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the RAS/MAPK pathway: oncogenic NRAS and upregulation of wild-type EGFR signaling.

Materials and Methods

Cell lines and reagents

LC-2/ad cells were obtained from Sigma (catalog no. 94072247), TPC1 cells obtained from R.E. Schweppe (University of Colorado; ref. 17), and H2228 cells obtained from J.D. Minna (University of Texas Southwestern). HCC78-TR cells were derived as described previously (18). Cells were maintained in RPMI1640 (Invitrogen) with 10% FBS at 37°C in a humidified 5% CO_2 incubator. Fingerprint analysis of cell lines was performed bi-annually by the Molecular Biology Service Center at the Barbara Davis Center for Diabetes at the University of Colorado Anschutz Medical Campus (Aurora, CO) to ensure authenticity. Alectinib was provided by Chugai Pharmaceuticals. Ponatinib, cabozantinib, trametinib, gefitinib, afatinib, and foretinib were obtained from Selleck Chemicals. Pervanadate was generated by incubating hydrogen peroxide with 100 mmol/L sodium orthovanadate in distilled water. Antibodies used were as follows: pEGFR Y1068 (D7A5), pEGFR Y1173 (53A5), total RET (D3D8R), pERK1/2 XP T202/Y204 (D13.14.4E), total ERK1/2 (L34F12), pAKT S473 XP (D9E), total AKT (40D4), and pSHC1 Y239/Y240 (2434) from Cell Signaling Technology; pTYR (4G10 Platinum), GAPDH (6C5) and GAPDH (ABS16) from Millipore; pRET Y1062, α-tubulin (TU-02); and NRAS (F155) from Santa Cruz Biotechnology.

Cellular proliferation

Cells were plated in 96-well tissue culture plates and removed from ponatinib, if indicated, 24 hours prior to drug treatment or siRNA transfection for the time periods indicated. Cell numbers were assessed using CyQUANT Direct Cell Proliferation Assay (Thermo Scientific) according to the manufacturer's instructions.

FISH

FISH assays and analyses were conducted as described previously with minor modifications (19). The *RET* break-apart probe set includes a 3′ *RET* [Spectrum Red (R)] probe recognizing a genomic region 3′ end of exon 8, and a 5′ *RET* [Spectrum Green (G)] probe recognizing a genomic region 5′ end of exon 12. Samples were positive for *RET*-rearrangement if more than 15% of cells showed split signals, *3′ RET* and *5′ RET* apart by $\geq 2\times$ the signal diameter.

Immunoblotting

Immunoblotting was performed as described previously (18). Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) and diluted in 4× Protein Sample Loading Buffer (LI-COR). Total protein was separated by SDS-PAGE, transferred to nitrocellulose, and stained with the indicated primary antibodies and IRDye anti-mouse or anti-rabbit IgG (LI-COR). Membranes were scanned with an Odyssey Imager and Odyssey Image Studio Software (LI-COR). When appropriate, membranes were stripped with 3× NewBlot Nitro Stripping buffer (LI-COR) and reprobed.

RNA isolation and sequencing

RNA isolation from cells was performed using the RNeasy Kit (Qiagen) per the manufacturer's instructions. High-throughput mRNA sequencing (RNA-seq) of each sample (three replicates per cell line) was performed as described previously (18). Sequencing was performed on the Illumina HiSeq2000. On average, 50 million (45 million – 59 million) single-end 126 bp sequencing reads were obtained per sample. Reads were mapped against the human genome using Tophat (version 2.0.13; ref. 20), NCBI reference annotation (build 37.2) was used as a guide, allowing 3 mismatches for the initial alignment and 2 mismatches per segment with 25 bp segments. On average, 43 million (37 million – 49 million) of the reads aligned to the human genome. Transcripts were assembled using Cufflinks (version v2.2.1; ref. 21) using the RefSeq annotation as the guide, but allowing for novel isoform discovery in each sample. The data were fragment bias corrected, multi-read corrected, and normalized by the total number of reads. Differentially expressed genes were identified by Cuffdiff (version v2.2.1) after merging the transcript assemblies. Samtools (version 1.2; refs. 22, 23) was used to convert sequence alignment map (SAM) files to binary alignment map (BAM) files, and variant calling was performed for each sample (a variant is defined as minimum 5 reads detected in 20 reads depth). Integrative Genomics Viewer (IGV) was used to visualize the aligned reads and variants (24). The RNA sequencing data can be accessed from the Gene Expression Omnibus (GEO) with the accession number of GSE98030.

