Original Article

Detection of ROS1 translocation in lung adenocarcinoma by IHC, FISH and RT-PCR and its clinicopathologic features

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Abstract: Recently, the translocation of c-ros oncogene 1, receptor tyrosine kinase gene (ROS1) has been demonstrated in lung adenocarcinoma (ADC), but the rate of this translocation is relatively low (~1%). In the present study, data from 473 patients with lung ADC were collected from archived records in the Department of Pathology, Sichuan Academy of Medical Sciences & Sichuan Provincial People’s Hospital, China. The original data were obtained between July 2007 and April 2014. ROS1 translocations were confirmed in 11 patients (2.32%) by screening with immunohistochemistry (IHC) staining and verification with reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). The sex ratio was significantly different in the ROS1-translocated group compared with the non-ROS1-translocated group, with the former having a higher proportion of females ($P = 0.032$). The 11 patients harboring ROS1 translocations had no history of smoking (100%). Histomorphological analysis revealed that the tumors were mostly papillary, with micropapillary components being observed in the majority of positive cases (7/11, 64%). Fewer cases exhibited extracellular mucus secretion and signet-ring components. ROS1 translocations represent another unique molecular subtype of lung ADC with clinical significance. The incidence of ROS1 translocation in lung ADC patients is 2.32%. IHC staining can be used to screen for ROS1 translocations, and positive cases should be verified by FISH or RT-PCR.

Keywords: Adenocarcinoma, lung, ROS1 translocation, fluorescence in situ hybridization, immunohistochemistry, real time PCR, clinicopathological features

Introduction

Lung cancer is the main cause of cancer-related deaths worldwide, and its fatality rate ranks first among all malignant tumors. Over the past decade, multiple potential targeted treatment sites have been identified among the molecular phenotypes of non-small-cell lung carcinoma (NSCLC). ROS1 translocations in lung cancer were first identified by Rikova et al. in 2007 [1]. Subsequently, various ROS1 gene translocations were detected by other researchers. Therefore, ROS1 translocations have come to be acknowledged as a new molecular marker of a lung cancer subtype, similar to EGFR mutations and ALK translocations. Notably, a significant treatment effect has been achieved with ALK tyrosine kinase inhibitors. ROS1 translocations are commonly detected using fluorescence in situ hybridization (FISH), reverse transcription-polymerase chain reaction (RT-PCR), and immunohistochemistry (IHC). Compared with other methods, FISH has a high specificity and can detect all types of ROS1 translocations. However, FISH is expensive and it cannot distinguish between different types of ROS1 translocations. Although RT-PCR can identify specific types of ROS1 translocations, it requires high quality RNA samples. The cost of IHC is low, and this method is easy to carry out. Rimkunas et al. found that an anti-ROS1 rabbit monoclonal antibody (D4D6) can be used to detect the protein product of ROS1 translocations with a high sensitivity [2].

In this study, data from 473 Chinese patients with lung adenocarcinoma (ADC) were collect-
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Materials and methods

Case collection

Four hundred and seventy-three consecutive samples of primary lung ADC surgical resection specimens were collected from the Department of Pathology, Sichuan Provincial People’s Hospital, from July 2007 to April 2014. The electronic case histories of the cohort were reviewed to extract data on clinical information, including sex, age, smoking history, clinical stages, treatment and follow-up. The approval from the hospital’s ethics committee and the informed consent for this study were obtained.

Histological analysis

All the specimens were fixed in 10% formalin solution and embedded in paraffin blocks. Sections were cut and stained with hematoxylin and eosin (H&E) for microscopy. All hematoxylin and eosin (HE) slides were histologically evaluated by 2 pathologists independently according to the European Respiratory Society (ERS)/American Thoracic Society (ATS)/International Association for the Study of Lung Cancer (IASLC) multidisciplinary classification of lung ADC. Some cases with a discrepancy between the 2 reviewers were reviewed again, and a consensus result was reached.

