Role and Relevance of TrkB Mutations and Expression in Non–Small Cell Lung Cancer

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Abstract

Purpose: TrkB has been involved in poor cancer outcome. TrkB mutations have been reported in non–small cell lung cancer. In this study, we aimed at characterizing the role of three potentially sensitizing TrkB mutations previously reported in lung cancer.

Experimental Design: We characterized three activation loop mutants of TrkB (M713I, R715G, and R734C) in terms of pathway activation/phosphorylation, migration, anchorage-independent growth, and sensitivity to a Trk inhibitor, using NIH3T3 cells and Baf3 cells. We also sequenced the tyrosine kinase domain of TrkB in a large number of lung cancer samples of East-Asian origin and cell lines.

Results: None of the mutants were constitutively active in NIH3T3 transformation and migration assays. M713I and R734C mutants showed low levels of autophosphorylation in comparison with wild-type TrkB. Although R715G showed similar level of autophosphorylation to wild-type TrkB on brain-derived neurotrophic factor stimulation, the mutant was not as competent as wild-type TrkB in supporting interleukin-3–independent growth of Baf3 cells. In addition, the Trk inhibitor AZD6918 inhibited wild-type TrkB-induced cell migration and cell growth, whereas the mutants were relatively resistant to the Trk inhibitor compared with wild-type TrkB. We could not confirm the presence of nonsynonymous mutation in 78 lung cancer samples and 29 cell lines.

Conclusions: Wild-type, but not mutant, TrkB enhances cell migration and transformation. Our study suggests that TrkB mutations should not be used for selection of patients with lung cancer treated with Trk inhibitors. High expression of wild-type TrkB might be beneficial for studies of Trk inhibitors.

Introduction

Receptor tyrosine kinases (RTK) regulate critical cellular processes, such as cell proliferation, metabolism, and migration. Deregulation of RTKs has been reported in various types of cancer, and RTK-targeted therapies, such as inhibition of epidermal growth factor receptor (EGFR) in NSCLC, have been successfully developed (1).

NTRK1-3 gene family encodes tropomyosin receptor kinases (TrkA, B, and C), which are activated by neurotrophins. Nerve growth factor, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 bind to TrkA, TrkB, and TrkC, respectively. On ligand binding, the tyrosine kinase and its downstream signaling are activated. Members of the Trk family are highly expressed in cells of neural origin, and are involved in neural maintenance and development (2).

Although functions related to neural cells have been extensively examined, the Trk receptor was originally described as an oncogene. Oncogenic Trk was reported as a fusion gene between the 5′ region of the tropomyosin and the tyrosine kinase domain of TrkA derived from Inv(1q) inversion. The fused protein resulted in constitutive activation of the tyrosine kinase. This type of constitutively active TrkA fusions were reported in a subset of papillary thyroid cancers and colon cancers (3, 4).

Overexpression of TrkB has been reported in several malignancies, such as neuroblastoma (5), prostate cancer, pancreatic ductal adenocarcinoma (6), multiple myeloma (7), and lung cancer (8). High levels of TrkB correlate with poor outcome (9). In vitro, TrkB has been shown to be involved in cancer cell proliferation, anoikis, cell migration/invasion, and epithelial-mesenchymal transition (10, 11). These results suggest that TrkB may have a significant impact on the malignant phenotype of tumors in vivo.
Accumulating evidence suggests that TrkB is a potential target for cancer therapy. Several small molecules that inhibit Trk signaling have been developed recently and are being tested in phase I and phase II trials (12).

Besides fusion genes, activating mutations in the ATP binding site of tyrosine kinase domains have been shown to result in constitutively activate tyrosine kinases (13, 14). Two reports described TrkB mutations in non–small cell lung cancer (NSCLC; refs. 15, 16). Some of these reported mutations are located in the tyrosine kinase domain. However, the significance of these mutations has not been elucidated. In this study, we characterized the function of 3 reported mutations, which by their localization might be activating mutations. We also have sequenced the TrkB tyrosine kinase domain in a large number of lung cancers and cell lines.

