Cigarette smoke induces the expression of Notch3, not Notch1, protein in lung adenocarcinoma

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Abstract. The aim of the present study was to determine the effect of cigarette smoke on the expression of Notch proteins in lung adenocarcinoma (LAC). Protein expression levels of Notch1 and Notch3 were analyzed using immunohistochemistry in 102 human LAC specimens. Of these, 52 were obtained from smokers and 50 from non-smokers. In addition, cigarette smoke extract (CSE) at varying concentrations (1, 2.5 and 5%) was administered to A549 cells. The expression of Notch1 and Notch3 protein was then detected by western blot analysis at different time points (0, 8, 24 and 48 h). Of the 102 LAC specimens, 42 (41.2%) were positive for Notch1 and 63 (61.8%) were positive for Notch3. There was no significant difference in the level of Notch1 expression between smokers and non-smokers with LAC (P>0.05). The positive rate and staining intensity of Notch3 expression were increased in the smokers compared with the non-smokers (P<0.05). The expression of Notch3 protein in A549 cells increased in a time- and dose-dependent manner following treatment with CSE, whilst the expression of Notch1 protein appeared stable. The results suggested that cigarette smoke was able to induce the expression of Notch3, not Notch1, protein in LAC. The data revealed an upregulation of Notch3 in LAC following cigarette smoke exposure. Such findings may provide a novel therapeutic target for the treatment of LAC.

Introduction

Lung cancer is a common cause of cancer-associated mortality in men and women worldwide (1,2). Lung adenocarcinoma (LAC), which is classified as a non-small cell lung cancer (NSCLC), is a prevalent subtype, accounting for ~25% of lung cancers (2,3). Cigarette smoke remains to be a major etiological risk factor for lung cancer (1,4); a previous study reported that an increase in the incidence of LAC was correlated with cigarette smoking (5). In addition, several signaling pathway abnormalities in lung cancer have been found to be associated with smoking (6,7). Therefore, further studies that aim to identify the signals activated by smoking-associated carcinogens may aid in the development of targeted therapies for lung cancer patients with a history of smoking.

Notch signaling pathways have been identified to have an important role in the regulation of cell differentiation, proliferation and apoptosis. At present, four Notch receptors (Notch1-4) have been identified in mammals. Of these, a previous study found that an abnormality in the Notch1 and Notch3 signaling pathway contributed to the pathogenesis of lung cancer (8).

Certain studies (9‑11) have produced controversial findings concerning the expression of Notch1 protein in NSCLC; Donnem et al (9) and Jiang et al (10) concluded that the overexpression of Notch1 was associated with a poorer prognosis in patients with NSCLC. By contrast, a study by Huang et al (11), which focused on LAC, demonstrated opposing results. With respect to Notch3, Haruki et al (12) found that the positive expression rate was ~37% (32/87). However, Zhou et al (13) and Ye et al (14) identified that the level of Notch3 increased in NSCLC tissues. The deviations in the expression of Notch1 and Notch3 may be due to the heterogeneity of the lung cancer samples. Although the study by Huang et al (11), discussed the association between Notch1 and smoking, this correlation remains uncertain due to the relatively insufficient number of smokers with LAC who were recruited to the study. Therefore, a requirement exists to analyze the effect of cigarette smoke on Notch expression in LAC.

In the present study, the association between Notch1 or Notch3 and smokers with LAC was analyzed by immunohistochemistry. In addition, cigarette smoke extract (CSE) was administered to LAC A549 cells and the expression of Notch1 and Notch3 were then detected by western blot analysis.

Materials and methods

Ethics statement. Ethical approval for the present study was granted by the Institutional Ethics Committee of Zhongnan Hospital, Wuhan University (Wuhan, China) and written informed consent was obtained from all patients.
**Patients and tissue samples.** In total, 102 LAC samples were obtained from patients diagnosed with pathological stage II LAC at Zhongnan Hospital, Wuhan University between July 2010 and February 2014. Following surgery (consisting of lobar or sublobar resection), these patients were interviewed to determine their smoking history. The tumors were staged according to the seventh edition of the International Association for the Study of Lung Cancer (15) and the histological subtype was graded according to guidelines provided by the World Health Organization (16). The clinical characteristics of the patients are shown in Table I.

The criteria used for the smoker group was as follows (17): i) A smoker prior to the diagnosis of lung cancer; ii) a smoking history of ≥10 packs/year; and iii) a smoking habit of ≥10 cigarettes per day during recent years. The non-smokers were defined as patients who had smoked <100 cigarettes during their lifetime and who had been exposed to passive smoking for <0.5 h everyday. Ex-smokers were excluded from the present study. All patients had not received chemotherapy or radiotherapy prior to their surgery.

