p190A RhoGAP is involved in EGFR pathways and promotes proliferation, invasion and migration in lung adenocarcinoma cells

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Abstract. Overcoming acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) is an emerging issue in lung cancer treatment. We report evidence that a GTPase-activating protein, p190-A RhoGAP (p190), is a potential molecular target for the treatment of lung adenocarcinoma. We documented inhibition of phosphorylation of p190 by EGFR-TKI treatment in lung adenocarcinoma cell lines. Small interfering RNA-mediated knockdown of p190 leads lung adenocarcinoma cells to growth suppression and to inhibition of invasion/migration through inducing cell cycle arrest but not apoptosis. These findings were observed not only in EGFR-TKI-sensitive cells but also in EGFR-TKI-resistant cells; even in cell lines harboring K-ras mutations. The mechanism of this inhibitory effect on growth and invasion/migration was Ras inactivation through disrupting the p190-A RhoGAP/p120RasGAP complex. In addition, a high level of p190 mRNA expression was observed in majority of surgically obtained tissue from lung adenocarcinoma patients. Overexpression of p190 mRNA associated with poor disease-free survival. The results suggest that overexpression of p190 mRNA may be involved in the carcinogenesis of lung adenocarcinoma. These findings indicate that p190 is a possible molecular target for treatment of lung adenocarcinoma.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, with a 5-year survival rate of only 20%. Although patients diagnosed with early-stage disease have the potential for cure with complete surgical resection, only 25% of lung cancer cases are diagnosed at an early stage.

Small molecule inhibitors of epidermal growth factor receptor tyrosine kinase (EGFR-TKI), such as gefitinib and erlotinib (1,2), induce dramatic responses in certain patients with non-small cell lung cancer (NSCLC) (3). EGFR mutation (short deletion in exon 19, L858R) positive patients have shown an impressive 60% response rate, which exceeds the response rate for conventional chemotherapy (4). However, the clinical success of treatment with EGFR-TKIs is uniformly limited by the development of acquired drug resistance. Two major mechanism of acquired resistance have been identified as T790M mutation in EGFR gene and gene amplification of MET oncogene (5,6). Hence, new therapeutic strategies, such as the development of more effective or alternative molecular-targeted agents against lung cancer are eagerly awaited.

Recently, large-scale screening methods have been applied to identify novel molecular targets. Mass spectrometry targeting phosphorylated tyrosin on lung adenocarcinoma cells harboring EGFR kinase domain mutation has been reported and revealed that phosphorylation of several proteins, which have not been identified in EGFR pathway, is strongly controlled by EGFR protein (7). We performed similar analysis and found that p190-A RhoGAP (p190) was one of common proteins which include functional tyrosine controlled by EGFR protein.

p190 is a 172-kDa protein that is encoded by the GRLF1 gene located on 19q13.3. The direct action of p190 has been reported to be a RhoGAP; converting Rho-GTP to Rho-GDP and inactivating the Rho pathway (8). Therefore, p190 has been thought to be a potent inhibitor of RhoA (9). On the other hand, indirect action of p190 has been reported to control the Ras pathway by binding to p120RasGAP protein. p190 has a binding activity to p120RasGAP and the activity is promoted by phosphorylation of tyrosine (Y¹¹⁰⁵) of p190 (10). The activity of p120RasGAP is reduced when p190 forms a complex with p120RasGAP (11). So far, p190 has been reported to inhibit cell migration and invasion through negatively regulating RhoA in some cell lines (12). On the other hand, EGF-induced p190 phosphorylation has been reported to play essential roles in Ras activation and cell proliferation using different cell lines (13). To date, only few reports are available on the function of...
p190 in lung cancer. We investigated the roles of p190 in lung adenocarcinoma cells and clarified whether p190 could be a potential therapeutic target for lung cancer.

