The Correlation between Aberrant Connexin 43 mRNA Expression Induced by Promoter Methylation and Nodal Micrometastasis in Non-Small Cell Lung Cancer

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ABSTRACT

Reduced connexin (Cx) 43 gene expression has been shown in most of lung tumors and cancer cell lines. Although aberrant Cx43 gene expression was linked with lung tumorigenesis, our understanding to the mechanism was still limited. We hypothesized that the evidence of aberrant Cx43 gene expression was gradually intensified from adjacent normal lung tissues surrounding tumors toward tumor tissues. In this study, 90 lung tumors and adjacent normal tissues were collected to examine Cx43 mRNA expression by reverse transcription-PCR (RT-PCR). Our data showed that Cx43 mRNA expression in adjacent normal lung tissue was significantly correlated with nodal involvement \( (P = 0.03) \), but the similar trend was not observed in tumor tissues. To verify whether lack of Cx43 mRNA expression resulted from promoter methylation, PCR-based methylation assay was performed for Cx43 promoter methylation analysis. A higher frequency of promoter methylation was observed in Cx43 mRNA-negative patients (21 of 33, 63.7%) compared with Cx43 mRNA-positive patients (3 of 57, 5.3%, \( P < 0.0001 \)). To elucidate whether aberrant Cx43 gene expression originated from adjacent normal lung tissues, 25 lung tumors and each of five adjacent normal tissues at various distances from tumor tissues were collected to examine Cx43 mRNA and protein expression by RT-PCR and Western blot, respectively. The results show that Cx43 mRNA and protein expressions gradually decreased from adjacent normal lung tissues to tumor tissues with a positive correlation to the distance from the tumor tissues. Gel-shift assay data also revealed that shifted band binding with API was only observed in adjacent normal tissues, which were far from the tumor tissues. These results indicate that promoter methylation may interfere with API binding to the promoter to cause aberrant Cx43 gene expression. Thus, Cx43 mRNA in adjacent normal tissue surrounding lung tumor simply detected by RT-PCR may act as a molecular marker of nodal micrometastasis in non-small cell lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer death in many developed countries, including Taiwan, in which 20% of cancer deaths have been caused by lung cancer. Late diagnosis in lung metastasis has resulted in <20% of lung cancer patients having a 5-year survival (1). Thus, suitable molecular markers for early diagnosis of lung cancer metastasis are helpful for reducing lung cancer mortality rates. Currently, the presence of lymph node metastasis along with extent of primary tumor (T) and distant metastasis (M) status represents the most accurate factor available for the prediction of prognosis in patients who undergo complete surgical resection. However, tumor recurrence has occurred in ~30% of patients with pathological stage I NSCLC and ultimately led to death, despite complete surgical resection. This suggests that occult micrometastatic tumor cells, which are not detected by current clinical staging examinations and conventional histopathologic methods, have already spread to the regional lymph node or distant mesenchymal organs at the time of surgery. Therefore, for an accurate prediction of prognosis, it is necessary to assess the lymph node status and take account of nodal micrometastasis.

Many human tumors, including lung cancer, have been reported to be deficient in expression of Cx43 mRNA and protein levels (2–7). Moreover, the decreased connexin 43 mRNA is correlated with its protein levels (8). This finding suggests that reduced Cx43 gene expression in human lung cancer cell lines and lung tumors may be caused by promoter methylation. After the transfection of Cx43 cDNA into a human lung carcinoma cell line deficient in Cx43 gene expression, the Cx43 transfectants show a reduced growth rate and inhibition of tumorigenicity (9). Similar findings were also observed in human glioblastoma cells (10). In contrast, when rat glioma cells...
were transfected by a mutant Cx43 cDNA, the growth capacity of the transfected clones was restored, and the tumorigenicity of these cells was reinforced (11). Furthermore, some evidence supports the hypothesis that Cx43 may be a tumor suppressor gene, which suggests that reduced Cx43 gene expression is involved in tumor progression, including invasion and metastasis (9–13), e.g., a mouse skin carcinogenesis experiment showed that clear reduction of Cx43 was observed in squamous cell carcinomas at invasive sites, and additionally, when squamous cell carcinomas metastasized into lymph nodes, few carcinomas cells expressed Cx43 (14, 15). This suggests that quantitative and qualitative changes in Cx43 expression are associated with tumor progression, including the loss of differentiation and invasion and metastasis, during multistage mouse skin carcinogenesis (15). In this study, we found that Cx43 mRNA expression in adjacent normal lung tissues from lung cancer patients was related with nodal micrometastasis. Moreover, promoter methylation was responsible for the aberrant Cx43 gene expression.