**Notch1 and Notch3 immunohistochemistry.** Immunostaining of the tumor samples was performed using an avidin-biotinylated horseradish peroxidase complex (ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer’s instructions. Samples were prepared as follows: Samples (~100 mm²) were cut from each tissue and immersed in 10% neutral formalin. Following fixation for 24 h, the tissue block was washed in dH₂O and embedded in paraffin. Prior to experimentation, the samples were cut using a microtome to ~5-µm thickness and affixed onto the slides which had been coated by amino-propyl-tri-ethoxy-silane. The slides were then incubated the slides at 60˚C for 2 h, then placed in a rack and monitored under a microscope until <0.5 h everyday. Ex-smokers were excluded from the present study. All patients had not received chemotherapy or radiotherapy prior to their surgery.

**Cell culture and reagents.** The human LAC A549 cell line (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen Life Technologies). The stock environment was maintained at 37˚C in a humidified 5% CO₂ incubator.

**Cell treatment and measurement of cell viability.** CSE was prepared as previously described (18). In brief, the smoke obtained from four full-strength Marlboro cigarettes (Marlboro Red; Phillip Morris USA, Pittsburgh, PA, USA) with the filters removed was passed through 100 ml of RPMI-1640 medium. The percentage of CSE was referred to as to the undiluted solution and was considered to be 100%. Subsequently, CSE was adjusted to pH 7.4, filtered through a 0.22-µm filter (Sigma-Aldrich, St. Louis, MO, USA) and used within 30 min of preparation. In total, three different concentrations (1, 2.5 and 5%) of CSE diluted with the culture medium were used. Normal RPMI-1640 without CSE was used as a negative control. The A549 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen Life Technologies) for 12 h at 37°C in a humidified 5% CO₂ incubator.

**Western blot analysis.** The samples of the A549 cells were harvested. Equal amounts (20 µg) of the proteins were subjected to SDS-PAGE (6%; Boster Biological Technology, Ltd.) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Sigma-Aldrich). The PVDF membrane was then selected high-power fields. The sections with <5% positive cells were regarded as negative for protein expression. For the positively stained sections, the staining intensity (gray level) of positive granules was assessed using a MIAS-300 image analyzer (Nanjing Aokang Biotechnology Analytical Co., Ltd., Nanjing, China).

**Table I. Characteristics of all patients with lung adenocarcinoma.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Smokers</th>
<th>Non-smokers</th>
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</thead>
<tbody>
<tr>
<td>Total, n</td>
<td>52</td>
<td>50</td>
</tr>
<tr>
<td>Males/females, n</td>
<td>40/12</td>
<td>24/26</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>61.2 (44-74)</td>
<td>62.4 (45-76)</td>
</tr>
<tr>
<td>Smoking history, packs/year</td>
<td>48.3±5.6</td>
<td>0</td>
</tr>
</tbody>
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Smoking history values are presented as the mean ± standard deviation.
blocked with phosphate-buffered saline containing 0.1% Tween 20 (Sigma-Aldrich) and 5% low-fat milk (Boster Biological Technology, Ltd.) and incubated overnight at 4°C with goat polyclonal IgG antibodies against human Notch1 (dilution, 1:400), Notch3 (dilution, 1:400) and GAPDH (I-19; catalog no. sc-48166; dilution, 1:2,000), which were all purchased from Santa Cruz Biotechnology, Inc. PVDF membranes were then incubated with a horseradish peroxidase-conjugated chicken anti-goat IgG secondary antibody (catalog no. sc-2953; Santa Cruz Biotechnology, Inc.; dilution, 1:2,000) for 1 h at room temperature. Immunoreactive bands were visualized using Luminol reagent (Boster Biological Technology, Ltd.) and a ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Clinical information is expressed as the median (range) for the morphological data. The group data are expressed as the mean ± standard deviation. Differences between groups were analyzed using the χ² test and a one-way analysis of variance for the functional data. P<0.05 was considered to indicate a statistically significant difference between values. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).
Results

Immunostaining of Notch1 and Notch3 proteins in LAC. Notch1 and Notch3 were detected by immunohistochemistry (Fig. 1). The gray positive granules of Notch1 and Notch3 were predominantly located in the cell membrane and cytoplasm of the tumor cells. Of the 102 lung cancer specimens, 42 (41.2%) were positive for Notch1 and 63 (61.8%) were positive for Notch3.

Correlation between Notch1 and Notch3 expression and cigarette smoking. As shown in Table II, there was no significant difference in the expression of Notch1 (positive rate and staining intensity) between smokers and non-smokers with LAC. The positive rate of Notch3 expression was higher in smokers compared with non-smokers. By comparing the intensities of positive staining, Notch3 was found to be more highly expressed in the smoking group than in the non-smoking group (134.7±70.4 vs. 82.6±44.6, respectively; P=0.0012). The effects of cigarette smoke on the expression of Notch1 and Notch3 according to gender and histological LAC subtype were not analyzed due to the limited sample size.