Materials and methods

Cell lines and culture. A549, H520 and H1975 were obtained from American Type Culture Collection; ATCC (Manassas, VA, USA). PC-14 was obtained from DS Pharma Biomedical Co., Ltd. (Suita, Osaka, Japan). LK87, LCSC#1 and II-18 were obtained from Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). All cells were maintained in RPMI-1640 (Invitrogen, San Diego, CA, USA) supplemented with 10% FBS in a humidified 5% CO₂ incubator at 37°C. A549 and LK87 have wild-type (WT) EGFR and K-ras mutation (codon 12), PC-14 has EGFR mutation (exon 19 del), LCSC#1 and II-18 have EGFR mutation (L858R) and H1975 has EGFR mutation (L858R and T790M). H520, with squamous cell carcinoma histology, has both WT EGFR. A549, H1975 and H520 have been reported to be EGFR-TKI-resistant, whereas LCSC#1, PC-14 and II-18 are EGFR-TKI-sensitive. NHBE (normal human bronchial epithelial cell) was obtained from Takara Bio Inc. (Otsu, Shiga, Japan) and was grown in BEGM Bullet kit (Lonza Walkersville Inc., Walkersville, MD, USA) according to the manufacturer's instructions.

Western blot and immunoprecipitation analyses. Protein samples were obtained from cells and transferred onto an Immune-Blot PVDF Membrane (Bio-Rad, Hercules, CA, USA). Proteins on the membranes were incubated with recommended concentration of primary antibodies, horse-radish peroxidase-labeled secondary antibodies and were visualized on ImageQuant LAS 4000 mini (GE Healthcare, Buckinghamshire, UK) by means of the ECL Western blot detection system.

For immunoprecipitation, the p190-A RasGAP antibody was bound on to Protein A Dynabeads® (Invitrogen Dynal, Oslo, Norway). The Protein A Dynabeads-p190-A RasGAP complex was incubated with the cell lysates of lung cancer cells. Obtained bead-protein complexes were eluted and analyzed by SDS-PAGE and western blotting. Antibodies to EGFR, phospho-EGFR, p190, phosphotyrosine, Src, phospho-Src family kinase, MEK, phospho-MEK, Erk, phospho-Erk and β-actin were purchased from Cell Signaling Technology, whereas antibodies to Ras, Rho and p120 were from Abcam.

RNA interference (RNAi). Cells were transfected with 5 nM siRNA directed to p190-A RhoGAP (p190 siRNA; Silencer® Select Validated siRNA, Applied Biosystems, Austin, TX, USA) or negative control siRNA (NR siRNA; non-related siRNA, Applied Biosystems) using the Lipofectamine RNAiMAX® (Invitrogen), according to the manufacturer's instructions. The target sequence of siRNA for p190 was GGCUGAUUGUAUCUGCGATT.

cDNA synthesis and real-time PCR. cDNA synthesis using TaqMan® Gene Expression Cells-to-C®kit was performed according to the manufacturer's instructions. Semi-quantitative real-time RT-PCR was performed by using the ABI PRISM 7000 HT sequence detection system (Applied Biosystems). The Assays-on-Demand products purchased from Applied Biosystems contained TaqMan minor groove binder probes (6-FAM dye-labeled) combined with the primers for p190 (Hs00534180_m1). An Assay-on-Demand product for 18s-rRNA (4319413E) was used as endogenous control.

Cell proliferation, migration and invasion assays. Cell proliferation assay was performed using CellTiter 96® AQUEous One Solution Cell Proliferation assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Transwell migration assay using CytoSelect™ 24-well Cell Migration assay (Cell Biolabs, San Diego, CA, USA) and Transwell invasion assay using CytoSelect™ 24-well Cell Invasion assay (Cell Biolabs) were performed according to the manufacturer's instructions.

Cell cycle analysis. Cells were fixed with 70% ice cold ethanol and stored at -20°C. Then cells were incubated with 0.5% RNase followed by propidium iodide. Histograms were obtained by FACS Canto II (BD). Doublets were excluded by scattergrams. Data were analyzed using ModFit software.

Quantitative assay for GTP-bound Ras and GTP-bound Rho. The levels of GTP-bound Ras and GTP-bound Rho were detected with the Raf-1 Ras binding domain agarose (Cell Biolabs) and GST-rohtekin pull-down assays (Cell Biolabs), respectively.

