Clinical Research Paper

Expressions of connexin 32 and 26 and their correlation to prognosis of non-small cell lung cancer

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Background and Objective: Gap junction intercellular communication (GJIC) plays an important role in regulating homeostasis and differentiation in many tissues. Connexins are gap junction proteins, whose expressions directly affect the function of GJIC. This study was to investigate expressions of connexin 32 and 26 proteins in non-small cell lung cancer (NSCLC), and their correlation to clinicopathological characters of NSCLC. Methods: Immunohistochemistry was applied to detect expressions of connexin 32 and 26 in 77 NSCLC tissues. Correlations of connexin 32 and 26 expressions to smoking, tumor size, histological type, the degree of differentiation, lymph node metastasis and prognosis were analyzed. Results: The positive rates of connexin 32 and 26 were 51.9% and 40.3% in the 77 samples, which were significantly higher than 20.3% and 30.5% in alveolar epithelium (p = 0.000, r = -0.322; p = 0.013, r = -0.215). Positive expression of connexin 32 was positively correlated with the differentiation degree of NSCLC tissues (p = 0.010, r = 0.345). The one- to five-year survival rates were higher in patients with positive connexion 32 expression than those without (p = 0.005). Moreover, the positive rate of connexion 26 was not correlated to smoking, tumor size, histological type, the degree of differentiation, lymph node metastasis and the postoperative survival time (p > 0.05). Conclusions: Expression of connexion 32 is closely correlated to the differentiation of NSCLC and affects the prognosis of NSCLC patients. Increasing the expression of connexion 32 may improve the prognosis of NSCLC.

Recent studies have found that cell proliferation and differentiation is regulated by gap junction intercellular communication (GJIC), the loss of which often leads to abnormal cell proliferation and tumorigenesis.1 Connexin is a basic protein subunit of GJIC and its expression influences the function of GJIC directly. Connexin has been found to closely relate to the size of lung cancer in animal experiments.2 In the present study, the protein expressions of connexion 32 and 26 were detected in 77 cases of non-small cell lung cancer (NSCLC) using immunohistochemistry, in order to investigate the correlation of connexion 32 and 26 to smoking, tumor size, histological type, differentiation degree, lymph node metastasis and post-operative survival time of NSCLC.

Patients and Methods

Patients. Patients with pathologically confirmed NSCLC treated by exaeresis in the Department of Thoracic Surgery, the Second Affiliated Hospital of Sun Yat-sen University from 1990 to 1999 were followed up by telephone or mail. Up to October 2005, 307 complete follow-up data of patients who did not receive radiotherapy or chemotherapy before surgery were obtained. Data of 77 cases were chosen randomly using the SAS 8.0 random number generator. The survival time was 7-80 months, with a mean of 19 months. Thirty-one (40.3%), 16 (20.8%), five (6.5%) and four (5.2%) patients died within the first, second, third and fourth year after surgery, respectively. Twelve (15.6%) and four (5.2%) patients were alive in the fourth and fifth year, and five (6.5%) were alive after the fifth year. There were 57 males and 20 females, aged 29–79 years (median 63 years). Fifty-seven cases had smoking history, among which 44 were males and 13 were females. There were 25 cases of squamous cell carcinoma, 47 cases of adenocarcinoma (including seven cases of bronchioalveolar carcinoma), four cases of large cell undifferentiated carcinoma and one case of mukoepidermoid carcinoma. There were 35 cases of well-differentiated carcinoma, 15 cases of moderately differentiated carcinoma and 27 cases of poorly-differentiated and undifferentiated carcinoma. Forty-two cases were complicated with lymph node metastasis. According to clinical classification proposed by WHO (2004), 30 cases were stage I, 23 cases were stage II, 18 cases were stage III and six cases were stage IV. The maximum diameter of the tumor described in the surgery record was considered as the tumor size. The tumor sizes of 32 cases were ≤ 3 cm while 45 cases were > 3 cm. All specimens were fixed in 10% formalin, imbedded in paraffin and cut into 4 μm thick sections.
Methods. Monoclonal anti-connexin 32 (M12.13) antibody was the product of Chemicon Company and multiclonal anticonnexin 26 (N-19) antibody was the product of Santa Cruz Company. The dilutions of anti-connexin 32 and anti-connexin 26 were 1:150 and 1:50, respectively. SP kit (ready-to-use) was purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. Antigen repair was performed in a microwave oven. Sections were incubated with primary antibodies at 4°C overnight, followed by sequential incubation with biotin labeled secondary antibodies and the streptomycin egg protein biotin complex at 37°C for 30 min and 45 min, respectively. Then the sections were stained with diaminobenzidine (DAB) for 3–10 min. After a rinse with tap water, the nuclei were counterstained with hematoxylin and the sections were mounted with neutral gum. Phosphate buffered saline (PBS) instead of primary antibody was used as negative control. Known positive endometrial tissue sections were used as positive control.

