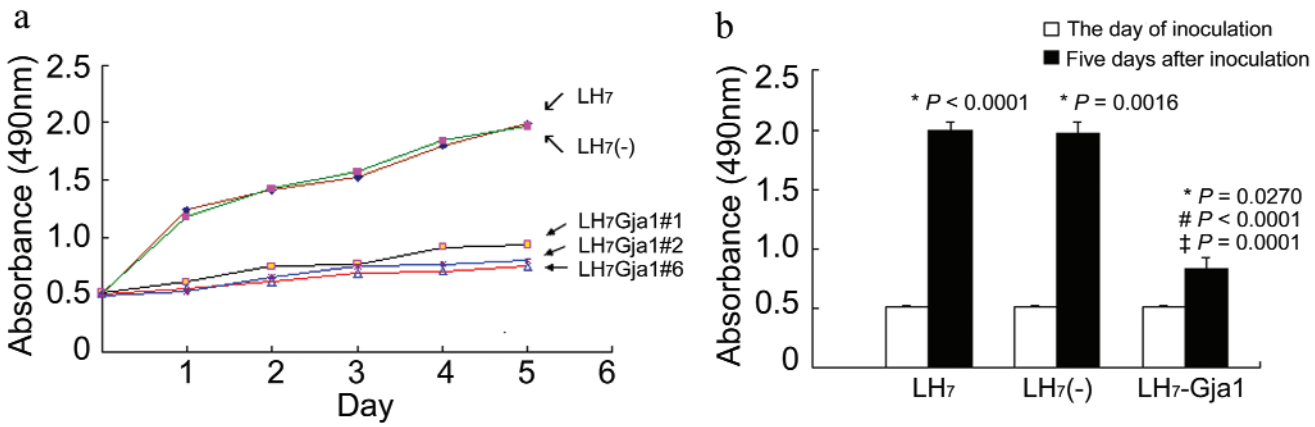

Fig. 5. (**a**) Growth curves of LH_7, LH_7(-) and LH_7-Gja1 clones. The absorbance at 490 nm represents viable cells. (**b**) Comparison of viable cells among LH_7, LH_7(-) and LH_7-Gja1 cells on the day of inoculation and at five days after inoculation. * vs. the day of inoculation, # vs. LH_7 cells and ‡ vs. LH_7(-) cells. The results are the average of three independent experiments.

in S and G2 phases were significantly lower than those of LH_7 or LH_7(-) cells ($P < 0.01$, Fig. 4). Further experiments showed that the growth rates of LH_7-Gja1 cells were also significantly lower than LH_7 or LH_7(-) cells over one-week period in culture ($P < 0.001$, $n = 3$) (Fig. 5).

Discussion

The reduction of connexin 43 or E-cadherin has been reported in several human cancers [7-9]. Our study demonstrates that the concurrent reduction of connexin 43 and E-cadherin is significantly related to differ-

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entiation and progression in 107 lung cancer samples. Furthermore, overexpression of connexin 43 not only restores the level of E-cadherin but also hampers proliferation of human lung cancer cells.

Connexin 43 and E-cadherin expression and their clinical/pathological correlations have not been well documented in human lung cancers [8,18,19]. In a previous study of 24 cases, Jinn et al. reported that connexin 43 was significantly decreased in poorly-differentiated lung cancer. Immunostaining for E-cadherin shows a heterogeneous decrease in expression [19]. Our study not only confirms this early report, but also demonstrates that concurrent reduction of connexin 43 and E-cadherin is significantly related to poor differentiation, advanced TNM staging and lymph node metastasis. These results indicate that concurrent reduction of connexin 43 and E-cadherin may reflect cellular activity in cancer cells and progression of lung cancer.

Earlier reports indicated that potential connections exist between connexins and cadherins, and suggest that cadherins might be a prerequisite for gap junction formation [20,21]. Other reports suggest that E-cadherin regulates gap junction intercellular communication (GJIC) and involves posttranslational regulation of connexin 43 [22,23]. Our results show that connexin 43 and E-cadherin are concurrently expressed in 77.6% of human lung cancer samples (including positive and negative expression of both proteins). Furthermore, our study demonstrates that transfection with connexin 43 gene in LH_7 lung cancer cells increases the expression of E-cadherin. The results revealed that E-cadherin not only regulates expression and function of connexin 43, but it is also induced by connexin 43. The correlation and coordination between E-cadherin and connexin 43 may have important implications for regulating progression of lung cancer.

Connexin 43 suppresses proliferation of cancer cells by inhibiting cell cycle progression [24,25]. Zhang *et al.* [2,3,26] demonstrated that after transfection of connexin 43, the cell cycle transition from G1 to S phase of tumour cells was inhibited, whether GJIC was present or not. These results further show that connexin 43 increases the synthesis and decreases the degradation of p27, and inhibits the expression of S-phase kinase-associated protein 2 (Skp2). Consistent with these findings, our study shows that although gap junctions and cell adhesions were not clearly observed, the proportion of cells in G1 is significantly increased and the cells in S and G2 phases are significantly decreased in LH_7-Gja1 cells. These results indicate that increased expression of cytoplasmic connexin 43 could still regulate cell cycle and inhibit proliferation of lung cancer cells. Shima *et al.* [27] also observed similar results in the cytoplasm of connexin 43-transfected basaloid squamous cell carcinoma cells, suggesting that connexin 43 may play a role as a tumour suppressor.

In summary, we have shown that Concurrent reduction of connexin 43 and E-cadherin may reflect cellular activity in cancer cells and progression of lung cancer. Connexin 43 may induce E-cadherin expression and inhibit cell proliferation and progression of lung cancer.

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