IHC stains and scorings

For each case, 1 block containing the representative tumor cells and less necrosis were chosen for the IHC analysis. The sections were deparaffinised in xylene and graded alcohols to distilled water. Endogenous peroxidases were blocked by placing the sections in a solution of 3% hydrogen peroxide for 20 minutes in the dark. Heat-induced antigen retrieval was performed by immersing the sections in a solution of 1 m methylene diamine tetraacetic acid, pH 9.0, at 100°C for 25 minutes. Sections were then incubated at 37°C with anti-ROS1 antibody (clone D4D6, 1:200; Cell signaling, USA) for 2 hours. IHC stains were performed by the 2-step Envision procedure using a DAKO Autostainer (Dakopatts, Copenhagen, Denmark) with 3, 3’-diaminobenzidine as a chromogen. Positive ROS1 staining was scored as weak or faint (1+), moderate (2+) or strong (3+) staining in the cytoplasmic granular pattern. For those IHC positive cases, ten consecutive 4-μm-thick sections were cut for FISH and RT-PCR analysis.

FISH detection for ROS1 translocation

FISH detection was performed using the LSI ROS1 Dual Color Break Apart Translocation Probe (Abbott-Vysis, Downers Grove, IL). Sections were observed under a ×100 objective with a fluorescence microscope (Olympus BX-UCB, Japan). There were 2 patterns of ROS1 translocation FISH signals noted in lung ADC: the classic break-apart (BA) and isolated green signal (IRG) patterns. A minimum of 100 non-overlapping tumor cell nuclei should be scored for each case. Positivity was defined as > 15% of tumor cells demonstrating a BA and/or IRG pattern.

Real-time PCR detection for ROS1 translocation

Total RNA was extracted from paraffin-embedded blocks using a DNeasy kit (Qiagen, Dusseldorf, Germany). RNA was then reverse transcribed into cDNA, using the ROS1 translocation detection kit (AmoyDx, Xiamen, People’s Republic of China). The reverse transcription conditions were as follows: 42°C, 60 minutes; 95°C, 5 minutes. Then, quantitative PCR was performed to screen for ROS1 gene translocations on an ABI 7500 Real-Time PCR System (Applied Biosystems Inc, Foster City, Calif) according to the manufacturer’s instructions.

Confirmation procedure

For the ROS1 IHC staining positive cases, both FISH and RT-PCR was performed to confirm the existence of ROS1 translocation.

Table 1. IHC, FISH and RT-PCR detection results

<table>
<thead>
<tr>
<th>IHC score (number)</th>
<th>RT-PCR Positive</th>
<th>RT-PCR Negative</th>
<th>FISH Positive</th>
<th>FISH Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (382)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1+ (28)</td>
<td>0</td>
<td>28</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>2+ (45)</td>
<td>0</td>
<td>45</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>3+ (18)</td>
<td>11</td>
<td>7</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>
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Statistical analysis

SPSS version 16.0 (Chicago, IL) was used for the statistical analysis, which included the Pearson χ² test or Fisher exact test. A P value less than .05 was considered statistically significant.

Results

Comparison of IHC, FISH and PCR for the detection of ROS1 translocations

The IHC, RT-PCR, and FISH results are shown in Table 1. ROS1-IHC detected 382, 28, 45, and 18 cases with 0, 1+, 2+, and 3+ staining scores, respectively (Figure 1). Among the IHC-positive cases, a ROS1 translocation was confirmed in 11 cases by RT-PCR. Ten of the 11 cases were positive for FISH, including 7 cases with the classical split signal, 2 cases with a single green signal, and 1 case in which both types of signal were detected (Figure 1). Additionally, one case had a FISH-positive percentage of 10%, which was interpreted as negative according to the criteria (Figure 2). This case showed a narrow distance between the split red and green signals (distance > 1 signal point). Moreover, an increase in the copy number of ROS1 was observed in some cases that were positive for ROS1-IHC staining but negative for ROS1 translocation (Figure 3).

