Druggable Oncogene Fusions in Invasive Mucinous Lung Adenocarcinoma

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Abstract

**Purpose:** To identify druggable oncogenic fusions in invasive mucinous adenocarcinoma (IMA) of the lung, a malignant type of lung adenocarcinoma in which KRAS mutations frequently occur.

**Experimental Design:** From an IMA cohort of 90 cases, consisting of 56 cases (62%) with KRAS mutations and 34 cases without (38%), we conducted whole-transcriptome sequencing of 32 IMAs, including 27 cases without KRAS mutations. We used the sequencing data to identify gene fusions, and then performed functional analyses of the fusion gene products.

**Results:** We identified oncogenic fusions that occurred mutually exclusively with KRAS mutations: CD74-NRG1, SLC3A2-NRG1, EZR-ERBB4, TRIM24-BRAF, and KIAA1468-RET. NRG1 fusions were present in 17.6% (6/34) of KRAS-negative IMAs. The CD74-NRG1 fusion activated HER2:HER3 signaling, whereas the EZR-ERBB4 and TRIM24-BRAF fusions constitutively activated the ERBB4 and BRAF kinases, respectively. Signaling pathway activation and fusion-induced anchorage-independent growth/tumorigenicity of NIH3T3 cells expressing these fusions were suppressed by tyrosine kinase inhibitors approved for clinical use.

**Conclusions:** Oncogenic fusions act as driver mutations in IMAs without KRAS mutations, and thus represent promising therapeutic targets for the treatment of such IMAs. *Clin Cancer Res; 20(12); 3087–93. ©2014 AACR.*

Introduction

Oncogene fusions have recently been identified as driver mutations and (possible) therapeutic targets in lung adenocarcinoma (LADC), a major histologic type of lung cancer (1). Such fusions include EML4- or KIF5B-ALK, KIF5B, or CCDC6-RET, and CD74-, EZR-, or SLC34A2-ROS1 (2–9). These oncogenic fusions occur mutually exclusively with one another, and with other targetable oncogene aberrations such as EGFR, KRAS, BRAF, and HER2 mutations. Therefore, molecular targeted therapy combined with the identification of driver oncogene aberrations represents a powerful and promising approach to personalized treatment of LADC (10, 11).

Invasive mucinous adenocarcinoma (IMA) of the lung is composed predominantly of goblet cells. IMA is morphologically characterized by tall columnar cells with basal nuclei and a pale cytoplasm containing varying amounts of mucin (12, 13). IMAs, which constitute 2% to 10% of all LADCs in Japan, the United States, and European countries (14–16), are indicated as being more malignant than more common types of LADC, such as acinar or papillary adenocarcinoma. The KRAS mutation is the only driver aberration commonly detected in IMAs (in 50%–80% of cases). To date, no driver gene aberrations have been detected in KRAS-negative IMAs; these aberrations must be identified to facilitate the development of effective treatments for such cancers. Therefore, we performed whole-transcriptome sequencing (RNA sequencing) of IMAs lacking KRAS mutations to identify novel chimeric fusion transcripts that represent potential targets for cancer therapy.
Translational Relevance

Oncogene fusions, such as the ALK, RET, and ROS1 fusions, have recently been revealed as therapeutic targets in lung adenocarcinoma (LADC). We identified multiple druggable oncogene fusions, including those involving the NRG1, ERBB4, and BRAF genes, in invasive mucinous adenocarcinoma (IMA), a malignant type of LADC. The fusions occurred mutually exclusively with KRAS mutations, a common driver oncogene aberration in IMA. These fusions represent potentially clinically relevant targets for treatment of IMAs that lack KRAS mutations.

