

Beyond *ALK* and *ROS1*: *RET*, *NTRK*, *EGFR* and *BRAF* gene rearrangements in non-small cell lung cancer

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Abstract: The discovery of gene rearrangements involving the receptor tyrosine kinase genes *ALK* and *ROS1* has revolutionized management of the subset of non-small cell lung cancers characterized by these alterations. The oncogenic fusion proteins expressed in these tumors drive cancer cell growth and survival, and targeted inhibition of this signaling can lead to dramatic and durable responses in patients. While the best characterized gene fusions in non-small cell lung cancer (NSCLC) involve *ALK* and *ROS1*, fusions involving other kinases including *RET*, *NTRK*, *EGFR* and *BRAF* are now established as additional targetable drivers. Here we review data supporting the roles of these fusions as oncogenic drivers, and the potential for targeting these fusions for improved clinical outcomes. These discoveries should encourage multiplexed molecular profiling of lung cancers using next-generation platforms which identify these gene fusions in order to expand treatment options for patients.

Keywords: *RET*; *NTRK*; *EGFR*; *BRAF*; non-small cell lung cancer (NSCLC)

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Introduction

Over the past decade, molecular characterization of non-small cell lung carcinomas (NSCLCs) has uncovered molecularly defined subsets. Somatic molecular alterations in NSCLCs can lead to oncogene activation through multiple mechanisms, including point mutations, insertions, deletions and gene rearrangements. These alterations are generally mutually exclusive, with no more than one predominant driver in any given cancer (1,2). The hallmark of all of these alterations is *oncogene addiction*, in which cancers are driven primarily, or even exclusively, by aberrant oncogene signaling. Oncogenic gene rearrangements in NSCLC can lead to expression of oncogenic fusion proteins when a 5' partner forms an in-frame gene fusion with a 3' proto-oncogene (3-7). These fusions retain the kinase domain of the proto-oncogene, with expression and dimerization

generally promoted by 5' partner. The result of this structure is expression of a constitutively activated fusion protein whose downstream signaling drives cell proliferation and survival in a ligand-independent manner (*Figure 1*) (8).

Targeted inhibition of oncogenic fusion proteins can result in potent cancer growth inhibition and regression of tumors in patients. To date, there are drugs approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for NSCLCs containing gene fusions involving anaplastic lymphoma kinase (*ALK*) and *ROS1* proto-oncogene receptor tyrosine kinase (*ROS1*) (9-12). However, other gene rearrangements involving other kinase-encoding genes, including *RET*, *NTRK1*, *EGFR* and *BRAF* have been described in NSCLC. Emerging data support the hypothesis that these fusions, like those involving *ALK* and *ROS1*, lead to oncogene dependency, and therefore may also offer an opportunity