Assessment of results. Both connexin 32 and 26 were expressed in cytoplasm and stained as brown (yellow) particles. In total 200 cancer cells in four different high power fields were counted. The percentage of positive cells greater than 10% was considered as positive, otherwise negative. Observation and assessment of the results were performed by three pathologists independently.

Statistical analyses. Statistical analyses were performed using SPSS 8.0 software package. The tumor size was compared using the t-test, and the rate was compared using the rank sum test. Survival analysis was performed with the Kaplan-Meier curve and the logrank test. Correlation coefficient was calculated using the Pearson method. p < 0.05 was considered statistically significant.

Results

Expression of connexin 32 and 26 in NSCLC tissues. The positive rates of connexin 32 and 26 were 51.9% (40 cases) and 40.3% (31 cases) out of 77 NSCLC tissues. Both connexin 32 and connexin 26 were expressed in cancer cells, but not in interstitial substances or blood vessels (Fig. 1A and 1B).

Normal alveolar epithelium adjacent to cancer tissues were found in 59 cases, among which 23 were accompanied with ciliated columnar epithelium. Connexin 32 and 26 were present in 12 (20.3%) and 18 (30.5%) specimens of alveolus tissues, while in nine (39.1%) and 10 (43.5%) specimens of bronchia ciliated columnar epithelium (Fig. 1C and 1D). The positive rates of connexin 32 and 26 in alveolus epithelium were significantly lower than those in cancer tissues ($Z = -3.745, p = 0.000, r = -0.322$, and $Z = -2.496, p = 0.013, r = -0.215$).

Relationship of connexin 32 expression with clinical pathological characteristics of NSCLC. Twenty-four out of 35 (68.6%) highly differentiated, eight out of 15 (53.3%) moderately differentiated and eight out of 27 (29.6%) poorly differentiated NSCLC were positive for connexin 32. The positive rate of connexin 32 was decreased with the decrease of differentiation degree of NSCLC, which showed significant difference ($\chi^2 = 9.153, p = 0.010, r = 0.345$).

The diameter of connexin 32-positive NSCLC was (4.09 ± 2.02) cm, while that of connexin 32-negative NSCLC was (4.73 ± 2.67) cm. The difference was not significantly different ($t = -1.172, p = 0.245$). The positive rate of connexin 32 in 32 cases of NSCLC specimens whose maximum tumor diameter was not greater than 3 cm was 62.5% (20/32), while that in 45 cases whose maximum tumor diameter was greater than 3 cm was 44.4% (20/45). There was no significant difference between the two groups ($Z = -1.553, p = 0.121$).

There was no statistical correlation of connexin 32 to age, gender, smoking history, histological type, lymph node metastasis and clinical stage of NSCLC patients ($p > 0.05$).

Relationship of connexin 26 expression with clinical pathological characteristics of NSCLC. The diameter of connexin 26-positive NSCLC was (4.73 ± 2.86) cm, while that of connexin 26-negative NSCLC was (4.18 ± 1.97) cm, which were not significantly different ($t = -0.997, p = 0.322$). The positive rate of connexin 26 in 32 cases whose maximum tumor diameter was not greater than 3 cm was 40.6% (13/32), while that in 45 cases whose maximum tumor diameter was greater than 3 cm was 40.0% (18/45). There was no significant difference between the two groups ($Z = -0.055, p = 0.956$).

There was no statistical association between connexin 26 and age, gender, smoking history, histological type, differentiation degree, lymph node metastasis or clinical stage of NSCLC ($p > 0.05$).

Relationship of connexin 32 and 26 expressions with prognosis of NSCLC patients. The one-, two-, three-, four- and five-year survival rates were all significantly higher in connexin 32-positive patients (40 cases) than in connexin 32-negative patients (37 cases), which were 75.0% vs. 43.2%, 55.0% vs. 21.6%, 45.0% vs. 18.9%, 15.0% vs. 8.1% and 12.5% vs. 0, respectively ($p = 0.005$). The survival curve is shown in Fig. 2A. Survival analysis revealed that there was no statistical association between prognosis and differentiation degree of 77 patients with NSCLC ($p > 0.05$). The one-, two-, three-, four- and five- year survival rates of connexin 26-positive patients (31 cases) were 48.4%, 32.3%, 25.8%, 6.5% and 6.5%, while those of connexin 26-negative patients (46 cases) were 67.4%, 43.5%, 36.9%, 15.2% and 6.5%, respectively. The differences among the two groups had no statistical difference ($p = 0.230$). The survival curve is shown in Figure 2B.

Correlation of connexin 32 expression to connexin 26 expression in NSCLC tissues. Out of 40 connexin 32-positive NSCLC tissues, 15 were positive for connexin 26. Out of 37 connexin 32-negative NSCLC tissues, 16 were positive for connexin 26. There was no statistical association between connexin 32 and 26 expressions ($p = 0.610$).

