Original Article

Co-expression of delta-catenin and RhoA is significantly associated with a malignant lung cancer phenotype

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Abstract: Delta-catenin, a member of the p120-catenin subfamily, and the Rho GTPase RhoA both have roles in the regulation of the cytoskeleton. In this study, we found that delta-catenin positive expression and RhoA over-expression is consistently found in non-small cell lung cancer, but not in normal lung tissue, and that their co-expression was significantly associated with histological type, differentiation, pTNM stage, lymphatic metastasis and a poor prognosis. We also demonstrate that delta-catenin can directly interact with RhoA and regulate its activity, which in turn mediates tumor invasion and metastasis.

Keywords: RhoA, delta-catenin, non-small cell lung cancer, prognosis, GTPase, co-expression

Introduction

Delta-catenin is an important member of p120-catenin (p120ctn) subfamily, which also includes p120ctn, ARVCF (armadillo repeat protein deleted in velo-cardio-facial syndrome), p0071, and plakophilins1/2/3 [1]. It is mainly expressed in the central nervous system and promotes synaptic growth. Delta-catenin contains ten Armadillo repeats, and is similar to p120 in terms of both structure and function [2, 3]. It can directly bind to the juxtamembrane domain (JMD) of E-cadherin, forming an E-cadherin/catenin complex that modulates intercellular adhesion [4], and it can also interact with presenilin-1 that is associated with Alzheimer’s disease [5].

In addition, delta-catenin has been shown to modulate the cytoskeleton by regulating Rho GTPase (including RhoA) activity [6, 7]. RhoA is a core member of the Rho GTPase family and a Rho GTP-binding protein of the Ras super-family, and alternates between activated (GTP-bound) and inactivated (GDP-bound) states. RhoA as a molecular switch dynamically regulates actin cytoskeleton assembly [8], and functions in a number of key cellular activities, including morphological changes, chemotaxis and motility [9, 10]. Therefore, we hypothesized that there may be a relationship between delta-catenin and RhoA in lung cancer.

In this study, we examined the expression of delta-catenin and RhoA in 128 cases of non-small cell lung cancer (NSCLC), and discussed the relationship between their expression and clinicopathological factors. We also found that delta-catenin in lung cancer cell lines regulated the expression and activity of RhoA, thereby influencing the invasion and metastasis of cancer cells.

Materials and methods

Materials

Primary pulmonary squamous cell carcinoma and adenocarcinoma samples were obtained from 128 patients (surgical resection specimens at the First Hospital of China Medical University between 1998 and 2005). There were 68 male and 60 female patients, with a mean age of 58 years. Based on the WHO histological classification of lung cancer, 65 cases were squamous cell carcinomas, and 63 were adenocarcinomas. Thirty-one cases were well differentiated, 60 cases were moderately dif-
Delta-catenin and RhoA in lung cancer

Table 1. Primer sequences and reaction conditions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length</th>
<th>Tm</th>
</tr>
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<tbody>
<tr>
<td>delta-catenin</td>
<td>5'-TACTCCGCAAGACGACTGACC-3'</td>
<td>284 bp</td>
<td>59°C</td>
</tr>
<tr>
<td></td>
<td>5'-CATCACACTCTGCTCTCATTCTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RhoA</td>
<td>5'-CATCGGAAGAACTGTG-3'</td>
<td>182 bp</td>
<td>53°C</td>
</tr>
<tr>
<td></td>
<td>5'-TCCCAAGGACACTC-3'</td>
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<tr>
<td>GAPDH</td>
<td>5'-AGAAGGTCTCGGGCTGATTG-3'</td>
<td>258 bp</td>
<td>57°C</td>
</tr>
<tr>
<td></td>
<td>5'-AGGGGCCATCCACAGCTTC-3'</td>
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</tr>
</tbody>
</table>

ferentiated, and 37 cases were poorly differentiated. Tumors were staged using the TNM staging standard revised version produced by the International Union Against Cancer (UICC) in 2002, on the basis of which there were 22 cases of stage I, 43 cases of stage II, 57 cases of stage III and 6 cases of stage IV disease. Seventy of these cases had complete follow-up records.

In addition, 30 samples of fresh lung cancer and adjacent normal lung tissue were obtained for the extraction of protein and RNA.

**Immunohistochemistry**

The expression of delta-catenin and RhoA was evaluated using streptavidin peroxidase (SP) immunohistochemistry in all 128 NSCLC samples. All specimens were cut into 6-µm-thick sections. After de-waxing and hydration, antigen retrieval was performed by heating under pressure in citrate buffer (pH 6.0), endogenous peroxidase was blocked with 3% H_2O_2, and then non-specific binding sites were blocked using non-immune serum. The sections were incubated with monoclonal antibodies against delta-catenin (1:50, Santa, USA) and RhoA (1:100, Santa, USA) at 4°C overnight. The secondary antibody and DAB were then successively added.