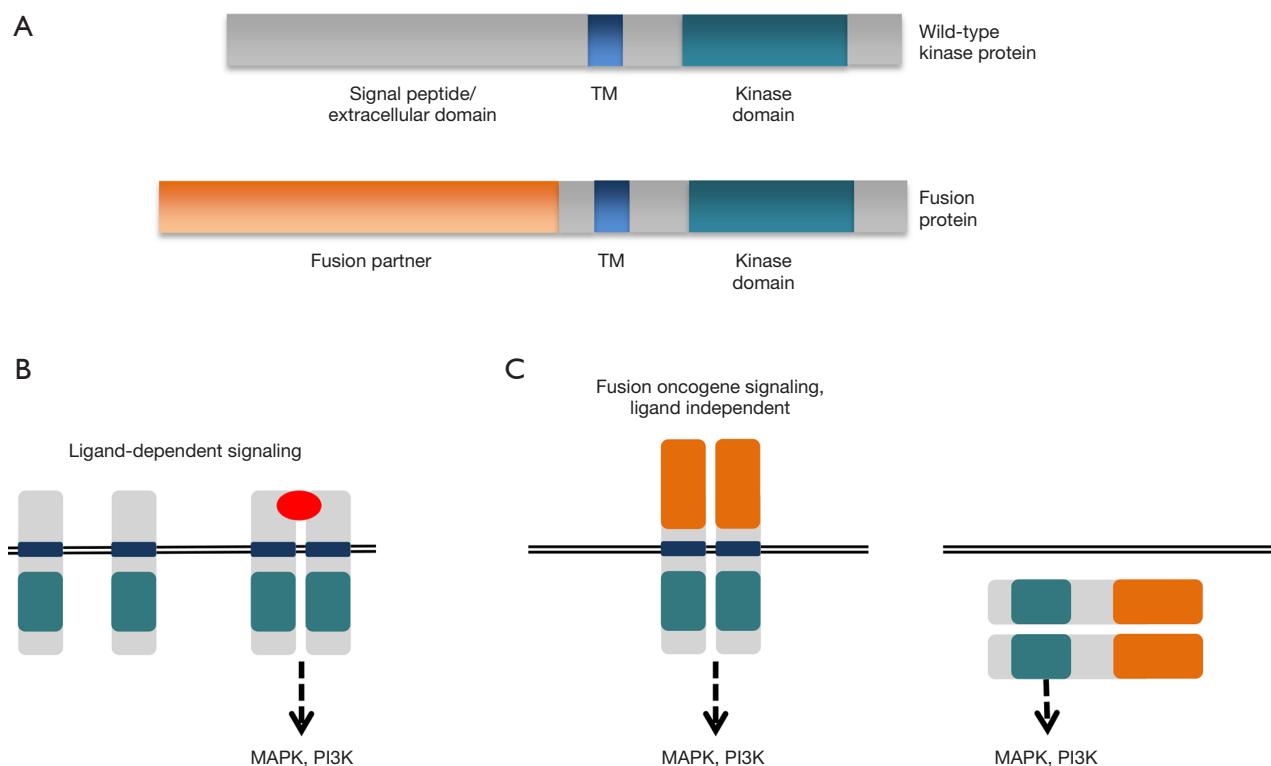


Figure 1 Schematic representation of oncogenic fusion proteins. (A) Wild-type proto-oncogene schematic of a RTK (above) and fusion protein produced by an in-frame gene rearrangement (below); (B) schematic of normal signaling by RTK, which occurs in a ligand-dependent manner to activate downstream signaling pathways such as MEK and PI3K; (C) fusion oncogene signaling occurs in a ligand independent manner, and can involve fusions that span the membrane (left) or are fully intracellular (right). Figure is adapted and modified from reference (8). TM, transmembrane domain; RTK, receptor tyrosine kinase.

for targeted and effective anti-tumor therapy.

Rearranged during transfection (*RET*)

The *RET* proto-oncogene encodes a receptor tyrosine kinase that is expressed on tissues of neural crest origin and functions in development of the kidneys and enteric nervous system (13,14). Rearrangements involving *RET* have oncogenic potential, and occur in a subset of thyroid papillary carcinomas (15,16). Gene rearrangements involving *RET* have also been observed in approximately 1–2% of NSCLCs and are mutually exclusive with other oncogenic driver mutations such as *ALK* or *ROS1* rearrangements or *EGFR* mutations (7,17–19). Multiple fusion partners with *RET* have been described. A recent global registry of patients with *RET* rearranged NSCLC reported that among 81 cases with identifiable fusion partners, 72% involved the kinesin family 5B gene (*KIF5B*), supporting previous observations that *KIF5B* is the most

common *RET* fusion partner in NSCLC (7,17,19,20). The second most common fusion partner is *CCDC6* (23%), though others including *NCOA4* (2%), *EPHA5* (1%) and *PICALM* (1%) have been reported (20,21).

The results from a global multicenter registry of 165 *RET* rearranged NSCLCs provide several valuable insights into the clinical and pathological features of this subtype of lung cancer (20). *RET* rearrangements were observed in males and females in equal proportions. Among patients in the global registry, 63% were never smokers, 24% were former smokers, and 10% were current smokers. 98% of cases were adenocarcinoma. The majority of patients (72%) had stage IV disease at the time of diagnosis, suggesting that *RET* rearranged NSCLCs may have high metastatic potential. Alternatively, it is possible that *RET* rearrangements are more readily identified in patients with metastatic disease because non-metastatic cases may less commonly undergo molecular testing.

Extrapolating from the paradigm of fusion oncogene

dependence observed in *ALK* and *ROS1*-rearrangement NSCLCs, efforts have been made both preclinically and clinically to target RET in *RET* rearranged NSCLCs. In cell line models expressing exogenous *KIF5B-RET*, RET undergoes ligand independent tyrosine phosphorylation, and expression of the fusion construct has transforming activity (17). To date, no highly selective RET inhibitor has been tested clinically, and the majority of preclinical and clinical development has focused on use of multi-targeted kinase inhibitors. In preclinical cell line models expressing *KIF5B-RET* fusion transgenes, treatment with the multi-targeted kinase inhibitors vandetanib, sorafenib and sunitinib suppressed RET phosphorylation and inhibited cell viability in a dose-dependent manner (17,18).

