A model of KIF5B-RET fusion-dependent lung tumorigenesis

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Oncogenic fusion of the RET (rearranged during transfection) gene was recently identified as a novel driver gene aberration not only for the development of thyroid carcinoma but also of lung adenocarcinoma, the most frequent histological type of lung cancer. This study constructed and analyzed transgenic mice expressing KIF5B-RET, the predominant form of RET fusion gene specific for lung adenocarcinoma, under the control of the SPC (surfactant protein C) gene promoter. The mice expressed the KIF5B-RET fusion gene specifically in lung alveolar epithelial cells, and developed multiple tumors in the lungs. Treatment of the transgenic mice with vandetanib, which is a RET tyrosine kinase inhibitor approved by the U.S. Food and Drug Administration for the treatment of thyroid carcinoma, for 8 or 20 weeks led to a marked reduction in the number of lung tumors (3.3 versus 0 and 6.5 versus 0.2 per tissue section, respectively; P < 0.01, t-test). The results suggest that the RET fusion functions as a driver for the development of lung tumors, whose growth is inhibited by RET tyrosine kinase inhibitors.

Introduction

Lung adenocarcinoma (LADC) is the most common histological type of lung cancer and its incidence is increasing in both Asian and Western countries (1). Recent genome-wide sequencing analyses identified somatic genomic aberrations associated with LADC. Activating mutations in several protein kinase genes, including EGFR, BRAF, HER2/ERBB2, PIK3CA and MET, function as drivers for LADC development and are potential therapeutic targets (2). Oncogenic fusions of protein tyrosine kinase genes, such as ALK and ROS1, are also drivers of carcinogenesis (3,4); indeed, their ability to drive tumorigenesis in vivo has been shown by constructing and analyzing transgenic mice expressing the EMLA-ALK and EZR-ROS1 genes (predominant forms of ALK and ROS1 fusions, respectively) specifically in lung alveolar epithelial cells (5–7).

We and others recently identified oncogenic fusions of the RET (rearranged during transfection) gene as a novel driver for the development not only of papillary thyroid carcinoma but also of LADC (8–11). KIF5B-RET is the predominant form of RET fusion in LADC. Other fusions, including CDDC6-RET, NCOA4-RET and TRIM33-RET, which are detected in papillary thyroid carcinoma, are also found in LADC, although they are minor forms (8,12,13). Previous studies used a conventional NIH3T3 cell assay to demonstrate the transforming activity of the KIF5B-RET fusion gene (11,14); however, the ability of KIF5B-RET fusion gene to drive tumorigenesis in vivo has not been proved.

Abbreviations: CT, computed tomography; HPF, high power field; LADC, lung adenocarcinoma; LPF low power field; SPC, surfactant protein C.

Here, we constructed a transgenic mouse model to examine the oncogenic potential of the KIF5B-RET fusion in lung alveolar epithelial cells in vivo. The KIF5B-RET transgene was expressed in lung epithelial cells under the control of the SPC (surfactant protein C) promoter; SPC is a gene that is specifically expressed in lung alveolar epithelial cells (5,7). Analysis of this mouse model revealed that the RET fusion generates lung tumors in vivo, and that tumor formation is suppressed by vandetanib, a RET tyrosine kinase inhibitor approved by the U.S. Food and Drug Administration (FDA).