DNA isolation and sequencing

Mutations identified by mRNA sequencing were confirmed using standard Sanger sequencing of genomic as described previously (18).

Retroviral constructs and transduction

TPC1 cells were stably transduced with retroviral particles containing pBabe N-Ras 61K, a gift from Channing Der (Addgene plasmid # 12543), or an empty vector control and subject to puromycin selection.

RNAi-mediated silencing

Cells were transfected with 20 nmol/L siRNA targeting *RET* (catalog no. 4392420, Ambion), *NRAS* (L-003919-00, GE Dharmacon), or a nontargeting control (SIC001, Sigma) using DharmaFECT 1 transfection reagent (T-2001, GE Dharmacon).

NRAS activation assay

NRAS activation was assessed using the Ras Pull-down Activation Assay (Cytoskeleton) according to the manufacturer's protocol, using an NRAS-specific antibody.

Results

Ponatinib inhibits RET signaling and cell growth in the RET-rearranged NSCLC cell line LC-2/ad

Ponatinib is a multi-kinase TKI originally designed as a second-generation ABL inhibitor, which is an FDA-approved second-line therapy for patients with Philadelphia-positive leukemia (25), that inhibits wild-type RET and the gatekeeper mutant, RET p.V804M/L, with low-nanomolar affinity (26). Clinical trials of ponatinib in *RET* fusion–positive NSCLC patients are ongoing (NCT01935336, NCT01813734). The

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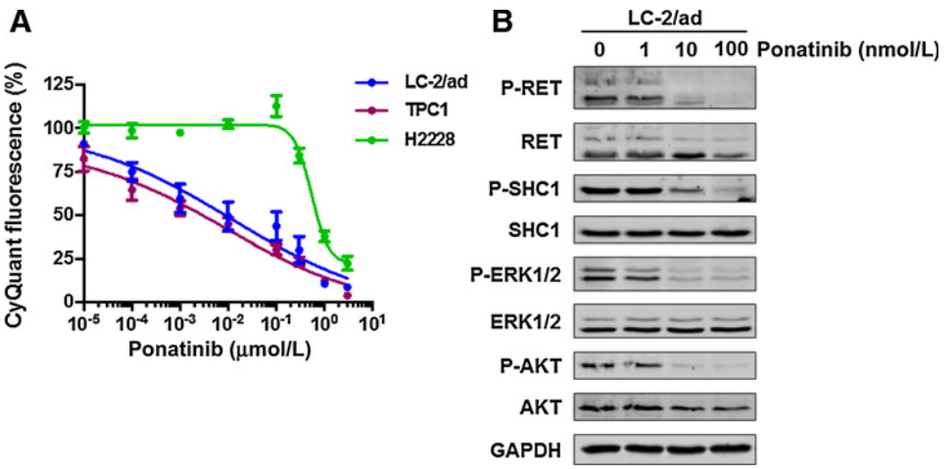

Figure 1.
Ponatinib inhibits RET and decreases cell proliferation of LC-2/ad cells. **A,** Cell viability of LC-2/ad (blue), TPC1 (pink), and H2228 (green) cells treated with increasing doses of ponatinib for 72 hours. Error bars, mean ± SEM for three triplicate experiments ($n = 9$). **B,** Western blot analysis of LC-2/ad cells treated with increasing doses of ponatinib for 2 hours. Lysates were analyzed via western blotting with the antibodies indicated, with P-TYR used to assess P-RET.