Clinical activity of RET-directed therapy was first reported in 2013, when three patients with *RET* rearranged NSCLC were treated with cabozantinib (21). Two of these patients experienced partial responses by RECIST 1.1 criteria, and a third had prolonged stable disease. Extending from this work, a phase 2 trial was conducted to assess the activity of cabozantinib among patient with *RET* rearranged lung adenocarcinoma. Among 25 patients who were assessable for response, there were seven partial responses [overall response rate (ORR) 28%, 95% CI, 12–49]. The median duration of treatment was 4.7 months, and the median duration of response was 7.0 months. The median progression-free survival (PFS) was 5.5 months (22).

Similarly, vandetanib has shown single agent activity in *RET* rearranged NSCLC. A case reported in 2014 demonstrated activity of vandetanib in a patient with a *CCDC6-RET* rearrangement NSCLC (23). More recently, among 19 *RET* rearrangement NSCLC patients enrolled in a Japanese phase 2 single arm study, there was a response rate of 47% and a median PFS of 4.7 months (24).

Lenvatinib has also shown single agent activity, with an objective response rate of 16% among 25 *RET*-fusion positive adenocarcinoma patients, including 3 of 4 responses in patients who had received prior *RET*-directed therapy (25). In this study, a median PFS was 7.3 months (95% CI, 3.6–10.2) and the median duration of treatment was 16 weeks.

Other multi-target kinase inhibitors have also been used in *RET*-rearrangement NSCLC, including sunitinib (26), sorafenib (27), alectinib (28), nintedanib, ponatinib and regorafenib (20). Data for these agents are generally limited to case reports. There is no clear gold standard for treatment, and no direct comparison of *RET* inhibitors has been performed. Thus, it is difficult to identify the most active *RET* inhibitor based on the currently available

clinical data.

One challenge facing clinical application of these *RET* inhibitors is toxicity. In the phase 2 trial of cabozantinib, dose reductions from the starting dose of 60 to 40 mg daily was required in 73% of patients, and to 20 mg daily in 15% of patients (22). Among *RET* rearrangement NSCLC patients, the most common reasons for dose reduction were palmar-plantar erythrodysesthesia, fatigue, and diarrhea (22). Such toxicities may be attributable to cabozantinib's inhibitory activity of VEGFR2 and other kinases. Vandetanib is associated with diarrhea, rash, nausea, hypertension, fatigue, and electrocardiogram QT prolongation, as well as other effects (29).

It is unclear whether the multi-kinase activity of the *RET* inhibitors is an advantage, perhaps by affecting vasculature or other elements of the tumor micro-environment, or whether it is a liability, contributing to toxicity without improving activity, and potentially limiting clinical activity by preventing maximal *RET* inhibition. Development of highly-selective *RET* inhibitors, such as BLU-667 which is currently in clinical development (NCT03037385), may provide insights.

RXDX-105 is a small molecule *RET* inhibitor with potent inhibition of wild-type *RET*, *RET* fusions, and *RET* activating mutations in cell lines and in xenograft models (30). It also has activity against BRAF V600E (30-32). RXDX-105 spares VEGFR2 and VEGFR1, and therefore may also spare some of the toxicities seen with other multikinase inhibitors (30). In a patient with *RET* rearranged NSCLC with brain metastases, treatment with RXDX-105 led to a confirmed partial response and near complete resolution of previously noted subcentimeter brain metastases (30). A phase 1 trial of RXDX-105 is ongoing (NCT01877811).

Metastasis to the central nervous system (CNS) represents an important clinical challenge in *RET* rearrangement NSCLC. Several studies have specifically focused on this need. Vandetanib is thought to have limited blood-brain barrier penetration, but Minocha and colleagues hypothesized that this penetration may be improved by modulation of P-gp/Abcb1- and Bcrp1/Abcg2-mediated efflux through the use of an mTOR inhibitor (33,34). Combining vandetanib and the mTOR inhibitor everolimus in *RET* rearranged NSCLC, the authors have observed promising systemic activity, with partial responses in 5 of 6 NSCLC patients with *RET* rearrangements identified by next generation sequencing (NGS) (33,35). Additionally and importantly, antitumor activity was also observed in the CNS, including in a patient with cabozantinib resistant

disease (33,35). However, the combination of vandetanib and everolimus is associated with toxicity, including grade 3 diarrhea, rash and QTc prolongation (35). There is an ongoing phase 1 trial studying the combination in patients with advanced cancer (NCT01582191).