Materials and Methods

Samples

Ninety IMAs were identified among consecutive patients with primary adenocarcinoma of the lung who were treated surgically at the National Cancer Center Hospital, Tokyo, Japan, from 1998 to 2013. Histologic diagnoses were based on the most recent World Health Organization classification and the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) criteria for LADC (13, 17). Total RNA was extracted from grossly dissected, snap-frozen tissue samples using TRIzol (Invitrogen). The study was approved by the Institutional Review Boards of the participating institutions.

RNA sequencing

RNA sequencing libraries were prepared from 1 or 2 μg of total RNA using the miRNA-Seq Sample Prep Kit or TrueSeq RNA Sample Prep Kit (Illumina). The resultant libraries were subjected to paired-end sequencing of 50 or 75 bp reads on a Genome Analyzer Ix (GAIIx) or HiSeq 2000 (Illumina). Fusion transcripts were detected using the TopHat-Fusion algorithm (18). Experimental conditions for RNA sequencing are described in Supplementary Table S1.

Examinations of oncogenic properties of fusion products

To construct lentivectors for expression of the CD74-NRG1, EZR-ERBB4, and TRIM24-BRAF fusion proteins, full-length cDNAs were amplified from tumor cDNA by PCR and inserted into pLenti-6/V5-DEST plasmids (Invitrogen). The integrity of each inserted cDNA was verified by Sanger sequencing. Expression of fusion products of the predicted sizes was confirmed by Western blot analysis of transiently transfected and virally infected cells (Supplementary Fig. S1A). Details of plasmid transfection, viral infection, Western blot analysis, and soft agar colony and tumorigenicity assays are described in Supplementary Materials and Methods.

Results and Discussion

We prepared an IMA cohort of 90 cases consisting of 56 (62%) cases with KRAS mutations and 34 (38%) cases without. The 34 KRAS-negative cases included two, one, and one cases with BRAF mutation, EGFR mutation, and EML4-ALK fusion, respectively; the remaining 30 were "pan negative" for representative driver aberrations in LADCs. Thirty-two cases, consisting of 27 pan-negative and five KRAS mutation-positive cases, were subjected to RNA sequencing (Supplementary Table S1). Analysis of >2 x 10^7 paired-end reads obtained by RNA sequencing and subsequent validation by Sanger sequencing of reverse transcription PCR (RT-PCR) products revealed five novel gene-fusion transcripts detected only in the pan-negative IMAs: CD74-NRG1, SLC3A2-NRG1, EZR-ERBB4, TRIM24-BRAF, and KIAA1468-RET (Fig. 1A and B; Table 1; details in Supplementary Materials and Methods; Supplementary Fig. S2 and Supplementary Table S2). RT-PCR screening of these fusions in the remaining 58 IMAs that had not been subjected to RNA sequencing revealed one additional pan-negative case with the CD74-NRG1 fusion. Thus, the CD74-NRG1 fusion, detected in five of 34 (14.7%) cases negative for KRAS mutations, was the most frequent fusion among KRAS mutation-negative IMAs. Fusions of CD74 or SLC3A2 with NRG1 were present in 17.6% (6/34) of cases. The five novel fusions were mutually exclusively with one another and were not present in any of the KRAS mutation-positive cases (Table 2).