Materials and methods

Generation of transgenic mice

KIF5B-RET (K15: R12) DNA bearing a FLAG-tag at the N-terminus was ligated into a plasmid vector carrying the SPC promoter along with splicing polyadenylation signals (kindly provided by Dr K Hagawara of Saitama Medical University (7)). The RET cDNA in this plasmid was derived from the longer splicing isoform, RET51 (15). The expression cassette was then injected into pronuclear-stage embryos from C57BL/J6 mice (Unitech Japan). The copy numbers of the transgene were measured by Southern blot analysis of DNA isolated from the tails of the founder mice. Total RNA was isolated from several organs of KIF5B-RET transgenic mice (line #1, 25 weeks-of-age) using the RNeasy Mini Kit (Qiagen) and subjected to reverse transcription with SuperScript III reverse transcriptase (Invitrogen) and oligo(dt) primer. The PCR primers used to confirm KIF5B-RET cDNA expression were as follows: forward, 5′-ATAGGTGGGAACCTGTAGAACC-3′, and reverse, 5′-GAGGCCCCATACAAATTGTGAT-3′. The primers used for GAPDH expression were as follows: forward, 5′-AACTTTTGCGATTGGAAGGG-3′, and reverse, 5′-CCC TGT TGCGTAGCCGTAT-3′. Whole cell lysates were prepared from several organs of #1 line mouse (25 weeks-of-age). The KIF5B-RET protein was detected by Western blotting with anti-RET (ab134100, Abcam) and anti-β-actin (1:35, #4970, Cell Signaling Technology) antibodies (16).

Mice were maintained in a standard air-conditioned and specific pathogen-free animal room. F2 mice were generated by in vitro fertilization using an F1 mouse according to a standard method and then subjected to examination. All animal experiments were approved by the Committee for Ethics of Animal Experimentation of the National Cancer Center, Tokyo, Japan.

Gross anatomical and histopathological examination

Examination of lung tumors in living animals was performed by micro-CT (R-mCT2, Rigaku). All mice were anesthetized with isofluran prior to and throughout the scans. Gross examination of organ morphology was performed after scheduled killing. Moribund mice showing weight loss or difficulty in moving or breathing were also immediately killed and similarly necropsied. Whole lung tissues were fixed in 10% formalin, embedded in paraffin, sectioned and then stained with hematoxylin and eosin (H&E). Pulmonary proliferative lesions were categorized as hyperplasia, adenomas and adenocarcinomas according to previously established criteria (17).

Immunohistochemical staining (IHC)

Serial paraffin sections of lung tissue were immunostained using an EnVision System-HRP (DAB) (Dako) as previously described (16,18). Primary antibodies against RET (1:250, clone 3454_1; Epitomics, Burlingame) and Ki-67 (1:2000, ab15580, Abcam) were used for IHC.

Treatment with a RET inhibitor

KIF5B-RET transgenic mice were administered vandetanib (50 mg/kg body weight, gavage, once daily; V-9401, LC Laboratories, MA) or vehicle alone. Treatment started at 39 weeks-of-age (when the KIF5B-RET transgenic mice began to develop lung tumors). At 8 (47 weeks-of-age) or 20 (59 weeks-of-age) weeks of treatment, the KIF5B-RET transgenic mice were killed and necropsied (n = 6 per group; treated and non-treated). One transgenic mouse of vandetanib treatment group accidentally died at week 8.
Results

Generation of KIF5B-RET transgenic mice

To investigate the role of KIF5B-RET in lung tumorigenesis, we generated transgenic mice that specifically expressed the fusion gene in the lung. The transgene construct comprised KIF5B-RET (K15; R12) cDNA, the most frequently occurring RET fusion variant (13,19), the SPC promoter, an RNA splicing cassette, and a polyadenylation signal (Figure 1A). We obtained nine independent F1 mouse lines, each carrying 5–7 copies of the transgene (Figure 1B, Supplementary Table S1, available at Carcinogenesis Online). To investigate lung-specific expression of the transgene, we performed RT-PCR and immunoblot analysis to detect KIF5B-RET mRNA and protein, respectively, in transgenic mice from the #1 line carrying approximately six copies of the transgene. KIF5B-RET mRNA and protein were specifically detected in the lungs, but not in several other organs (Figure 1C and D).