Alectinib is an orally available inhibitor of ALK, and also has RET inhibitory activity in cell-based and xenograft models of *KIF5B-RET* rearranged NSCLC (36). Alectinib also has a more favorable toxicity profile than many of the other multi-kinase RET inhibitors. A recent study reported that among four patients with metastatic *RET* rearranged NSCLC who were treated with alectinib, including three who had received prior RET TKIs, there were two objective radiographic responses (28). One patient with CNS metastases had improvement in both intracranial and extracranial disease on an escalated dose of 900 mg twice daily of alectinib (28).

Mechanisms of acquired resistance to RET inhibitors in patients are currently poorly understood. Repeat biopsies or analysis of circulating tumor DNA at the time of acquired resistance to RET inhibitors may be informative.

Neurotrophic tyrosine receptor kinase (*NTRK*)

The *NTRK* genes, *NTRK1*, *NTRK2* and *NTRK3*, encode the tropomyosin receptor kinases (TRK), TRKA, TRKB and TRKC, respectively. The TRK proteins function during normal physiology as receptors for nerve growth factors (37-40). They signal through PI3 kinase, RAS/MAPK/ERK, and PLC-gamma pathways to mediate neuronal development (41-44).

A somatic chromosomal rearrangement resulting in expression of oncogenic fusion involving *NTRK1* was first described in 1986 in a colorectal tumor sample (45). Since then, fusions involving *NTRK1* have been described in a variety of other cancer types, including intrahepatic cholangiocarcinoma (46), papillary thyroid cancer (47), spitzoid neoplasms (48), glioblastoma (49) and sarcoma (50,51). Additionally, fusions involving *NTRK2* and *NTRK3* have been described in a variety of cancer types [reviewed in (44)]. Interestingly, the frequency of *NTRK* fusions is low (<5%) in common cancer types, but *NTRK3* fusions can be frequent or nearly uniform in uncommon cancer types such as secretory breast carcinoma (52), mammary analog secretory carcinoma (53), and congenital fibrosarcomas (54,55).

Oncogenic fusions involving *NTRK1* were first described in NSCLC in 2013. Using a targeted next-generation

DNA sequencing (NGS) assay on a collection of 36 lung adenocarcinoma samples without other known oncogenic drivers, Vaishnavi and colleagues identified 2 cases with in-frame fusions involving *NTRK1* (56). One fusion involved the 5' end of the myosin phosphatase-Rho-interacting protein gene (*MPRIP*) and the 3' end of *NTRK1*, including the tyrosine kinase domain. The second fusion involved the 5' end of *CD74* and the 3' end of *NTRK1*, again with the tyrosine kinase domain intact. These fusions were tumorigenic in xenograft models. Additionally, Ba/F3 cells engineered to express a *MPRIP-NTRK1* cDNA construct demonstrated auto-phosphorylation of TRKA and activation of downstream signaling pathways, while inhibition of TRK signaling with tyrosine kinase inhibitors blocked downstream signaling and reduced cell viability (56).

The frequency of *NTRK* fusions in NSCLC is not well established. Estimates range from 0.1% among all NSCLCs to 3% among patients without other identified driver mutations (8,56). Furthermore, the clinical and pathologic features of these patients are also not well characterized.

Several TRK inhibitors are now in clinical development. Larotrectinib (LOXO-101) is an oral tyrosine kinase inhibitor that has high selectivity and potency for TRKA, TRKB, and TRKC. Data from the phase 1 study of larotrectinib in solid-tumor patients was presented in late 2016 (57). The study had enrolled 59 patients. Of these, 8 patients had tumors harboring an *NTRK* gene fusion. The drug was well tolerated overall with the most common adverse effect (AE) being fatigue (37%). The most common grade 3/4 AE was anemia (8%). Among 7 *NTRK* fusion patients with evaluable disease, 6 had partial response and one had stable disease by RECIST assessment. The majority of *NTRK* fusion patients have remained on study for greater than 12 months (57). Larotrectinib is now being further investigated in an ongoing phase 2 study in subjects with *NTRK* fusion positive solid tumors (NCT02576431) and a pediatric phase 1/2 study (NCT02637687).