LC-2/ad cell line, which expresses CCDC6-RET and is the only published patient-derived NSCLC cell line driven by a RET fusion (27), is exquisitely sensitive to ponatinib *in vitro* (IC_{50} = 25 nmol/L; Fig. 1A). This is comparable with the previously demonstrated effect of ponatinib on cell proliferation in the TPC1 cell line (a CCDC6-RET–expressing PTC cell line; ref. 28). Notably, LC-2/ad and TPC1 cells are considerably more sensitive to ponatinib than H2228 cells (IC_{50} > 1 μmol/L; Fig. 1A), which harbor an oncogenic *EML4-ALK* fusion (29). Ponatinib does not inhibit ALK kinase activity *in vitro* at concentrations less than 1 μmol/L (30); thus, the high IC_{50} in the H2228 cell line likely reflects the accumulation of known kinase targets inhibited by ponatinib at concentrations greater than 1 μmol/L, supporting the conclusion that the low-nanomolar sensitivity of LC-2/ad cells is due to RET inhibition. Furthermore, when LC-2/ad cells were treated with increasing doses of ponatinib for 2 hours, tyrosine phosphorylation of RET was decreased in a dose-dependent manner (Fig. 1B). This was accompanied by decreased phosphorylation of SHC1, which mediates activation of PI3K-AKT and MAPK signaling (31), and decreased phosphorylation of AKT and ERK1/2 (Fig. 1B).

Generation of ponatinib-resistant derivatives of the LC-2/ad cell line

In an attempt to generate two discrete *in vitro* models of resistance to RET inhibition, LC-2/ad cells were cultured in increasing concentrations of ponatinib using two dose escalation strategies (Fig. 2A). The PR1 (ponatinib resistant-1) cell line was generated by initially culturing LC-2/ad cells in a fixed dose of 200 nmol/L ponatinib that was increased to 400 nmol/L after resistant clones successfully proliferated at a normal rate. The PR2 (ponatinib resistant-2) cell line was generated using a traditional dose escalation strategy, with LC-2/ad cells initially treated with a subnanomolar concentration of ponatinib that was incrementally increased to 400 nmol/L over 10 months. The LC-2/ad derivatives were deemed resistant when proliferation at 400 nmol/L ponatinib matched that of parental cells. Unless otherwise noted, PR1 and PR2 cells were subsequently maintained in 200 nmol/L ponatinib.

PR1 and PR2 cell proliferation was significantly less sensitive to ponatinib when compared with parental LC-2/ad cells, with IC_{50} values of approximately 660 nmol/L and approximately 400 nmol/L, respectively (Fig. 2B). This dramatic shift in IC_{50}, as opposed to a complete loss of sensitivity to ponatinib, is likely due to the cumulative inhibition of multiple kinase targets by ponatinib at high concentrations (30). Furthermore, the resistance phenotype extends to two additional RET TKIs, cabozantinib and alectinib (Supplementary Fig. S1), both of which are currently being assessed in clinical trials accruing RET fusion–positive NSCLC patients (32–34).

Break-apart FISH analysis revealed that the PR1 and PR2 cell lines still harbor the *RET* fusion gene with no notable change in copy number (Supplementary Fig. S2). DNA sequencing revealed a wild-type *RET* kinase domain in the parental LC-2/ad, PR1, and PR2 cell lines, establishing that a *RET* kinase domain mutation does not account for the resistance phenotype of the PR1 or PR2 cells; this was confirmed by next-generation RNA sequencing. This lack of a kinase domain mutation was not entirely surprising as ponatinib is active against the RET gatekeeper mutations V804L/M (26).