Materials and Methods

Cell lines and reagents

We used 29 lung cancer cell lines in this study (A549, NCI-H1355, NCI-H1373, NCI-H1466, NCI-H1944, NCI-H2077, NCI-H2087, NCI-H2122, NCI-H23, NCI-H2347, NCI-H3122, NCI-H854, NCI-H322, NCI-H358, NCI-H820, NCI-H720, NCI-H1299, NCI-H1725, NCI-H460, NCI-H1173, NCI-H128, NCI-H211, NCI-H592, NCI-H620, NCI-H678, NCI-H69, NCI-H82, NCI-H1717, Cori23). NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. Baf3 and WEHI3B cells were grown in RPMI supplemented with 10% FBS and 10% WEHI3B conditioned media. Baf3 and WEHI3B cells were a kind gift from Dr. Meyerson (MGH, Boston, MA). The Trk inhibitor AZD6918 was obtained from Astrazeneca. Antibodies for phospho-TrkB (pY516), pErk, pAkt, and α-tubulin were purchased from Cell Signaling Technology. Anti–pan-Trk (C14) was purchased from Santa-Cruz Biotechnology. Anti-phosphoryrosine (4G10) antibody was purchased from Millipore. BDNF was from Sigma-Aldrich.

Tumor samples and DNA sequencing

Tumor samples were obtained from Aichi Cancer Center, Kyushu University Hospital and Vrij Universiteit Medical Center in Amsterdam, through protocols approved by the institutional review boards of their facility (17). A list of the tumor pathologic diagnosis is reported in Table 1. Diagnosis of large cell neuroendocrine carcinoma was based on both morphologic appearance and immunohistochemical demonstration of at least one of the neuroendocrine-specific markers such as chromogranin A, synaptophysin, and CD56. DNA was extracted from paraffin-embedded slides. Sections containing more than 70% tumor cells were dissected and processed with DNase and blood kit (Qiagen). Exons 17 to 21 including tyrosine kinase and phospholipase C-γ (PLC-γ) binding domains of TrkB were sequenced by PCR-direct sequencing. Primers are described in Supplementary Table S1.

Plasmid construction and retroviral infection

Full-length TrkB cDNA was obtained by PCR by using a pBabeinm/TrkB plasmid as a template. The TrkB plasmid was a kind gift from Dr. Peeper (NKI, Amsterdam, The Netherlands). The TrkB ORF was subcloned into pQCXIN retrovirus vector (Clontech). TrkB mutants were generated by using QuickChangell site-directed mutagenesis kit (Stratagene) with wild-type pQCXIN/TrkB vector as a template according to the manufacturer’s instruction. Primers for mutagenesis are shown in Supplementary Table S1. The mutations were confirmed by sequencing. NIH3T3 and Baf3 cells were infected with retrovirus according to standard protocols as described previously (18). Briefly, TrkB-expressing retroviruses were produced by cotransfection of GP2-293 cells with pVSV-G (Clontech) by using nanofect transfection reagent (Qiagen). Cells were infected with TrkB retroviruses in the presence of 4 μg/mL polybrene (Sigma). Stable populations were obtained by selection in medium containing 800 μg/mL of G418 (Invitrogen).

Wound healing assay

NIH3T3 cells were plated in 12-well tissue plates and maintained in DMEM. At 80% to 90% confluency, the tip of a micropipette was used to create a linear scratch. The cells were then washed with PBS to remove floating cellular debris and fed for an additional 8 hours with DMEM without FBS. Cell migration was judged by photographs taken immediately after scratching and at designated times after scratching by a digital camera. Using Image J software (Rasband, W.S., ImageJ, U.S. NIH, Bethesda, MD), the wound area was measured, and the wound closure area was calculated as follows: wound closure area = area of wound at time 0 hour – area of wound at time 8 hours.

Cell proliferation assay

Anchorage-independent cell growth/Anoikis assay. A total of 5,000 NIH3T3 cells were plated onto 24-well low-attachment tissue plate (Corning), and maintained for 2 weeks. Pictures were taken by a digital camera.
**Cell growth assay for Baf3 cells.** A total of 3,000 cells were plated onto 96-well plate with/without interleukin-3 (IL-3) or BDNF stimulation. Cells were collected and counted at the indicated time points by Cellometer Auto T4 (Nexcelom Bioscience).

**MTS assay.** Changes in Baf3 cells proliferation were examined by the addition of AZD6918 at various concentrations. The number of surviving Baf3 cells was determined after 72 hours of treatment by measuring the dissolved formazan products after the addition of MTS as described by the manufacturer (Promega).

**Results**

**Mutation analysis**

We sequenced the tyrosine kinase domain of TrkB in 29 lung cancer cell lines, 68 NSCLC samples [28 large cell neuroendocrine carcinomas (LCNEC), 29 adenocarcinomas, 6 squamous cell carcinomas, and 5 large cell carcinomas], and 10 small cell lung cancer (SCLC) samples. Although TrkB mutations have been reported in NSCLC by others (Fig. 1; refs. 15, 16), we could not detect mutations in our samples. The results of our study and previous studies are summarized in Table 1.