MATERIALS AND METHODS

**Study Subjects.** Between 1994 and 1997, 90 consecutive patients with operable NSCLC underwent surgery at the Department of Thoracic Surgery, Veterans’ General Hospital-Tai Chung, Taiwan, ROC. Noncancer patients with lung disease, including pneumothorax, tuberculosis, chest wall deformity, and cryptococcal infection, who underwent thoracic surgery at Cheng-Kung University Hospital, Taiwan or Changhua Christian Hospital, Changhua, served as control subjects. None of the subjects received radiation therapy or chemotherapy before surgery. After surgery, pathological material from different areas of the tumor was processed by conventional histological procedures, and the fresh samples were immediately stored at −70°C until further use.

**Preparation of RNA and RT-PCR.** Total RNA was extracted by homogenizing the tissues in 1 ml of TRIzol reagent (Life Technologies, Inc., Grand Island, NY), followed by chloroform re-extraction and isopropanol precipitation. The preparation of cDNA was performed in a reaction with a total volume of 20 μl, containing 5 μg of total RNA in the reverse transcriptase reaction buffer (Life Technologies, Inc.), 10 mM DTT, 100 pmol of oligo d(T)18, 0.5 mM each of deoxynucleotide triphosphates, and 200 units of M-MLV reverse transcriptase (Life Technologies, Inc.), containing 5 μM of total RNA in the reverse transcriptase reaction buffer (Life Technologies, Inc.), 10 mM DTT, 100 pmol of oligo d(T)18, 0.5 mM each of deoxynucleotide triphosphates, and 200 units of M-MLV reverse transcriptase (Life Technologies, Inc.). The reactions were incubated at 42°C for 50 min and then terminated by heating at 75°C for 10 min followed by quick chilling on ice. Target sequences were amplified in a 50-μl reaction mixture containing 20 pmol of each of the following primers: Cx43-S (5′-GGTGAGGGAGAAGTAACCAAC-3′) and Cx43-AS (5′-CCGTTGGGACTCTAAAGGAGG-3′), 2.5 units of Taq polymerase (TaKaRa, Shiga, Japan), 0.5 mM deoxynucleotide triphosphates, 5 μl of PCR reaction buffer, and 1 μl of cDNA preparation. An initial cycle was performed for 5 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 54°C, and 1 min at 72°C. The PCR products were analyzed using 1% agarose gel electrophoresis.

**PCR-based Methylation Assay.** The methylation status of genomic DNA was analyzed using the modified PCR methylation assay. Genomic DNA (1 μg) was digested overnight with 10 units of methylation-sensitive (MspI) and methylation-insensitive (HindIII) enzyme. For the assessment of the methylation status of the promoter of the Cx43 gene, 100 ng of digested DNA were amplified by PCR using primers flanking the restriction sites. Undigested and digested DNA were used as controls and included for every site examined. To rule out the possibility of incomplete digestion, all samples were digested twice with each of the enzymes in independent experiments. PCR amplifications from each of the duplicate digests were repeated at least twice to ensure reproducibility of the results.

**Gel-shift Assay.** Nuclear extract was prepared as described previously. DNA binding was measured using a gel retardation assay. A complementary pair of synthetic oligonucleotides containing the sequence 5′-CCCAGTTGAGTCAGTGCGTTG-3′ of the AP1 binding site was synthesized. The oligonucleotides were 32p-labeled at the 5′-end using T4 polynucleotide kinase and [α-32p]dATP. Nuclear extract (15 μg) was incubated with 1 μl of poly(dI-dC) and 20 ng of 32p-labeled AP1 binding site at room temperature for 20 min. The reaction mixture was loaded onto a 5% polyacrylamide gel and electrophoresed at 120 V for ~3 h in 25 mM Tris to 195 mM glycine. Gel was dried and exposed on X-ray film overnight.