Entrectinib (RXDX-101) is an oral tyrosine kinase inhibitor with potent activity against ROS1, ALK, and TRKA, TRKB and TRKC (58). Among 119 patients treated on two phase 1 studies, entrectinib was very well tolerated (59). Among three patients with extra-cranial, TKI-treatment naive tumors harboring *NTRK* gene fusions, all had objective responses by RECIST criteria (59). One of these was a patient with NSCLC with a *SQSTM1-NTRK1* fusion, who remained on treatment for over 18 months. Notably, in addition to having extensive extra-cranial disease, this patient had brain metastases at baseline and

had a rapid and durable response to entrectinib in the brain (8,59). An additional patient with a glioneuronal tumor with a *BCAN-NTRK1* fusion had a clinical and volumetric response to entrectinib (60). This promising activity within the CNS is highly relevant to patients with NSCLC, where CNS metastases are common. Entrectinib is now being further investigated in an ongoing basket phase 2 trial in subjects with solid tumors harboring *NTRK1/2/3*, *ROS1* or *ALK* gene rearrangements (NCT02568267).

Several other TKIs have activity against TRKA/B/C. Others in clinical development include TSR-011 (NCT02048488), DS-6051b (NCT02279433), PLX7486 (NCT01804530). Notably, although crizotinib has some TRK inhibitory activity in preclinical models (56) its activity in patients with NTRK rearrangement tumors has not been well validated. While several multi-kinase inhibitors have some TRK inhibitory activity, this is likely more of a liability than a strength, given that highly potent and specific TRK inhibition is sufficient to induce dramatic clinical responses (57,59).

Although *NTRK* rearranged tumors have been highly sensitive to selective TRK inhibitors, several cases of acquired resistance to TRK inhibitors have been reported (61,62). In these cases, patients' tumors initially responded well to the TRK inhibitor, but subsequently progressed, indicating outgrowth of a resistant subclone. Acquired resistance to entrectinib was described in a patient with colorectal cancer harboring an *LMNA-NTRK1* rearrangement, and in a patient with a mammary analogue secretory tumor harboring an *ETV6-NTRK3* fusion. In each case, resistant subclones had new mutations in the *NTRK* kinase domain, altering the protein structure to create steric hindrance to drug binding in the ATP binding pocket. These cases indicate that these tumors remain dependent on TRK signaling, and that resistance is mediated by preventing drug binding. Thus, strategies to overcome steric hindrance mutations may be effective in treating select cases of NTRK-rearrangement tumors with acquired resistance to first generation TKIs. It is not yet known how frequently *NTRK* rearranged tumors will develop resistance via alternative mechanisms, such as activation of a bypass signaling pathway or otherwise achieving TRK-independent resistance, as can be seen in *ALK* rearranged NSCLCs (63,64).

EGFR

The majority of clinically relevant *EGFR* mutations in lung

cancer are exon 19 deletions or exon 21 missense mutations, which were first discovered in 2004 by sequencing *EGFR* in tumor biopsies from patients with response to the *EGFR* tyrosine kinase inhibitors gefitinib, or erlotinib (65). This discovery led to refined drug usage and development which over the past 13 years has improved outcomes for patients with lung cancer and activating/sensitizing *EGFR* mutations such that median overall survival is now measured in years (66). Next generation molecular pathology platforms have since detected *EGFR* gene fusions which involve a breakpoint somewhere in *EGFR* exon 23—intron 25 fused with either *RAD51* or *PURB*, and the fusions are both activating and sensitizing. These gene fusions would not be discovered using conventional PCR-based methods for detecting classical *EGFR* mutations. A seminal paper in 2016 reported 5 cases of *EGFR* gene fusions, 4 of whom were treated with *EGFR* tyrosine kinase inhibitors with documented clinical and radiologic responses (67). Their technique utilized a next-generation sequencing assay that interrogated the entire coding region of *EGFR* (as well as introns 7, 15, 24, 25, and 26). The investigators surveyed over 10,000 lung cancers using this technique and found *EGFR* fusions in 5 cases (0.05%).

We at Massachusetts General Hospital Cancer Center are currently treating a 36-year-old man with virulent, chemorefractory lung cancer metastatic to brain, pleura and peritoneum who was found to have an *EGFR* exon 24-*RAD51* exon 4 gene fusion using our next-generation sequencing platform (68). After discovery of the fusion, the patient demonstrated both clinical and radiologic improvement on erlotinib therapy and has remained on erlotinib for over 2 years with ongoing clinical and radiologic response.

In addition to *EGFR* fusions, an *EGFR* exon 18–25 kinase domain duplication has been reported in a 33-year-old male with lung cancer. This patient's tumor responded to therapy with the *EGFR* inhibitor afatinib (69).