Clinicopathological features of ROS1-translocated lung ADC

The clinical features were compared between 11 patients with ROS1-translocated lung ADC and 462 lung ADC patients harboring wild-type ROS1 (Table 2). Female patients were significantly predominant in the ROS1-translocated group compared with the wild-type ROS1 group (P = 0.032). The 11 ROS1-translocated cases had no history of smoking (100%). With respect...
to the clinical stage of the positive cases, 6 (55%) were stage I, 2 (18%) were stage II, and 3 (27%) were stage III. The mean tumor diameter was 3.19 cm (range, 1.2-6 cm); no significant differences in staging were observed compared with the ROS1 translocation-negative group.

All 11 patients with ROS1-translocated lung ADC had tumors that were poorly to moderately differentiated. The predominant type was papillary growth in 7 cases (64%), solid growth in 3 cases (27%), and acinar growth in 1 case (9%). Two (18%) of these cases were associated with characteristics of extracellular mucus secretion; 1 case (9%) was associated with signet-ring cell components, but the proportion was small, i.e., less than 5% of tumor tissues (Figure 3).

Among the 11 patients with ROS1-translocated lung ADC, 8 (73%) underwent surgery combined with platinum-based chemotherapy, 3 (27%) underwent surgery alone, and none underwent crizotinib treatment. As of January 31, 2015, follow-up data were obtained from 6 patients, including 2 deaths (survival times of 10 and 0.5 months) and 4 survivors.

**Discussion**

In this study, 473 patients with lung ADC were included and ROS1 translocations were detected in 11 cases (2.32%) by IHC screening combined with RT-PCR and FISH verification. The incidence of ROS1 translocation was quite low in lung ADC, similar to the results reported in previous studies performed in China and other countries. In 2007, the incidence of ROS1 translocation in NSCLC was initially estimated to be 0.06% (1/150 cases) by Rikova et al., who used RT-PCR and DNA sequencing. Subsequently, the positive rate for ROS1 translocations in NSCLC was found to vary from 0.6 to 2.0% [1-10] by several research groups using

![Image](A: Strong staining (3+) by ROS1-IHC, with 100% positive tumor cells, ×200 magnification; B: ROS1-FISH showing split signal (narrow distance); C: ROS1 translocation confirmed by RT-PCR; D: Some cases showing an increase in the copy number of ROS1 normal fusion signals.)
different methods. Cases harboring ROS1 translocations were exclusively lung ADC, and only a small portion of patients were squamous or large cell carcinomas [2, 4, 9, 10]. Therefore, subsequent studies have more frequently focused on lung ADC, and the positive rate for ROS1 translocations in lung ADC ranges from approximately 1.0% to 3.9% [2-4, 7, 11-15]. The present study showed that there was no significant difference in the incidence of ROS1 translocations in lung ADC between Eastern and Western populations.

Both RT-PCR and FISH only detected ROS1 translocations in the cases with 3+ staining by ROS1-IHC. This suggests that ROS1-IHC is an effective screening tool for ROS1 translocations. Nine different ROS1 translocation genes have been identified in NSCLC, i.e., CD74-ROS1, SLC34A2-ROS1, TPM3-ROS1, EZR-ROS1, SDC4-ROS1, LRIG3-ROS1, FIG-ROS1, KDELR2-ROS1, and CCDC6-ROS1. Among these genes, CD74-ROS1 is the most common [16]. The majority of FISH-positive patients showed the classic split signal, with well-separated green and red signals that were easy to interpret. A few cases showed a single green signal or a combination of both split and single green signals. Only 1 case showed the split signal with a short distance between the green and red signals. In this case, the percentage of FISH-positive cells was 10%, which was interpreted as negative according to the positive threshold of 15%. However, ROS1 gene translocation was confirmed in this case by RT-PCR. The false negative result of FISH may be attributable to the difficulty in distinguishing the short-distance split mode from the negative signal, which results in the misreading of certain positive signals. Therefore, if the FISH results are uncertain or close to the positive threshold, the case should be verified by RT-PCR.