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<th>Table 1. Summary of TrkB mutations in lung cancer</th>
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**Abbreviations:** ADC, adenocarcinoma; LCC, large cell carcinoma; SCC, squamous cell carcinoma; TC, typical carcinoid; AC, atypical carcinoid; ND, not detected.

<sup>a</sup>Cell lines.

**Characterization of mutated TrkB**

M713I, R715G, and R734C are TrkB mutations recently reported in LCNECs (19). Protein alignment of TrkB and EGFR revealed that the 3 mutations were located in the activation loop of TrkB flanking the position corresponding to the EGFR mutation L858R (Fig. 1B). To study the functional significance of these 3 mutations, we stably introduced wild-type and mutant (M713I, R715G, and R734C) TrkBs into TrkB-negative NIH3T3 cells. Western blot analysis revealed that TrkB autophosphorylation was detectable in the wild-type and R715G TrkB-expressing cells, but not in the M713I and R734C TrkB cells (Fig. 2A). On BDNF stimulation, the level of phosphorylation was similar between wild-type and R715G cells, whereas it was lower in M713I and R734C than in wild-type cells. Downstream signaling, AKT, and extracellular signal regulated kinase (ERK) phosphorylation were preferably observed in wild-type TrkB-transfected cells (Fig. 2B). Similar TrkB phosphorylation and downstream signaling were observed in H322 and H2122 lung cancer cell lines (data not shown). In addition, wild-type TrkB-expressing 3T3 cells show greater migration ability than their mock-transfected counterpart. On the contrary, there were no significant differences between mutated TrkB- and
mock-transfected cells (Fig. 3A). A Trk inhibitor, AZD6918, is potent in vitro inhibition of pan-Trk kinases and in vivo antitumor growth in a neuroblastoma model (20). The drug inhibited wild-type TrkB-induced migration (Fig. 3A). Furthermore wild-type, but not mutated, TrkB-transfected NIH3T3 cells showed anchorage-independent growth (Fig. 3B).

To evaluate the transformation capacity of TrkB (M713I, R715G, and R734C) mutants, we introduced wild-type and mutant TrkBs into BaF3 cells (ref. 21; Fig. 4A). BaF3 cells are IL-3 dependent, and withdrawal of IL-3 results in cell death. TrkB-expressing BaF3 cells could not grow in the absence of IL-3 and BDNF stimulation, suggesting that wild-type and mutant TrkBs are neither constitutively active nor capable of overcoming IL-3 dependency. (Fig. 4A and B). Interestingly, in line with a previous finding in NIH3T3 transformation assay (22), wild-type TrkB-transfected BaF3 cells showed accelerated growth rate compared with mock transfectants on BDNF stimulation, whereas no difference in growth was observed between the mutant TrkB- and mock-transfected cells (Fig. 4B), suggesting that the mutant TrkBs are BDNF nonresponsive mutants. It is interesting to note that BDNF could rescue BaF3 cells from IL-3 withdrawal regardless of ectopic TrkB expression, suggesting that BDNF could activate cell survival/proliferation signaling through TrkB-independent pathway. Taken together, these results indicate that the observed outgrowth of wild-type TrkB-transfected BaF3 cells over mock-transfected and mutant TrkB-transfected cells in the presence of BDNF may be attributed to BDNF-stimulated TrkB signaling. AZD6918 inhibited BDNF/TrkB-induced cell proliferation at as low as 10 nmol/L with wild-type TrkB being the most sensitive; whereas there was no difference between TrkB-expressing cells and mock cells in IL3-induced cell growth on exposure to the Trk inhibitor (Fig. 4C). Inhibitory effects of the drug were confirmed by Western blotting (Fig. 4D). At 5 nmol/L, AZD6918 inhibited TrkB Y516 autophosphorylation, and the 3 mutants seemed to be relatively resistant to the drug. In addition, we evaluated endogenous TrkB expression in 17 NSCLC cell lines, and tested sensitivity to AZD6918 in 4 cell lines (Supplementary Fig. S1). Although 2 cell lines expressed detectable level of TrkB, IC50 of AZD6918 were more than 1 μmol/L in all tested cell lines.

**Discussion**

Overexpression of TrkB in cancers has been described, and is indicative of aggressive tumor behavior. Several mechanisms underlying overexpression of TrkB have been reported. In neuroblastoma, TrkB is often overexpressed in association with amplification of MYCN locus (23, 24). Overexpression of TrkB through an autocrine loop was reported in prostate cancer and malignant myeloma (7, 25). TrkA and TrkC, but not TrkB, oncogenic fusion proteins have been reported (3, 4). Although the mechanism for gain of oncogenic function is mainly because of activation of the tyrosine kinase, TrkB has a kinase-independent function. The kinase domain deficient splicing variant TrkB-T1 may enhance metastasis of pancreatic adenocarcinoma cells through activation of Rho-Rock pathway (26).