Development of lung neoplasms in KIF5B-RET transgenic mice

The postnatal growth of KIF5B-RET transgenic mice was indistinguishable from that of their littermate controls and they did not show any gross abnormalities. To investigate the oncogenic role of the RET fusion, we performed scheduled necropsy and histopathological examination of the lungs from 33 F2 mice harboring the transgene and 26 without the transgene. KIF5B-RET transgenic mice developed visible nodules on the surface of the lungs. Subsequent histological analysis revealed that the multifocal proliferative pulmonary lesions included hyperplasias, adenomas and adenocarcinomas. KIF5B-RET transgenic mice had a significantly higher incidence of lung tumors (adenomas and/or carcinomas) than control mice (P = 0.002; log-rank test) (Figure 2A). Computed tomography (CT) revealed progressive enlargement of multiple lung tumors in KIF5B-RET transgenic mice with aging, consistent with grossly visible nodules on the surface of the lungs (Figure 2B). At 83 weeks-of-age, all seven transgenic mice examined harbored adenomas and one mouse examined harbored adenocarcinomas. Immunohistochemical analysis using an anti-RET antibody revealed diffuse cytoplasmatic granular staining in the adenoma and adenocarcinoma cells (Figure 2C and D). These pulmonary lesions were positive for Ki-67, suggesting that KIF5B-RET fusion protein expression is associated with cell proliferative activities. The histopathological features of these lung tumors (e.g. a papillary growth pattern) are similar to those observed in human LADCs harboring the KIF5B-RET fusion(20) (Supplementary Table S1, available at Carcinogenesis Online).

An FDA-approved RET tyrosine kinase inhibitor suppresses the oncogenic activity of the KIF5B-RET fusion

We next examined whether a drug that inhibits RET tyrosine kinase suppressed lung tumorigenesis in KIF5B-RET transgenic mice. As shown in Figure 3A, KIF5B-RET transgenic mice were treated with vandetanib (50 mg/kg body weight per day) or with vehicle alone for 8 or 20 weeks. The mice in each group were subsequently necropsied and histopathologically evaluated for pulmonary proliferative lesions by examining a single cross section of lung from each mouse. The majority (83%) of vehicle-treated mice harbored multiple hyperplasias and adenomas compared with 20% of vandetanib-treated mice (Table 1). No adenocarcinomas were observed in both of the groups. The number of adenomas per cross section of lung in the vehicle-treated mice at 8 weeks was smaller than that at 20 weeks (3.3 ± 2.5 versus 6.5 ± 3.9), indicating increased occurrence with aging (Figure 3A and B). However, no adenomas were observed in vandetanib-treated mice at 8 weeks and only 0.2 ± 0.4 were observed at 20 weeks (Figure 3B and C), indicating that vandetanib effectively suppresses RET fusion-driven lung tumorigenesis.

Discussion

Here, we showed that the KIF5B-RET fusion gene is oncogenic in lung alveolar epithelial cells in vivo. Pulmonary proliferative lesions, including hyperplasias, adenomas and adenocarcinomas, developed in mice harboring a RET fusion transgene. The tumorigenesis was markedly suppressed by vandetanib, an FDA-approved RET kinase inhibitor. Thus, this line of transgenic mice is a suitable model for RET fusion-driven lung carcinogenesis.

We found that lung tumors developed more slowly in KIF5B-RET transgenic mice than in EML4-RET and EZR-ROS1 transgenic mice, which harbor the same SPC-driven expression system and a similar copy number of transgenes (three and four copies, respectively (5,7)). The reason for this difference in tumor growth is unknown. It may be that the transgenic mice used in this study might express lower levels of the transgene than EML4-RET and...