Four of the novel fusions, CD74-NRG1, SLC3A2-NRG1, EZR-ERBB4, and TRIM24-BRAF, involved rearrangements of genes encoding protein kinases or a ligand of a receptor protein kinase (NRG1/neuregulin/herégulin) for which oncogenic rearrangements have not been previously reported in lung cancer (Supplementary Fig. S3). The remaining fusion was a novel type involving the RET protein kinase (NRG1/neuregulin/herégulin) for which oncogenic rearrangements have not been previously reported in lung cancer (Supplementary Fig. S3). The remaining fusion was a novel type involving the RET oncogene; fusions with RET are observed in 1% to 2% of LADCs (4, 5, 7, 8, 11). In a screen of 315 LADCs without IMA features from Japanese patients and 144 consecutive LADCs from U.S. patients, all tumors were negative for all of the NRG1, BRAF, and ERBB4 fusions, as well as the novel RET fusion. Therefore, these fusions might be driver aberrations specific to LADCs with IMA features. The four novel gene fusions were likely to have been caused by interchromosomal translocations or paracentric inversion (Table 1 and Supplementary Fig. S3). Consistently, separation of the signals generated by the probes flanking the translocation sites of NRG1 in fusion-positive tumors was observed upon FISH analysis of CD74-NRG1 fusion-positive tumors (Supplementary Fig. S4). We also confirmed overexpression of NRG1, ERBB4, and BRAF proteins in tumor cells carrying the corresponding fusions by immunohistochemical analysis, using antibodies recognizing polypeptides retained in the fusion proteins; expression of NRG1, ERBB4, and BRAF proteins was also observed in some fusion-negative cases (Supplementary Fig. S5). IMAs harboring gene fusions were obtained from both male and female patients, although
Figure 1. Oncogenic fusions in invasive mucinous LDAC. A, schematic representations of the wild-type proteins (top rows of each section) followed by the fusion proteins identified in this study. The breakpoints for each variant are indicated by blue arrows. TM, transmembrane domain. Locations of putative cleavage sites in the NRG1 polypeptide are indicated by dashed green lines. B, detection of gene-fusion transcripts by RT-PCR. RT-PCR products for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown below. Six IMAs (T) positive for gene fusions are shown alongside their corresponding non-cancerous lung tissues (N); labels below the gel image indicate sample IDs (see Table 1). C, pie chart showing the fraction of IMAs that harbor the indicated driver mutations.
NRG1 fusion-positive cases were preferentially from female never smokers (Table 1). The CD74-NRG1 and SLC3A2-NRG1 fusion proteins, whose sequences were deduced from RNA sequencing data, contained the CD74 or SLC3A2 transmembrane domain and retained the EGF-like domain of the NRG1 protein (NRG1 III-b form; Fig. 1A). The NRG1 III-b3 protein has a cytosolic N-terminus and a membrane-tethered EGF-like domain, and mediates juxtacrine signals signaling through HER2:HER3 receptors (19). Because parts of CD74 or SLC3A2 replaced the transmembrane domain of wild-type NRG1 III-b3, we speculated that the membrane-tethered EGF-like domain might activate juxtacrine signaling through HER2:HER3 receptors. In addition, it was also possible that expression of these fusion proteins resulted in the production of soluble NRG1 protein due to proteolytic cleavage at sites derived from NRG1 (dashed green lines in Fig. 1A), as recently suggested for NRG1 type III proteins (20, 21). Exposing EFM-19 cells to conditioned media from H1299 human lung cancer cells expressing exogenous CD74-NRG1 fusion protein resulted in phosphorylation of endogenous ERBB2/HER2 and ERBB3/HER3 proteins, suggesting that autocrine HER2:HER3 signaling was activated by secreted NRG1 ligands generated from CD74-NRG1 polypeptides (Fig. 2A). Phosphorylation of extracellular signal—regulated kinase (ERK) and AKT, downstream mediators of HER2:HER3, was also elevated. HER2, HER3, and ERK phosphorylation was suppressed by lapatinib and afatinib, U.S. Food and Drug Administration (FDA)-approved tyrosine kinase inhibitors (TKI) that target HER kinases (22–24). Together, these observations indicate that NRG1 fusions activated HER2:HER3 signaling by juxtacrine and/or autocrine mechanisms.