Real-time PCR on clinical samples. A total of 133 cDNA samples of pulmonary adenocarcinomas (pathological stage IA-IIIB) and adjacent normal lung tissue, were obtained along with clinicopathological data from patients who had undergone surgery at the Miyagi Cancer Center or Tohoku University Hospital. Quantitative RT-PCR was performed by an ABI 7000 using TaqMan probes for p190, β-actin was used as an internal control. Threshold cycles of prier probes were normalized to β-actin and translated to relative values.

Immunohistochemical analysis. Expression of phospho-p190 protein in paraffin-embedded clinical tissues was examined using the Linked Streptavidin-Biotin method (Histofine SAB-PO, Nichirei Biosciences, Japan). Rabbit anti-phospho-p190 antibody (1:50 dilution; Abcam) was used as a primary antibody. Two independent investigators evaluated the immunohistochemical staining without knowledge of the clinicopathological parameters. For semi-quantitative assessment of the immunohistochemical results, the mean percentage of positive tumor cells was determined in ≥10 random fields at x400 magnification in each section. It was graded as focal (<10%), regional (11-50%), or diffuse (>50%). The intensity of the phospho-p190 immunoreaction was graded weak, moderate or strong. The mean percentage of positive tumor cells and the staining intensity were then combined to produce a phospho-p190-IHC result.

Survival and statistical analyses. Two-year disease-free survival (2-year DFS) was calculated from the time of surgery to the patient's last follow-up or recurrence. DFS of patients was analyzed using Kaplan-Meier survival curves and the log-rank test was used to examine the statistical significance. All
in vitro experiments were performed in triplicate. Comparisons between 2 groups were carried out using unpaired Student’s t-test; comparisons among 3 or more groups were carried out using one-way ANOVA. Survival and statistical analysis were carried out with the Prism 5 for Mac OS X (GraphPad Software, USA). All P-values <0.05 were considered as statistically significant.

Results

Western blotting showed that phosphorylation of EGFR was inhibited by treatment with EGFR-TKI in EGFR-TKI-sensitive cells; LCSC#1, PC-14 and II-18, but not in EGFR-TKI-resistant cells; A549, LK87, H1975 and H520. Similarly, immunoprecipitation analysis showed that phosphorylation of p190 was inhibited by administration of EGFR-TKI (Fig. 1).

Since no chemical inhibitor for p190-tyrosine phosphorylation is currently available, we performed p190 knockdown using siRNA (p190 RNAi). Before knockdown, we confirmed that all lung cancer cell lines in this study show high levels of p190 mRNA expression by RT-PCR, ~20-160-fold higher than that of NHBE (Fig. 2A). To test the effect on cell proliferation after p190 RNAi, an MTS assay was performed. The results showed that cellular proliferation of all lung adenocarcinoma cell lines in this study was significantly suppressed by p190 RNAi, while such suppression was not observed in H520 and NHBE (Fig. 2B).

To understand the mechanism of the growth suppression we further performed cell cycle analyses using lung cancer cells after p190 RNAi. A decrease in the S population was observed after p190 RNAi in all lung adenocarcinoma cell lines but not in the squamous cell carcinoma H520 (Fig. 3). These results indicate that p190 RNAi led the lung adenocarcinoma cells to cell cycle arrest.

p190 is a RhoGAP, and reportedly is involved in control of cell invasion and migration. To clarify this function in lung cancer cell lines, we knocked down p190 and tested invasion

Figure 1. Results of western blot analysis and immunoprecipitation of p190 and EGFR in lung adenocarcinoma cell lines. Phosphorylation of EGFR at Y1068 is inhibited by EGFR-TKI treatment in EGFR-TKI-sensitive cell lines; LCSC#1, PC-14 and II-18, but is retained in EGFR-TKI-resistant cell lines; A549, LK87, H1975 and H520. Phosphorylation of p190 was parallel to that of EGFR. Cells were pretreated with 0-0.1 µM EGFR-TKI for 3 h before stimulation by 50 ng/ml epidermal growth factor (EGF) for 10 min.