In contrast to parental LC-2/ad cells, which are dependent upon RET kinase activity, RET is expressed, but not phosphorylated, in the PR1 and PR2 cell lines (Fig. 2C). Interestingly, phosphorylation of RET can be restored in the PR1 and PR2 cell lines when incubated with the phosphatase inhibitor pervanadate (Supplementary Fig. S3), suggesting that suppression of RET phosphorylation in the resistant cell lines is due to a phosphatase-mediated mechanism. PR1 and PR2 cells express similar levels and activation of ERK1/2 and AKT to the parental LC-2/ad cells (Fig. 2C). However, unlike the parental LC-2/ad cells, ponatinib failed to perturb signaling through either ERK1/2 or AKT (Fig. 2D), further evidence that both the PR1 and PR2 cell lines have acquired a bypass signaling mechanism that drives PI3K/AKT and MAPK signaling independent of RET.

Oncogenic NRAS p.Q61K confers resistance to RET inhibition in the PR1 cell line

Next-generation RNA sequencing was performed on all three cell lines to identify differentially expressed genes and to detect mutations in known oncogenes or tumor suppressors. *EGFR*, *BRAF*, and *KRAS*, which are commonly mutated in NSCLC, were wild-type in all three cell lines; however, a previously described activating mutation in *NRAS* was identified in the PR1 cell line.

DNA sequencing confirmed that a single base pair substitution in *NRAS* encoding the NRAS p.Q61K mutant was present in the

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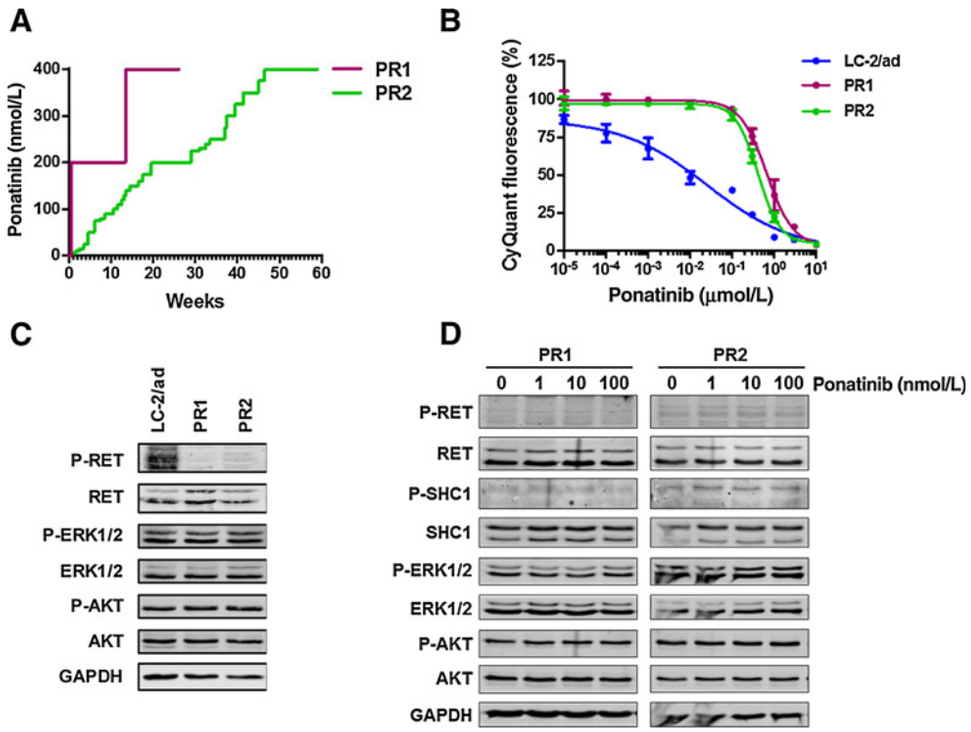

Figure 2.