The EZR-ERBB4 fusion protein contained the EZR coiled-coil domain, which functions in protein dimerization, and also retained the full ERBB4 kinase domain (Fig. 1A). These features indicated that the EZR-ERBB4 protein is likely to form a homodimer via the coiled-coil domain of EZR, causing aberrant activation of the kinase function of ERBB4, similar to the situation of EZR-ROS1 fusion (5). Indeed, when the EZR-ERBB4 cDNA was exogenously expressed in NIH3T3 fibroblasts, tyrosine 1258, located in the activation loop of the ERBB4 kinase site, was phosphorylated in the absence of serum stimulation, indicating that fusion with EZR aberrantly activated the ERBB4 kinase (Fig. 2B). Consistent with this, phosphorylation of a downstream

### Table 1. Characteristics of invasive mucinous LDACs with novel gene fusions

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Sex</th>
<th>Age (year)</th>
<th>Smoking habit</th>
<th>Gene fusion</th>
<th>Chromosome aberration</th>
<th>Oncogene mutation</th>
<th>Pathologic stage</th>
<th>TTF1</th>
<th>HNF4A</th>
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<tr>
<td>1</td>
<td>301T</td>
<td>M</td>
<td>55</td>
<td>Ever (47)</td>
<td>CD74-NRG1</td>
<td>t(5;8)(q32;p12)</td>
<td>None</td>
<td>1a</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>AD12-108T</td>
<td>F</td>
<td>68</td>
<td>Never</td>
<td>CD74-NRG1</td>
<td>None</td>
<td>2b</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>3</td>
<td>AD09-404T</td>
<td>F</td>
<td>78</td>
<td>Never</td>
<td>CD74-NRG1</td>
<td>None</td>
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<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>AD13-199T</td>
<td>F</td>
<td>47</td>
<td>Never</td>
<td>CD74-NRG1</td>
<td>None</td>
<td>1b</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
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<td>AD13-223T</td>
<td>F</td>
<td>53</td>
<td>Never</td>
<td>CD74-NRG1</td>
<td>None</td>
<td>1a</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>6</td>
<td>AD13-379T</td>
<td>F</td>
<td>66</td>
<td>Never</td>
<td>SLC3A2-NRG1</td>
<td>t(8;11)(p12;q13)</td>
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<td>1b</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
<tr>
<td>7</td>
<td>436T</td>
<td>M</td>
<td>61</td>
<td>Ever (41)</td>
<td>EZR-ERBB4</td>
<td>t(2;6)(q25;q34)</td>
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<td>8</td>
<td>AD08_127T</td>
<td>F</td>
<td>66</td>
<td>Never</td>
<td>TRIM24-BRAF</td>
<td>inv7(q33;q34)</td>
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<td>1a</td>
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<td>+</td>
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<td>9</td>
<td>AD12-119T</td>
<td>M</td>
<td>62</td>
<td>Current (63)</td>
<td>KIAA1468-RET</td>
<td>t(10;18)(q21;q11)</td>
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<td>1a</td>
<td>+</td>
<td>–</td>
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*EGFR, KRAS, BRAF, and HER2 mutations and ALK, RET, and ROS1 fusions.

### Table 2. Characteristics of 90 invasive mucinous LDACs

| Variable | All | KRAS | BRAF | EGFR | CD74-NRG1 or SLC3A2-NRG1 | EZR-ERBB4 | TRIM24-BRAF | EML4-ALK | KIAA1468-RET | None (%)
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<tr>
<td>Total</td>
<td>90</td>
<td>56</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Age (mean ± SD; y)</td>
<td>67.2 ± 9.7</td>
<td>68.1 ± 9.7</td>
<td>66.5 ± 3.5</td>
<td>50</td>
<td>61.2 ± 11.5</td>
<td>61</td>
<td>66</td>
<td>64</td>
<td>62</td>
<td>68.1 ± 9.6</td>
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<tr>
<td>Male (%)</td>
<td>39</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>8</td>
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<td>Female (%)</td>
<td>51</td>
<td>28</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>13</td>
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<tr>
<td>Smoking habit</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker (%)</td>
<td>51</td>
<td>29</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>13</td>
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<tr>
<td>Ever smoker (%)</td>
<td>39</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>8</td>
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</table>
mediator ERK was also elevated. Phosphorylation of ERBB4 and ERK was suppressed by lapatinib and afatinib, which inhibit ERBB4 protein (22–24).