We observed the expression of delta-catenin in tumor tissue, and calculated the percentage of positively stained cells in a field of 400 tumor cells. Staining intensity was scored as 0 for negative, 1 for weak, 2 for moderate, and 3 for strong, and the proportion of positive cells was scored as 0 for absent, 1 for 1-25%, 2 for 26-50%, 3 for 51-75%, and 4 for ≥76%. The final score was the product of the two ratings, and negative expression was defined as a score <2, and positive expression as a score ≥2 [11].

Based on a previous study of RhoA expression in ovary tumors by Horiuchi, the intensity of RhoA cytoplasmic staining was scored as strong (+++), moderate (++), faint (+), or negative (-). Scores of – or + were defined as normal expression, and higher scores were defined as over-expression [12].

The simultaneous occurrences of delta-catenin positive expression and RhoA over-expression were defined as co-expression.

**Cell culture, plasmids, and transfection**

The human lung cell lines H460 and LK2 were cultured in RPMI 1640 or DMEM culture medium containing 10% fetal bovine serum, 2.3 g/L NaHCO_3, and 100 U/ml green streptomycin, at 37°C in 5% CO_2. Every two days the cells were passaged using 0.25% trypsin digestion.

Lipofectamine 2000 (Invitrogen, USA) was used to transfect full-length human delta-catenin cDNA plasmids (pCMV5-FLAG/delta-catenin) into LK2 cells that only expressed endogenous delta-catenin at a low level. The empty vector was used as a negative control.

The designed delta-catenin siRNA (5'-CUACGU-UGACUUUCUCUAAUU-3', 5'-UGAGUAGAAGUCA-ACGUAGUU-3') was transfected into H460 cells that expressed a high level of delta-catenin, while non-specific siRNA was used as a negative control.

**Western blot**

Lung cancer tissue and cells were lysed in lysis buffer, sonicated on ice, and then centrifuged at high speed (16000 rpm, 4°C, 30 minutes). The supernatant was collected. The lysates were separated by SDS-PAGE, and then transferred to a PVDF membrane. The membrane was incubated with anti-delta-catenin antibody (1:50, Santa, USA) and RhoA (1:100, Santa, USA) at 4°C overnight. The secondary antibody and DAB were then successively added.

**RT-PCR**

Total RNA was extracted from lung tissue and cells using Trizol reagent (Invitrogen). RT-PCR was carried out using the RNA PCR (AMV) Ver.3.0 kit (TaKaRa Bio Inc., Dalian, Liaoning,
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China). Primer sequences and reaction conditions are shown in Table 1. RT-PCR products were separated on a 1.5% agarose gel, and then grey values were determined using Biolmaging Systems. GAPDH mRNA was used as an internal control.

RhoA activity assay

RhoA activity was determined using the RhoA G-LISA™ activation assay kit (BK124, Cytoskeleton, Denver, USA). GTP-bound RhoA activity was measured using a spectrometer (BD Transduction Laboratories) at the wave length of 490 nm.

Matrigel invasion assay (Transwell assay)

Matrigel basement membrane gel (100 μl, diluted 1:4) (BD Biosciences) was added to the upper chamber that was incubated at 37°C in 5% CO₂ for 2 hours. Then 100 μl of cell suspension was added to the upper chamber (3×10⁵ cells/ml). The lower chamber was filled with RPMI1640 medium containing 10% fetal bovine serum. Transwell microporous membrane (8 μm pore size, Corning, USA) was placed between the upper and lower chamber. After transfection (24 hours), tumor cells were seeded into the upper chamber and incubated at 37°C in 5% CO₂ for 24 hours. The cells on the lower surface of the membrane were fixed with 100% methanol for 30 minutes and stained with hematoxylin, and then counted under a microscope in five fields (×400).

Immunoco-localization

The lung cancer-derived cell lines H460 and LK2 were digested with 0.25% trypsin, and fixed with 4% paraformaldehyde for 30 minutes. The cells were treated with 0.5% Triton X-100 for 30 minutes at room temperature, and then incubated with anti-delta-catenin antibody or anti-RhoA antibody at 4°C overnight. The cells were then incubated with the fluorescently labeled secondary antibody at 37°C for 1 hour, stained with 5 pg/ml DAPI for 2 minutes, and then examined using laser scanning confocal microscopy.