**Protein Extraction and Western Blot.** Total protein extracts from fresh lung tumor tissues were prepared with a lysis buffer [100 mM Tris (pH 8.0) and 1% SDS]. The protein concentration was determined using the Bio-Rad protein assay kit, and this was followed by separation with SDS-PAGE (12.5% gel, 1.5 mm thick). After electrophoretic transfer to Hybond-C extra nitrocellulose, the nonspecific binding sites were blocked with 5% nonfat milk in TBS-Tween 20. Cx43 protein was detected by incubating the membrane with monoclonal antihuman Cx43 antibody (Zymed Laboratories, Inc., dilution 1:1000) for 60 min at room temperature, followed by extensive washing with TBS-Tween 20 and subsequent incubation with peroxidase-conjugated secondary antibody (1:500 dilution). The Cx43 band was visualized using enhanced chemiluminescence (NEL Life Science Products, Inc., Boston, MA).

**Statistical Analysis.** Statistical analysis was performed using the SPSS statistical software program version 10.0 (SPSS, Inc., Chicago, IL). Analyses of the associations between Cx43 mRNA expression and prognostic factors for lung cancer (including age, sex, T, N, M, tumor stage, tumor type, and tumor grade) and Cx43 gene methylation status were, respectively, performed using the Pearson χ2 test, Fisher’s exact test, and likelihood ratio test.

RESULTS

Our previous studies have shown that Cx43 protein was not detected in most of lung tumor tissues (151 of 165, 92%; unpublished data). In this study, 90 lung tumors and adjacent normal tissues were collected to examine the correlation of Cx43 mRNA expression with clinical-pathological parameters. Among the parameters, Cx43 mRNA expression in adjacent normal lung tissues was only associated with nodal involvement (Table 1; P = 0.03). To verify whether lack of Cx43 mRNA expression resulted from promoter methylation, PCR-based methylation assay for Cx43 promoter methylation was performed. Patients with Cx43 mRNA negative had a higher fre-
quency of promoter methylation (21 of 33, 63.7%) compared with those with Cx43 mRNA positive (3 of 57, 5.3%; Table 2; Fig. 1). Thus, promoter methylation may play an important role in aberrant Cx43 transcription in NSCLC.

To verify that down-regulated Cx43 gene expression in adjacent normal lung tissues gradually decreased with distance from lung tumors, Cx43 mRNA and protein expressions in 25 surgically resected adjacent normal lung tissues at various distances surrounding lung tumors were evaluated by RT-PCR and Western blot, respectively. Our data show that CX43 mRNA and protein were normally expressed in lung tissues of noncancer controls (Fig. 2A) but gradually decreased in adjacent normal tissues with being closer to the lung tissues. Similar decreases in Cx43 protein expression levels, after being adjusted with that of /H9252-actin, were also observed (Fig. 2B). To further elucidate the mechanism for aberrant Cx43 mRNA transcription, 5 of 25 lung cancer patients were used for gel-shift assays to examine the binding affinity between AP1 and the Cx43 gene promoter. Our results show that aberrant Cx43 mRNA expression in lung tumors and adjacent normal tissues were correlated to the reduction of promoter binding with AP1 (Fig. 3). Loss of AP1 binding was found in tumors with the absence of Cx43 mRNA expression and gradually decreased in adjacent tissues in direct relation to the decreasing distance from the tumor tissues. This result strongly indicates that the binding affinity of AP1 with the promoter region may be responsible for the gradual decrease in Cx43 gene expression. In conclusion, the gradual decrease in Cx43 gene expression in adjacent normal lung tissues strongly suggests that the absence of Cx43 mRNA may be useful as an early diagnostic molecular marker for nodal micrometastasis in NSCLC patients.