Bergethon, et al. reported that the ROS1-positive group in their study had a median age

![Figure 3. Morphological features of ROS1-translocated lung adenocarcinoma. A: Papillary structures (HE, ×200); B: Acinar structure (HE, ×200); C: Solid structure (HE, ×200); D: Significant extracellular mucus secretion (HE, × 200).](image-url)
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Table 2. Clinicopathological features of ROS1 translocation-positive and negative lung adenocarcinomas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ROS1-FISH + (n = 11)</th>
<th>ROS1-FISH - (n = 462)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>56</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>236</td>
<td>0.032</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>226</td>
<td></td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>11</td>
<td>297</td>
<td>0.020</td>
</tr>
<tr>
<td>Ever</td>
<td>0</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td>0.368</td>
</tr>
<tr>
<td>I</td>
<td>6</td>
<td>231</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td>0.192</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>3</td>
<td>214</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td>0.361</td>
</tr>
<tr>
<td>≤3</td>
<td>8</td>
<td>245</td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>3</td>
<td>194</td>
<td></td>
</tr>
</tbody>
</table>

of 49.8 years (32-79 years), while the ROS1-negative group had a median age of 62.3 years [5]. Yoshida et al. investigated 15 patients with ROS1 translocation and found that the percentage of female patients was significantly higher in the ROS1-translocated group compared with the ROS1-negative group (80% V.S. 45%) [3]. In our study, the patients with ROS1 translocations were relatively young, and there was a significant difference in the male-to-female ratio, a finding that was consistent with most other reports. However, other studies showed no significant difference in the sex ratio of patients with ROS1 translocations [11, 12]. Currently, most studies indicate that ROS1-translocated lung cancer is associated with no history of smoking or a history of light smoking [3, 5, 6, 11, 17]. It is worth noting that some ROS1-translocated patients still have a history of smoking. Therefore, lung ADC patients who smoke cannot be excluded for ROS1 translocation screenings.

Bergethon et al. reported 18 patients who were positive for ROS1 translocations, including 7 cases with tumors that were acinar predominant, 5 that were solid predominant, 5 that were papillary predominant and 1 that was lepidic predominant and exhibited mucus secretion [5]. Yoshida et al. summarized 15 patients who were positive for ROS1 translocations. Of these cases, six had tumors that were primarily papillary predominant, four tumors were solid predominant, three were lepidic predominant, and two were acinar predominant [3]. Moreover, Cha found that a solid tumor with mucus secretion was most common in 13 patients positive for ROS1 translocations [12]. In the present study, the 11 ROS1-translocated patients primarily showed papillary and solid growth. The discrepancy in the morphological results of the above studies may be attributable to the small sample sizes. It is also possible that ROS1-translocated lung ADC lacks a unique morphology.

As ROS1-translocated lung ADC was detected in a small number of patients by previous studies, only a few researchers have investigated the prognosis of patients with this subtype [3, 4, 7, 10, 13]. Cai et al. assessed the prognosis of 8 patients with ROS1 translocations and found that being positive for ROS1 translocation is a predictive factor for poor prognosis [10]. However, Warth et al. analyzed the prognosis of 9 patients with ROS1 translocations and found that being positive for ROS1 translocation is suggestive of a better prognosis [4]. Presently, no consensus has been reached on the relationship between ROS1-translocated lung cancer and prognosis. The effect of the mutation on prognosis may depend on the use of targeted therapy. The Food and Drug Administration of the United States has approved crizotinib as a medication for the treatment of ROS1-translocated NSCLC. However, the use of crizotinib is not approved in China. As the present study performed a retrospective analysis, none of the 11 patients with ROS1-translocated lung ADC received crizotinib treatment, and platinum drugs were used in their chemotherapy regimen.

Conclusions

We detected ROS1 translocations in lung ADCs at a rate of 2.3% among a large Chinese patient sample. ROS1 translocations represent another molecular subtype of lung cancer with independent clinical significance. These patients are relatively young, predominantly female and
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generally have no smoking history. IHC staining can be used to screen for ROS1 translocations, although IHC-positive cases should be verified by FISH or RT-PCR. If the FISH results are unclear or near the positive threshold, further confirmation should be performed by RT-PCR to provide better evidence for the use of targeted therapy.

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Disclosure of conflict of interest

None.

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References


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