Table 1. Correlation between delta-catenin and RhoA in lung cancer

<table>
<thead>
<tr>
<th></th>
<th>delta-catenin</th>
<th>P-value</th>
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<tr>
<td>RhoA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal expression</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>Over expression</td>
<td>12</td>
<td>71</td>
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</table>

Figure 1. Delta-catenin and RhoA expression in normal bronchial epithelium, lung squamous cell carcinoma and adenocarcinoma. Delta-catenin (A) and RhoA (B) was weakly expressed in the cytoplasm of normal bronchial epithelial cells. Positive delta-catenin expression was detected in the cytoplasm of squamous cell carcinoma (C), and RhoA was strongly expressed in these cells (D). Delta-catenin was expressed (E) and RhoA (F) was over-expressed in adenocarcinoma.
Delta-catenin and RhoA in lung cancer

The H460 and LK2 cells were digested with 0.25% trypsin, collected by centrifugation (12,000 rpm), lysed in lysis buffer for 1 hour, and then centrifuged at 14,000×g for 30 minutes at 4°C. The supernatant was extracted, and the protein concentration was determined. The extracts (containing 1 mg of total protein) were incubated with 1 μl of normal rabbit serum and 30 μl of protein G Plus/protein A agarose beads at 4°C for 1 hour, and then centrifuged at 12,000×g for 5 minutes to remove the beads. Anti-delta-catenin (or RhoA) antibody together with 30 μl protein G Plus/protein A agarose beads were added to the supernatant, and this was incubated at 4°C overnight. The mixtures were then centrifuged at 12,000×g for 3 minutes to remove the supernatant, and the beads containing immune complexes were washed five times with PBS buffer. Normal rabbit IgG was used as a negative control.

**Statistical analysis**

All data were analyzed with SPSS for Windows 18.0 (SPSS Inc., Chicago, IL, USA). Immunohistochemical results were analyzed using Pearson’s Chi-Square test. The results of the western blot and RT-PCR were analyzed by variance analysis and the t-test. The Kaplan Meier method was used to compare survival between different patient groups, and differences in survival were tested using the Log-Rank test. A value of $P<0.05$ was considered statistically significant.

**Results**

*Delta-catenin and RhoA co-expression is associated with a poor prognosis in NSCLC patients*

Among 128 lung cancer cases, delta-catenin was expressed in 64.06% (82/128) of cases; and RhoA was over-expressed in 64.84% (83/128) of cases. There was a significant association between RhoA over-expression and delta-catenin positive expression ($P<0.001$)

### Table 3. Relationship between co-expression and clinicopathological factors

<table>
<thead>
<tr>
<th>Clinicopathological factors</th>
<th>n</th>
<th>Delta-catenin positive expression</th>
<th>RhoA over-expression</th>
<th>Co-expression</th>
<th>$P$-value</th>
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<td>Age (years)</td>
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<tr>
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<td>39</td>
<td>39</td>
<td>31</td>
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<tr>
<td>≥58</td>
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<td>I–II stage</td>
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<td>15</td>
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<td>64</td>
<td>63</td>
<td>56</td>
<td></td>
</tr>
</tbody>
</table>
Delta-catenin and RhoA in lung cancer

We also analyzed the relationship between co-expression (concurrent delta-catenin positive expression and RhoA over-expression) and clinicopathological factors (Table 3). This revealed that the co-expression rate in lung adenocarcinoma (65.08%, 41/63) was significantly higher than in squamous cell carcinoma (46.15%, 30/65) (P<0.05). Furthermore, the co-expression rate in stage III-IV disease (65.08%, 41/63) was higher than in stage I-II disease (46.15%, 30/65) (P<0.05), and the co-expression rate in well to moderately differentiated tumors (70.27%, 26/37) was higher than in poorly differentiated tumors (49.45%, 45/91) (P<0.05). The co-expression rate in tumors with lymph node (LN) metastasis (62.92%, 56/89) was higher than in those without LN metastasis (38.46%, 15/39) (P<0.05). There were no significant differences in co-expression with respect to patient age and sex (P>0.05).

In cases where delta-catenin and RhoA were co-expressed in tumors, the average survival time and 5-year survival rate were significantly lower than when tumors did not co-express delta-catenin and RhoA (P<0.05 for both). The co-expression of these proteins was thus significantly associated with a poor prognosis (Figure 2).

Delta-catenin and RhoA expression was elevated in lung cancer at both the protein and RNA level

Western blot analysis revealed that the protein expression of both RhoA and delta-catenin in adjacent normal lung tissue was significantly lower than in lung cancer (Figure 3A & 3B). RT-PCR analysis also revealed that delta-catenin and RhoA RNA levels were significantly higher in lung cancer compared to normal lung tissue (Figure 3C & 3D).