Fig. 1. Lung tumorigenesis in KIF5B-RET transgenic mice. (A) Schematic representation of the transgene. A cDNA encoding FLAG-tagged KIF5B-RET was inserted between the SPC promoter and the splicing and polyadenylation (polyA) signal sequences. (B) Copy number analysis. Genomic DNA was obtained from the tails of transgenic mice (lines #1–9) generated from pronuclear-stage C57BL/6J embryos and analyzed by Southern blot analysis using a full-length KIF5B-RET cDNA probe. Controls comprised mouse genomic DNA with 1, 2, 5, 10, 20, 50 and 100 copies of the transgene per diploid genome. A line #1 mouse (marked in red) was selected to build the transgenic line. (C) KIF5B-RET mRNA expression. Oligo(dt)-primed cDNA was synthesized from total RNA isolated from lung (Lu), liver (Lv), kidney (Kd), spleen (Sp), stomach (St), small intestine (Si) and colon (Co) from #1 line mouse (25 weeks-of-age) and subjected to RT-PCR in the presence (+) or absence (−) of reverse transcriptase. RT-PCR was also performed using a human LADC cDNA probe. The most frequently occurring KIF5B-RET mRNA and protein expression is associated with cell proliferative activities. The histopathological features of these lung tumors (e.g. a papillary growth pattern) are similar to those observed in human LADCs harboring the KIF5B-RET fusion(20) (Supplementary Table S1, available at Carcinogenesis Online).

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EZR-ROS1 transgenic mice. Alternatively, the KIF5B-RET fusion may be a weaker driver for tumor proliferation than the EML4-RET and EZR-ROS1 fusions. In fact, the latter idea is supported by the results of a recent large-scale screening study that examined RET fusion-positive LADCs. The results showed that tumors harboring RET fusions were significantly smaller than those harboring ALK and ROS1 fusions (21). Differences in the ability to drive tumorigenesis might correlate with the time at which visible tumors appear in the lungs.

The utility of several RET tyrosine kinase inhibitors such as vandetanib, cabozantinib and E7080 as treatments for RET fusion-positive LADC is being examined in clinical trials (8,12), and promising results have been reported in a few patients (13,22). Thus, the therapeutic in vivo experiments described herein will complement these clinical trials.

**Fig. 2.** Development of pulmonary lesions in KIF5B-RET transgenic mice. (A) Kaplan–Meier tumor-free survival curves for F2 mice with and without the KIF5B-RET transgene. P values were calculated using the log-rank test. The number of mice other than those with detectable tumors at the time of necropsy is shown below the curves. (B) Computed tomography images of the lungs from four 75-week-old KIF5B-RET transgenic F2 mice. The tumor (T) and the heart (H) are indicated. (C) Representative gross findings in lungs isolated from 83-week-old KIF5B-RET transgenic F2 mice. (D) Representative histopathological images of lung hyperplasia, adenoma, and adenocarcinoma in the mice shown in (C). Low and high resolution images of hematoxylin and eosin (H&E)-stained and RET- and Ki-67-immunostained tissues are shown. Scale bar = 500 μm [H&E low power field (LPF)] and 100 μm [H&E high power field (HPF); RET and Ki-67].
A mouse model of KIF5B-RET

Fig. 3. Administration of vandetanib to KIF5B-RET transgenic mice. (A) Treatment schedule for the vandetanib-treated and vehicle (control) groups. The time of necropsy is also shown. One group was necropsied after 8 weeks of treatment (or not) and the other after 20 weeks. (B) Average number of adenomas in the treatment and control groups. Adenomas were counted by examining a single cross section of H&E-stained lung tissue. P values were calculated using an unpaired t-test. (C) Representative histopathological images showing lung tissue from the 20 week treatment group. Scale bar = 100 μm. Adenomas are observed only in lungs from control (vehicle-treated) mice.

Table I. Development of lung proliferative lesions in KIF5B-RET transgenic mice

<table>
<thead>
<tr>
<th>Group (according to administration time)</th>
<th>No. of mice examined</th>
<th>No. of mice with hyperplasia</th>
<th>No. of mice with adenoma</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>5 (83%)</td>
<td>5 (83%)</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>5</td>
<td>1 (20%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>20 weeks</td>
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<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
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<td>5 (83%)</td>
<td>5 (83%)</td>
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<tr>
<td>Vandetanib</td>
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<td>0 (0%)</td>
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Supplementary material

Supplementary Table S1 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

References


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