Mutations in the tyrosine kinase domain are a possible mechanism of activation of TrkB. TrkB mutations have been described in NSCLC (Table 1). Ding and colleagues reported 6 mutations out of 188 lung adenocarcinomas (15). Marchetti and colleagues reported 4 mutations out of 29 LCNECs; however, they failed to find mutations in 443 NSCLCs, including 228 adenocarcinomas, 184 squamous...
cell carcinomas, and 31 large cell carcinomas without neuroendocrine features (16). In this study, we could not detect nonsynonymous mutations in 78 lung cancer samples and 29 cell lines. In addition, another group has sequenced the entire TrkB gene in 51 lung cancer samples and 32 lung cancer cell lines and found no mutations (J. Brown, Astrazeneca, personal communication). Possible causes of the discrepancy in this series are as follows: (i) ethnic difference: our samples mainly (60 of 78) consist of East-Asian (Japanese) patients, and all of LCNEC samples were from Japanese patients whereas all patients in the previous reports were recruited in Western countries; striking differences in the frequency of EGFR mutations between Caucasians and East Asians are well known (27, 28); (ii) false negative: Marchetti and colleagues carried out single-strand conformation polymorphism (SSCP) as an initial screening, and only SSCP positive tumors were sequenced; it may be possible that SSCP may not be sensitive enough to resolve existing mutations; and (iii) false positive: it has been documented that DNA extracted from paraffin-embedded tissue easily led to PCR artifact (19). Taken together with the previous reports, the frequency of Trk mutation in NSCLC was quite low [10 of 722 (1.39%); Table 1]. In this study, though no nonsynonymous mutation was detected, we found 5 known silent single-nucleotide polymorphisms (I616I; rs2289657) out of 78 tumor samples (data not shown). A synonymous polymorphism in ERCC1 has reported to be correlated with ERCC1 expression, different prognosis, and response to chemotherapy (29–31). Meaning of the polymorphism in TrkB remains to be explored.

We selected 3 mutations reported before for further characterization. However, the mutations that we tested in this study were not activating mutations. Intriguingly,
although R715G showed comparable levels of phospho-TrkB to wild-type TrkB, the mutant TrkB failed to show transforming ability (Figs. 3 and 4). Our results suggest that mutations of TrkB cannot be used as a positive marker for patient selection. To date, small molecule inhibitors targeting kinase domain have been most successful in cases where the targeted kinases contain activating mutation such as in EGFR. It is unclear whether the Trk inhibitor will be effective as a single agent. Indeed, we tested sensitivity to AZD6918 in 4 lung cancer cell lines, all of their IC50 were as high as 1 to 10 μmol/L (Supplementary Fig. S1). However, our results suggested that the Trk inhibitor might inhibit wild-type TrkB-induced cell migration and proliferation in the presence of BDNF. Some studies reported high levels of BDNF and TrkB coexpression in lung cancers (8, 32, 33). The Trk inhibitor may be potentially effective in those cancer patients based on our data (Fig. 4C). Using the Trk inhibitor in combination with cytotoxic agents is another possible option. Several in vitro studies reported that inhibiting Trk signaling might enhances efficacy of cytotoxic agents (34, 35).

In conclusion, although mutations in the TrkB gene may not be used for patient selection, overexpression of wild-type TrkB may represent a potential target for treatment.

Figure 3. TrkB in cell migration and anchorage-independent growth. A, cell migration ability was determined with wound healing assay. Wild-type and mutated TrkB-expressing NIH3T3 cells were treated with 50 ng/mL of BDNF or 50 nmol/L of AZD6918. After 8 hours incubation, wound closure area was determined as described in Materials and Methods. Experiments were repeated at least 3 times. Representative pictures are shown. Results are expressed as relative wound closure area; nontreated mock is shown as 1. Bars, SD. *, P = 0.001; **, P = 0.011; ***, P = 0.007. Statistical significance (defined as P < 0.05) was determined by Student’s t test. B, anchorage-independent cell growth. The cells were seeded onto low-attachment plate, and maintained for 2 weeks. Photographs were taken by ×4 objective lenses.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We thank Dr. Meyerson (MGH, Boston, MA) for the Baf3 and WEHI3B cells and Dr. Peeper (NKI, Amsterdam, The Netherlands) for the TrkB ORF carrying plasmid.

References


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