A direct interaction between delta-catenin and RhoA

The co-localization of delta-catenin and RhoA in the cytoplasm of H460 and LK2 cells was confirmed using laser scanning confocal microscopy (Figure 4A-F). Furthermore, the co-immunoprecipitation analysis demonstrated that delta-catenin and RhoA could directly interact with each other in both cell types (Figure 4G & 4H).
Figure 4. Interaction of delta-catenin and RhoA in lung cancer cells. The sub-cellular location of delta-catenin (A) and RhoA (B) in H460 cells, and their co-localization (C). The sub-cellular location of delta-catenin (D) and RhoA (E) in LK2 cells, and their co-localization (F). Co-immunoprecipitation of delta-catenin and RhoA in H460 and LK2 cells (G, H).

Figure 5. RhoA and delta-catenin expression. RhoA expression was reduced after delta-catenin expression (A, B), and elevated after the forced expression of delta-catenin (C, D). Elevated delta-catenin expression reduced RhoA activity in LK2 cells (E), and RhoA activity increased after silencing delta-catenin in H460 cells (F). *P<0.05.
Delta-catenin and RhoA in lung cancer

Western blot and RT-PCR analysis revealed that the protein and mRNA expression of RhoA was reduced after delta-catenin knock-down (Figure 5A & 5B), and enhanced after the forced expression of delta-catenin (Figure 5C & 5D). We also measured RhoA activity after the forced expression or silencing of delta-catenin. G-LISA analysis showed that RhoA activity was markedly decreased after up-regulating delta-catenin in LK2 cells (P<0.05) (Figure 5E), whilst it was strongly increased after down-regulating delta-catenin in H460 cells (Figure 5F).

Delta-catenin promotes lung cancer cell invasion

The invasive ability of lung cancer cells after the forced expression or silencing of delta-catenin was evaluated using the Matrigel invasion assay (Figure 6). Forty-eight hours after the forced expression of delta-catenin, the average number of invasive cells (48.65) was significantly higher than in the control group (20.54), whilst 48 hours after the silencing of delta-catenin, the average number of invasive cells (14.96) was lower than in the control group (35.04). Hence, delta-catenin is required for cancer cell invasion.

Figure 6. Reduction of delta-catenin in H460 cells inhibited cell invasion, and the over-expression of delta-catenin in LK2 cells promoted cancer cell invasion. *P<0.05.
Delta-catenin and RhoA in lung cancer

Discussion

The expression of delta-catenin and RhoA has been studied in a number of malignant tumors, including those of prostate, ovary, and lung cancer, and glioma [11, 13-20]. However, an association between delta-catenin and RhoA expression has not been established in lung cancer, and it was also uncertain whether there was a relationship between their co-expression and the prognosis of NSCLC patients.

In this study, we found elevated RNA and protein delta-catenin expression in lung cancer cells compared to normal lung tissue. RhoA was also over-expressed in lung squamous cell carcinoma and adenocarcinoma, often concurrently with delta-catenin expression. Their co-expression was associated with a higher degree of malignancy (high stage, adenocarcinoma, poor differentiation and lymph node metastasis), and was closely associated with a poor prognosis for NSCLC patients.

RhoA has been shown to be expressed at a low level in normal tissue, and is mainly cytoplasmic. It is activated by extracellular stimulation, where upon it is transported to the plasma membrane, and once there it can stabilize intercellular junctions. Delta-catenin was more highly expressed in the cytoplasm of tumor cells, maintaining RhoA in an inactivate state but inducing its expression. This in turn interrupts adhesion signals, leading to the degradation of proteins that inhibit migration, including stress fibers and focal adhesions [12, 21, 22]. Therefore, the immunopositive staining in the cytoplasm of lung cancer cells might represent an accumulation of GDP-bound RhoA. RhoA activity was suppressed by delta-catenin, and the inactivated RhoA would enhance tumor invasion and metastasis.

We found a significant association between delta-catenin positive expression and RhoA over-expression, and that these proteins interact directly. Both proteins affect cell-cell adhesion, and thus delta-catenin was considered to be an important direct regulator of RhoA activity. It has also been reported that RhoA activity is regulated by proteins such as GEF, GAP and GDI [23], and that delta-catenin could indirectly regulate RhoA activity through interacting with these factors [24]. However, the exact nature of these regulatory interactions remains unclear, and further study is therefore needed.

Acknowledgements

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Disclosure of conflict of interest

None.

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