The TRIM24-BRAF fusion protein retained the BRAF kinase domain but lacked the N-terminal RAS-binding domain responsible for negatively regulating BRAF kinase. These features suggested that the fusion was constitutively active, as in the cases of the ESRP1-BRAF and AGTRAP-BRAF fusions in other cancers (25). When the TRIM24-BRAF cDNA was exogenously expressed in NIH3T3 cells, ERK, a downstream mediator of BRAF, was phosphorylated in the absence of serum stimulation, indicating that fusion with TRIM24 aberrantly activated BRAF kinase (Fig. 2C). ERK phosphorylation was suppressed by sorafenib, an FDA-approved drug originally identified as a RAF kinase inhibitor (26), and also by the MEK inhibitor U0126 (Fig. 2C).

Exogenous expression of fusion gene cDNAs induced anchorage-independent growth of NIH3T3 fibroblasts, indicating their transforming activities (Fig. 2D–F). This growth was suppressed by the kinase inhibitors that suppressed fusion-induced activation of signal transduction, as described above. NIH3T3 cells expressing EZR-ERBB4 or TRIM24-BRAF fusion cDNA formed tumors in nude mice (Fig. 3). Therefore, we concluded that these three fusions function as driver mutations in IMA development. We screened 200 commonly used human lung cancer cell lines, but all were negative for these three fusions (data not shown); thus, the oncogenic properties of these fusions remain unvalidated in human cancer cells.
The results here suggest that the NRG1, ERBB4 and BRAF fusions are novel driver mutations involved in the development of IMA of the lungs (Fig. 1C) and potential targets for existing TKIs. The recurrent NRG1 fusions were especially notable because NRG1 was previously identified as a regulator of goblet-cell formation in primary cultures of human bronchial epithelial cells (27); therefore, activation of the NRG1-mediated signaling pathway(s) might play a part in IMA development by contributing to both cell transformation and acquisition of goblet-cell morphology. In addition to a small fraction of known druggable aberrations (an ALK fusion and an EGFR mutation), more than 10% (11/90; 12.2%) of IMA harbored other druggable aberrations targeted by existing kinase inhibitors: these aberrations were represented by fusions involving NRG1, ERBB4, BRAF, or RET, or BRAF mutations (Table 2, Fig. 1C). To facilitate translation of these findings to the cancer clinic, it will be necessary to establish diagnostic methods, particularly using break-apart and fusion FISH methods, capable of detecting these aberrations. Such methods will also help identify additional fusions involving other partner genes and contribute to a greater understanding of the significance of gene fusions in lung carcinogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Nakaoku, K. Tsuta, M. Enari, A.J. Schetter, C.C. Harris
Writing, review, and/or revision of the manuscript: T. Nakaoku, K. Tsuta, H. Ogiwara, S.-I. Watanabe, H. Nokihara, K. Yasuda, M. Hiramoto, A.J. Schetter, C.C. Harris, Y.H. Kim, M. Mishima, T. Yoshida, T. Kohno
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Tsuta, K. Shirashi, M. Enari, H. Ogawa, S.-I. Watanabe, H. Okayama
Study supervision: K. Tsuta, J. Yokota

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Figure 3. Tumorigenicity of NIH3T3 cells expressing EZR-ERBB4 or TRIM24-BRAF fusion cDNAs. A, tumor growth in nude mice injected with NIH3T3 cells expressing empty vector, EZR-ERBB4 fusion, or TRIM24-BRAF fusion. Cells were resuspended with 50% Matrigel and injected into the right flank of nude mice. Tumor size was measured twice weekly for 5 weeks. Data are shown as mean ± SEM. B, representative tumors were photographed on day 21. The numbers in parentheses indicate the ratio of the number of mice with tumors to the number of mice receiving cell injection.
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