BRAF

BRAF is a RAF kinase which is downstream of RAS and signals via the MAPK pathway. The majority of activating *BRAF* mutations in cancer occurs within the kinase domain at amino acid V600, most commonly V600E. Genotyping studies of NSCLC have detected *BRAF* base substitutions in 2–5%, and about half of these mutations result in the *BRAF* amino acid substitution V600E. Most patients with

BRAF mutation are former smokers, and non-V600E mutations are more common in heavy smokers. In the era before targeted therapy for *BRAF*, there was no difference in overall survival in patients based on the presence of *BRAF* mutation, or mutation subtypes (70-74).

In contrast to base substitutions, *BRAF* gene fusions are extremely rare and biologically distinct from *BRAF* V600E. In a study of comprehensive genomic profiling using the FoundationOne assay, *BRAF* fusions involving the intact in-frame *BRAF* kinase domain were discovered in 0.2% (8/4,013) of NSCLCs (75). In contrast, *BRAF* fusions are detected in 3% of melanomas; 2% of gliomas; 1% of thyroid cancers; and 0.3% of pancreatic carcinomas. All lung cancers with *BRAF* fusions were adenocarcinomas, or had adenocarcinoma features, and none were detected in squamous or small cell lung cancers (75).

BRAF fusions are similar to other kinase fusions in that they tend to be mutually exclusive of other activating mutations in the MAPK pathway (75). The 5' fusion partners identified to date include *EPS15*, *NUP214*, *ARMC10*, *BTF3L4*, *AGK*, *GHR*, *ZC3H4V*, and *TRIM24* (75). Protein domain analysis shows that these fusions maintain the kinase domain of *BRAF* and the dimerization motif for *BRAF* at amino acids 506–509, however the RAS-binding domain is replaced by the various 5' partners (76). Early studies have determined that *BRAF* fusions are activating, and certain *BRAF* fusion variants can homodimerize with one another (77).

Distinguishing *BRAF* base substitutions from fusions may be particularly important with regards to selection of targeted drug therapy. Signaling through RAF/MEK/ERK is complex, dependent on RAF dimerization, and limited by feedback inhibition of RAS signal. *BRAF* V600E is unique in that it functions as an activated monomer independent of RAS signal and can be targeted with single-agent *BRAF* inhibitors. Prospective drug trials of single-agent *BRAF* inhibitors (vemurafenib or dabrafenib) in patients with metastatic NSCLC and *BRAF* V600E show response rates of 30–40% and median PFS of 5–7 months (78,79). Addition of the MEK inhibitor trametinib to dabrafenib in patients with *BRAF* V600E improves ORR to 63% and median PFS to 10 months (80). In contrast, all other activating *BRAF* mutants function as constitutive RAS-independent dimers not susceptible to single-agent RAF inhibitors (which do not inhibit dimers because their binding to one site in the dimer significantly reduces their affinity for the second site) (81). Thus, based on preclinical

information, *BRAF* inhibitors may not be effective in *BRAF* fusion-driven malignancies, and in fact may actually promote cancer growth. Case reports of activity of single-agent MEK inhibitors in melanomas with *BRAF* fusion have been described (76).

Conclusions

RET, *NTRK*, *EGFR* and *BRAF* fusions in NSCLC represent recently discovered molecular subsets, and preclinical and early clinical data suggest that targeted inhibition of their activated protein products can cause tumor regressions. Further delineation of the most potent and selective kinase inhibitors, and the CNS activity of these drugs, is needed. However, these gene mutations are sufficiently rare that it may not be possible to conduct randomized trials to compare the activity of targeted therapies to traditional chemotherapy or immunotherapy for each subtype. There is no question that the overall survival of a patient with advanced lung cancer is improved when that patient has access to more than one effective therapy. There are now FDA approved drugs with different mechanisms of action for all lung cancer patients as first, second, and even third-line therapy (82). Discovery of actionable gene fusions opens doors for patients to try targeted therapies available by commercial supply, or to enroll them in an appropriate clinical trial. We anticipate that these expanded effective therapeutic options will lead to improved overall survival in these patients, as they have for patients with other molecularly targetable oncogenic alterations.

Taken individually, these genetic events are rare. However, taken together, and paired with universal comprehensive molecular profiling, there will be more and more patients who benefit from molecularly targeted therapy for their advanced lung cancer. Given the ever-increasing landscape of potentially actionable mutations in NSCLC, we advocate for multiplexed testing of all NSCLCs using next-generation gene sequencing platforms which identify both gene sequence alterations and gene fusions to increase discovery of targetable mutations in individual patients in the most efficient manner possible (68,83).

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Footnote

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