Cell viability following CSE treatment. The results of the Trypan blue exclusion test revealed that CSE significantly reduced the viability of A549 cells in a time- and dose-dependent manner at concentrations of 1% and 2.5% (Fig. 2; P<0.05). At a concentration of 5% CSE, the cell viability was markedly reduced at 24 h compared with at 0 and 8 h (P<0.05); however, there was no difference in the cell viability between 24 h and 48 h following the administration of 5% CSE (P>0.05).

Notch1 and Notch3 expression in A549 cells, as detected by western blot analysis. The expression of Notch1 and Notch3 in A549 cells treated with different concentrations of CSE was analyzed at continuous time points by western blot analysis. As shown in Fig. 3, the results revealed that the expression of Notch1 protein in A549 cells treated with CSE was relatively stable at different time points (P>0.05) and at various concentrations (P>0.05). As shown in Fig. 4, the expression of Notch3 in A549 cells increased in a time- and dose-dependent manner following treatment with CSE at concentrations of 1% and 2.5% (P<0.05). The earliest peak of Notch3 protein expression was observed at 24 h following treatment with 5% CSE.

Discussion

It is estimated that >300 million people smoke cigarettes in China (20). Certain individuals do not stop smoking following the onset of respiratory symptoms due to an addiction to the tobacco; in addition, numerous patients continue to smoke cigarettes until a diagnosis of lung cancer has been established. In the present study, in order to identify the signaling pathways that are affected by cigarette smoke, stage II LAC samples were obtained from smokers and non-smokers; in addition, the effects of CSE on A549 cells were investigated. A previous study revealed that abnormalities in the Notch signaling pathway were associated with cigarette smoke in smokers and patients with chronic obstructive pulmonary disorder (COPD) (21). Recent studies have demonstrated that Notch1 and Notch3 have important roles in the pathogenesis...
of LAC (9-14). Therefore, the present study chose to analyze the expression of Notch1 and Notch3 proteins.

Zheng et al (22) reported that overexpression of Notch1 inhibited the growth of A549 cells and interfered with their ability to form tumors in nude mice. The results of a further study by Kluk et al (23) revealed that Notch1 was rarely activated in NSCLC specimens (detailed clinical data concerning LAC was not provided). Wael et al (24) demonstrated that blocking Notch1 in A549 cells resulted in increased cell proliferation. Furthermore, Huang et al (11) observed that negative Notch1 expression was significantly associated with advanced clinical stage and lymph node metastasis in LAC patients. However, increasing evidence indicates that Notch1 acts as an oncogene in LAC. A number of studies have investigated the association between the expression of Notch1 and its clinical significance and found that Notch1 may be used as a predictable biomarker for poor LAC prognoses (9,10). In addition, Westhoff et al (25) established that the activation of Notch1 was correlated with poor clinical outcomes in NSCLC patients not harboring TP53 mutations. Microenvironment hypoxia is common in LAC, where it supports cancer stem cell survival and results in poor responses to anticancer therapies (26-28). A study by Chen et al (29) demonstrated that hypoxia dramatically elevated the expression of Notch1 in lung tumor cell lines and that Notch1 was required for LAC cell survival under hypoxia. It has been reported that under a hypoxic microenvironment, Notch-1 activates Akt-1 through the inhibition of phosphatase and tensin homolog expression and the induction of the insulin-like growth factor 1 receptor (30). Several studies have revealed that A disintegrin and metalloproteinase (ADAM)17 (31), ADAM10 (32) and Galectin-1 (33) may contribute to the migration and invasion of LAC cells via the activation of Notch1. The activation of the Notch1 signaling pathway also has downstream effects on protein kinase cascade kinase 2α (34), Ras (35) and tribbles homolog 3 (36). The activation of Notch1 may contribute to drug resistance in LAC, since the downregulation of Notch1 has been found to be effective during treatment with 6-tocotrienol (37-39), gefitinib (40,41), cisplatin (42) or pterostilbene (43). Blocking Notch1 has been identified as one of the cues to promote migration and invasion of A549 cells in a time- and dose-dependent manner. Therefore it may be hypothesized that cigarette smoke promotes the pathogenesis of LAC via the Notch3 pathway.

In conclusion, the present study revealed that cigarette smoke promoted the expression of Notch3 protein, not Notch1 protein, in LAC. This differed to results obtained from patients with COPD and healthy smokers (21). This may be due to the fact that the Notch signaling pathway has different roles in different diseases. Further studies should be conducted in order to validate these results. Cigarettes contain >60 chemicals that have been identified as carcinogens (48,49); therefore, studies that aim to identify the chemicals in cigarettes that may affect Notch3 are required. In addition, specific inhibitors of the Notch3 pathway may be investigated in future studies in order to clarify the effects of cigarette smoke on Notch3 expression.

Acknowledgements

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