Discussion

It has been reported that abnormal expression of connexin exists in many kinds of tumors, accompanied by abnormal cell proliferation and canceration. In addition, abnormally expressed connexin affects tumor infiltration and progression.4-6 Udaka et al.2 reveal that expressions of connexin 32 and 26 in mouse lung cancer is closely related to the tumor size. When the tumor was relatively small (0.5–1.5 mm), the expressions of connexin 32 and 26 were similar as those in normal lung cancer tissues, whereas the expressions of connexin 32 and 26 were significantly lower in tumor tissues with larger size.
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tissues. When the tumor became relatively large (\( \geq 2.5 \) mm), the expression of connexin 32 was significantly decreased, and that of connexin 26 could not be detected. However, in this study, we did not find significant difference in the tumor size between the positive group (either connexin 32 or connexin 26) and the negative group. Furthermore, the positive rates of connexin 32 and 26 were not significantly different between groups with the maximum tumor size \( \leq 3 \) cm in diameter and \( > 3 \) cm. The inconsistency between our findings with Udaka et al.\(^2\) may be explained by different experimental specimens. The tumors from experimental mice were small, but such small tumors could hardly been noticed in human lung cancer. Fan et al.\(^3\) demonstrate that abnormal expression of connexin exists in many kinds of cancers. They found that among eight lung adenocarcinoma, three (37.5\%) were positive for connexin 32 and two (25.0\%) were positive for connexin 26; among 11 paracancerous lung tissues, nine (81.8\%) were positive for connexin 32 and eight (72.7\%) were positive for connexin 26. The positive rates of connexin 32 and 26 were significantly lower in cancer tissues than in paracancerous lung tissues. We showed that the positive rates of connexin 32 and 26 in alveolar epithelium, 20.3\% and 30.5\%, were significantly lower than those in NSCLC tissues, which were 51.9\% and 40.3\%, respectively. This might be due to different study methods and sample size. Fan et al.\(^3\) used tissue microarrays, which could easily be influenced by tumor heterogeneity. In addition, the sample size in Fan’s study was small, and it was not clearly stated that whether paracancerous lung tissues were alveolar epithelium or bronchiolar ciliated columnar epithelium.

The expression of connexin is correlated with prognosis of lung cancer patients. Ito et al.\(^7\) reported that connexin 26 is mainly expressed in cancer cells adjacent to interstitial substances and tumor capsules in lung squamous cell carcinoma; furthermore, the positive rate of connexin 26 is higher in cancer cells metastasizing to lymph nodes than in the primary focus, suggesting that connexin 26-positive cells have strong invasive capacity and metastatic potential. In addition, the five-year survival rate in connexin 26-positive group is significantly lower than that in connexin 26-negative group. On the contrary, connexin 32 is considered as a tumor inhibitory factor in adenocarcinoma of the lung. Connexin 32 can inhibit growth and invasiveness of cancer cells and impede tumor progression.\(^8\) It can also downregulate the expression of multidrug resistance gene-1 (MDR-1) and induce cytotoxicity reaction targeting tumor cells.\(^9\) We found that the expression of connexin 32 in NSCLC tissues was positively related with differentiation degree of tumor tissues, implying that decreased expression of connexin 32 is accompanied with increased malignancy of tumor cells. This result confirms that connexin 32 acts as a tumor inhibitory factor in NSCLC. The one-, two-, three-, four- and five-year survival rates of patients in connexin 32-positive group were higher than those in negative group, suggesting that increased expression of connexin 32 may

![Figure 1. Immunohistochemical staining of connexin 32 and 26 in lung adenocarcinoma and paracancerous tissues (SP × 200). Positive expressions of connexin 32 (A) and 26 (B) appear in the cytoplasm of cancer cells (brown), but not in interstitial cells. (C) Positive expression of connexin 32 is shown in the cancer cells (brown, white arrow), but not in the alveolar epithelium and bronchiolar epithelium of paracancerous tissues (black arrow). (D) Expression of connexin 26 is shown in the alveolar epithelium of paracancerous tissues (brown).](image1.png)

![Figure 2. Correlation of connexin 32 (A) and connexin 26 (B) expressions to prognosis of non-small cell lung cancer](image2.png)
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improve the survival rate of patients. Survival analysis showed that there was no significant association between prognosis and the differentiation degree of NSCLC patients in this study. Whether the correlation of connexin 32 to prognosis of NSCLC is related to decreased MDR-1 expression and induction of cytotoxicity reaction needs further investigation. We found no relation between connexin 26 and histological type, differentiation degree, lymph node metastasis and prognosis of NSCLC patients. This may be because that a high percentage of patients with non-squamous cell carcinoma, such as adenocarcinoma, were included in the study.

References