Figure 2. Results of cell proliferation assays after p190 knockdown using siRNA (p190 RNAi) in lung adenocarcinoma cell lines. (A) Relative expression level of p190 mRNA in lung adenocarcinoma cell lines. The expression relative to normal human bronchial epithelial cells (NHBE) is significantly elevated in all lung cancer cell lines. (B) Results of MTS assays after p190 RNAi. Bar graph depicts cell proliferation (measured by SpectraMax M2e) after 3 days of growth in lung cancer cell lines following p190 RNAi. The proliferation of all lung adenocarcinoma cell lines is inhibited by p190 RNAi. A paired t-test identified statistically significant difference in cell growth (P<0.05). Error bars represent SD.
and migration of lung cancer cells with high levels of p190 mRNA. By p190 RNAi, both cell invasion and migration were significantly reduced in A549, LK87, LCSC#1, PC-14 and H1975 (Fig. 4). In II-18 cell and H520, the difference was not detected probably due to original nature of low activity of cell invasion/migration.

Phosphorylation of p190 at Y1105 is known to promote formation of a p190/p120 complex. Formation of the complex has been reported to downregulate the RasGAP activity of p120, leading to an elevation of Ras activity. These complexes upregulate the RhoGAP activity of p190, leading to a repression of Rho activity. We investigated the effect of both EGFR-TKI and p190 RNAi on the p190/p120 complex formation, Ras activation and Rho inactivation in lung cancer cell lines. Immunoprecipitation analysis showed that p190/p120 complex formation was inhibited in EGFR-TKI-sensitive cell lines by EGFR-TKI treatment in a dose-dependent manner, but not in EGFR-TKI-resistant cell lines. On the other hand, formation of the complex was inhibited by p190 RNAi in all lung cancer cell lines (Fig. 5A). Ras inactivation was parallel to disruption of p190/120 complex by EGFR-TKI or p190 RNAi in all adenocarcinoma cell lines, while Rho activation was inverse to disruption of p190/120 complex in the adenocarcinoma cell lines except II-18 (Fig. 5B). In II-18 cells, RhoA activation could not be evaluated due to low internal protein level of RhoA.

Phosphorylation of MEK and Erk, downstream molecules of Ras, were inhibited by p190 RNAi in all adenocarcinoma cell lines, although the effect is relatively small in A549. In contrast, phosphorylation of Src, a potential upstream molecule of p190, was inhibited by EGFR-TKI in EGFR-TKI-sensitive cells but not in EGFR-TKI-resistant cells, while no effect of p190 RNAi was observed (Fig. 6).

We also studied the expression level of p190 in clinical samples. Relative expression level of p190 mRNA in tumor to paired normal lung tissue was tested by RT-PCR in 133 human lung adenocarcinoma tissue samples (Table I). High level of p190 mRNA was detected in majority of samples of lung adenocarcinoma. Pathological stage III patients (n=18) had significantly higher mRNA level of p190 compared with stage IIA or IIB patients (P<0.05) (Fig. 7A). Whereas EGFR mutation existed in 56 samples, there was no association between p190 mRNA level and EGFR mutation of the tumors (P=0.703) (Fig. 7B). Furthermore, we analyzed a cohort of 133 patients with outcome data. By ROC curve analysis, we determined a threshold of p190 mRNA expression level as
Figure 4. Results of cell invasion and migration assays after p190 RNAi in lung adenocarcinoma cell lines. (A) Results of migration assays. After 24-h siRNA treatment, cell suspension was placed in the upper chamber. Four or 8 h later migratory cells that passed through polycarbonate membrane were lysed and quantified using fluorescent dye (measured by SpectraMax M2e). The migration of A549, LK87, PC-14 and H1975 are inhibited by p190 RNAi. A paired t-test identified statistically significant difference in cell growth (*P<0.05). (B) Results of invasion assays. Similar to the migration assays, the invasion of A549, LK87, PC-14 and H1975 are inhibited by p190 RNAi.