DISCUSSION

There is a growing body of evidence suggesting that connexin gap junction proteins act as tumor suppressors. Their tumor inhibitory effect is usually attributed to their main function of cell coupling through gap junctions (6–12, 16, 17). Aberrant Cx43 gene expression has been found in several types of tumor, including liver, prostate, kidney, skin, breast, and lung cancers (2–6). In our previous report, Cx43 protein expression was not detected in most of the lung tumors (unpublished data), and the findings were similar to previous studies. Ruch et al. (2) indicated that Cx43 mRNA and protein levels of various human

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**Table 1** Relationships between Cx43 mRNA expression in lung tumors and adjacent normal tissues from lung cancer patients and their clinical parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cx43 mRNA expression</th>
<th>Adjacent normal tissue</th>
<th>Tumor tissue</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor type</td>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>15</td>
<td>40</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>14</td>
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<td>13</td>
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<td>carcinoma</td>
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<td></td>
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<tr>
<td>Tumor stage</td>
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<tr>
<td>III</td>
<td>16</td>
<td>28</td>
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<tr>
<td>P</td>
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<td>T factor</td>
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<tr>
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<td>1 (n = 20)</td>
<td>1</td>
<td>17</td>
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<td>4</td>
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<tr>
<td>2 (n = 28)</td>
<td>14</td>
<td>14</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.030</td>
<td>0.103</td>
<td></td>
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</table>

**Table 2** Correlation between Cx43 mRNA expression and its promoter methylation in adjacent normal lung tissues from lung cancer patients

<table>
<thead>
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<th>Promoter methylation</th>
<th>Cx43 mRNA expression</th>
<th>P</th>
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<tbody>
<tr>
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<td>54</td>
</tr>
<tr>
<td>Positive</td>
<td>21</td>
<td>3</td>
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lung cell lines were all significantly reduced in comparison with nontransformed lung epithelial cells (2). The decrease in Cx43 mRNA levels in these lung carcinoma cells was correlated well with Cx43 protein expression (8). However, no direct evidence is available to demonstrate the involvement of Cx43 in lung tumorigenesis. In the present study, we report for the first time how gradual increases in aberrations of Cx43 gene expression through promoter methylation in adjacent normal lung tissues compare with corresponding tumors. Moreover, aberrant Cx43 mRNA expression in adjacent normal lung tissue is correlated to nodal micrometastasis of NSCLC. This finding may be used to support previous findings showing that aberrant Cx43 gene expression was involved in human lung tumorigenesis.

In a multistage mouse chemical-induced skin carcinogenesis model, the expression of Cx43 in squamous cell carcinomas was significantly decreased compared with surrounding nonmalignant epidermis and papillomas (14, 15). Moreover, Cx43 was expressed in only few squamous cell carcinomas that had metastasized into lymph nodes (15). In human lung carcinoma cells, Cx43 mRNA was not detected in a highly metastatic lung carcinoma cell line, PG, by Northern blot (9). Furthermore, a transfection with exogenous Cx43 cDNA into PG cells could result in a marked suppression of growth in vitro and in vivo (9). This seems to reveal that aberrant Cx43 gene expression may be associated with lung tumor metastasis. A similar observation was made in breast cancer where a correlation was found between metastatic potential and the decrease of Cx43 gene expression (18).

It is well established that promoter methylation can result in decreased expression of tumor suppressor genes, such as p16, p15, and hMLH-1, contributing to increased tumorigenicity in various tumors (19). Promoter methylation has been shown to link with the aberrant transcription of Cx43 in rat liver cells (20). Additionally, treatment of Cx43-negative HeLa cells with 5-aza-2′-deoxycytidine resulted in expression of Cx43, suggesting gene silencing via DNA methylation (21). In the present study, the majority of Cx43 mRNA-negative samples was found to possess promoter methylation. The promoter methylation was not only detected by PCR-based methylation assay but also confirmed by Southern blot (data not shown). Moreover, the promoter methylation responsible for the decrease of Cx43 gene expression was further confirmed by gel-shift binding assay data. Previous reports have indicated that the API1 binding site was located at the region of Cx43 promoter methylation (21–24). Thus, we suggest that aberrant API1 binding with promoter region by methylation may be responsible for the aberrant Cx43 gene transcription in NSCLC.

In summary, gradually aberrant Cx43 gene expressions in adjacent normal lung tissues surrounding tumors mediated through promoter methylation strongly suggest that Cx43 mRNA in adjacent normal tissue simply detected by RT-PCR may be act as a molecular marker of nodal micrometastasis in NSCLC.

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