Figure 5. Western blot and immunoprecipitation analyses of Ras and Rho signal pathways in lung adenocarcinoma cell lines. (A) Results of immunoprecipitation with p120/p190 complex. p120 are not detected by western blotting after p190 RNAi in the lung adenocarcinoma cell lines. (B) Results of Ras and Rho activation assay. GTP-bound active form Ras is not detected after p190 RNAi in the lung adenocarcinoma cell lines or after administration of EGFR-TKI in PC-14 and II-18. GTP-bound active form Rho is detected after p190 RNAi in all the lung cancer cell lines, except II-18.
three times the level of corresponding normal tissue of the patients. High expression of p190 is associated with poor disease-free survival (P=0.003) (Fig. 7C).

Phosphorylated-p190 (phospho-p190) was evaluated immunohistochemically in 52 adenocarcinoma specimens. Immunohistochemical staining showed that 92% (48 of 52) of pulmonary adenocarcinoma samples were positive for phospho-p190 (Table II). The staining intensity was also stronger in tumor cells than that in normal cells (Fig. 8A). No statistically significant difference was found between IHC score and clinical outcome of lung adenocarcinoma patients (Table II). However, there were statistically significant association between histological features and phospho-p190 staining using Fisher's exact-test (P=0.013) (Fig. 8B).

**Discussion**

In this study, we found three novel findings. First, we clearly demonstrated that phosphorylation of p190 is involved in the
EGFR signal pathway. Previous mass spectrometry screening suggested that tyrosine (Y1105) phosphorylation of p190 is affected by EGFR-TKI. Here, we discovered that one point detected by previous mass spectrometry analysis is actually on the line of EGFR signaling pathway by detailed western blot analysis. We highlighted unique functional characteristics of p190RhoGAP, a sort of switch of Ras and Rho pathway and how EGFR-TKI affects this switch in lung adenocarcinoma cells. As briefly described in the introduction, p190 is one of the

Figure 7. Relative expression level of p190 mRNA in human lung adenocarcinoma specimens. Numbers indicate relative p190 mRNA expression normalized against β-actin. (A) Box plot of p190 mRNA expression levels in lung adenocarcinoma patients based on pathological stage IA/IB/II/III shows that average p190 expression correlates with pathological stage in lung adenocarcinoma. (B) Box plot of p190 mRNA expression levels in lung adenocarcinoma patients based on EGFR mutation status shows that average p190 expression does not correlate with EGFR mutation status of lung adenocarcinoma. There is no correlation between mRNA level and pathological stage of the patients. (C) Kaplan-Meier analysis shows that patients with high expression of p190 mRNA have a significantly poor disease-free survival probability (P=0.003).

Figure 8. Immunohistochemical staining analysis of human lung adenocarcinoma specimens. (A) Representative photomicrographs show phospho-p190 expression in normal lung and lung adenocarcinomas. IHC score is indicated in parenthesis. (B) Correlation analysis of histological features with the mean percentage of positive tumor cells in lung adenocarcinoma tumors (n=52 tumors). Gray box highlights a statistically significant association between histological features and the mean percentage of positive tumor cells using Fisher’s exact-test (P=0.013).
Comparisons of p190-A, a human tumor GTPases, such as RhoA, Rac1 and Cdc42 exists and control lung adenocarcinoma cells, cross-talk between Rho family of invasion/migration has been significantly inhibited in lung lines by p190 RNAi. Whereas, our study disclosed that lung adenocarcinoma cell lines, except II-18 by p190 RNAi. showed that GTP-binding active form RhoA existed in the six hepatic carcinoma (21) and pancreatic cancer (22). Our study associated with poor prognosis especially in breast cancer (20), is the first step in invasion and metastasis. RhoA expression is outcome in patients with lung cancer (19). Increased motility failure and the most significant predictor of poor clinical mutation type of lung cancer.

In conclusion, phosphorylation of p190 is regulated by EGFR in lung adenocarcinoma cells and p190 involves cell proliferation and migration/invasion through controlling the Ras signaling pathway. Studies on clinical specimens suggest that p190 is associated with aggressive nature of the tumors. p190 shows promise as a novel molecular target for treatment of lung adenocarcinoma including tumors with acquired resistance to EGFR-TKI or with K-ras mutation.

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