PR1 and PR2 cells are resistant to ponatinib. **A,** Dose escalation schema for the derivation of PR1 and PR2 derivatives of the LC-2/ad cell line. **B,** Cell viability of LC-2/ad (blue), PR1 (pink), and PR2 (green) cells treated with increasing doses of ponatinib for 72 hours. Error bars, mean ± SEM for 3 triplicate experiments ($n = 9$). **C,** Western blot analysis of untreated LC-2/ad, PR1 and PR2 cells. **D,** Western blot analysis of PR1 and PR2 cells removed from ponatinib for 24 hours prior to 2 hours treatment with increasing doses of ponatinib. Lysates were analyzed via western blotting with the antibodies indicated, with P-TYR used to assess P-RET.

PR1 cell line, but not in the parental LC-2/ad or PR2 derivative (Fig. 3A). siRNA knockdown of RET and/or NRAS protein expression in the LC-2/ad cells reinforced the RET-dependence of the LC-2/ad cells, with decreased cell proliferation seen with knockdown of RET, but not NRAS (Fig. 3B). In contrast, PR1 cell proliferation is more sensitive to silencing of NRAS than RET (Fig. 3D), consistent with a cell proliferation phenotype that is NRAS-dependent. Knockdown of RET and NRAS in combination did not further decrease cell proliferation above single knockdown of the dominant oncogene in either parental LC-2/ad or PR1 cells (Fig. 3B–E). Consistent with the acquisition of a RAS-driven phenotype, PR1 cells are more sensitive to the MEK inhibitor trametinib (ref. 35; $IC_{50} = 32$ nmol/L) compared with parental LC-2/ad cells ($IC_{50} = 195$ nmol/L; Fig. 3F).

We attempted to express mutant NRAS p.Q61K via both retroviral transduction and transient transfection in the parental LC-2/ad cells, but LC-2/ad cells did not tolerate expression of this oncogene. However, we were able to confirm that stable expression of NRAS p.Q61K can induce resistance to ponatinib in the CCDC6-RET–expressing TPC1 PTC cell line (Supplementary Fig. S4). Given the observed loss of phospho-RET in both PR1 and PR2 cells, we hypothesized that loss of RET signaling was critical in order for a switch in dependency to occur. Interestingly, resistance to ponatinib was only observed in NRASQ61K-expressing TPC1 cells maintained in 10 nmol/L ponatinib and further, NRAS activation as measured by a NRAS-GTP binding assay was only increased in TPC1-NRASQ61K cells following ponatinib incubation (Supplementary Fig. S4A). TPC1-NRASQ61K cell proliferation was less sensitive to ponatinib compared with empty vector–transduced cells (TPC1-EV) cultured in the same conditions (Supplementary Fig. S4B). Phosphorylation of ERK1/2 remained sensitive to RET inhibition in TPC1-EV cells, but not TPC1-NRASQ61K cells, ruling out the possibility that ponatinib treatment required to induce the switch to NRAS dependence was responsible for the resistance phenotype (Supplementary Fig. S4C).

NRAS p.Q61K persists as the dominant oncogene in the PR1 cell line in the absence of RET inhibition

To test the permanence of the oncogene switch in the PR1 cell line, we removed the cells from ponatinib for six weeks. PR1 cells removed from RET inhibition (PR1-Out of Ponatinib or PR1-OP) remain ponatinib-resistant, with no change in IC_{50} (Supplementary Fig. S5A). Interestingly, PR1-OP cells regained phospho-RET and phospho-SHC1, which were inhibited by ponatinib in a dose-dependent manner (Supplementary Fig. S5B). Ponatinib did not, however, inhibit phospho-AKT or phospho-ERK (Supplementary Fig. S5B), suggesting that while RET is phosphorylated and capable of signaling through SHC1, its ability to regulate downstream signaling is negated in the presence of oncogenic NRAS. siRNA knockdown of NRAS decreased cell proliferation in PR1-OP cells, whereas knockdown of RET had no effect (Supplementary Fig. S5C). This confirms that PR1 cells have undergone a stable switch to NRAS-dependent proliferation that is independent of RET.

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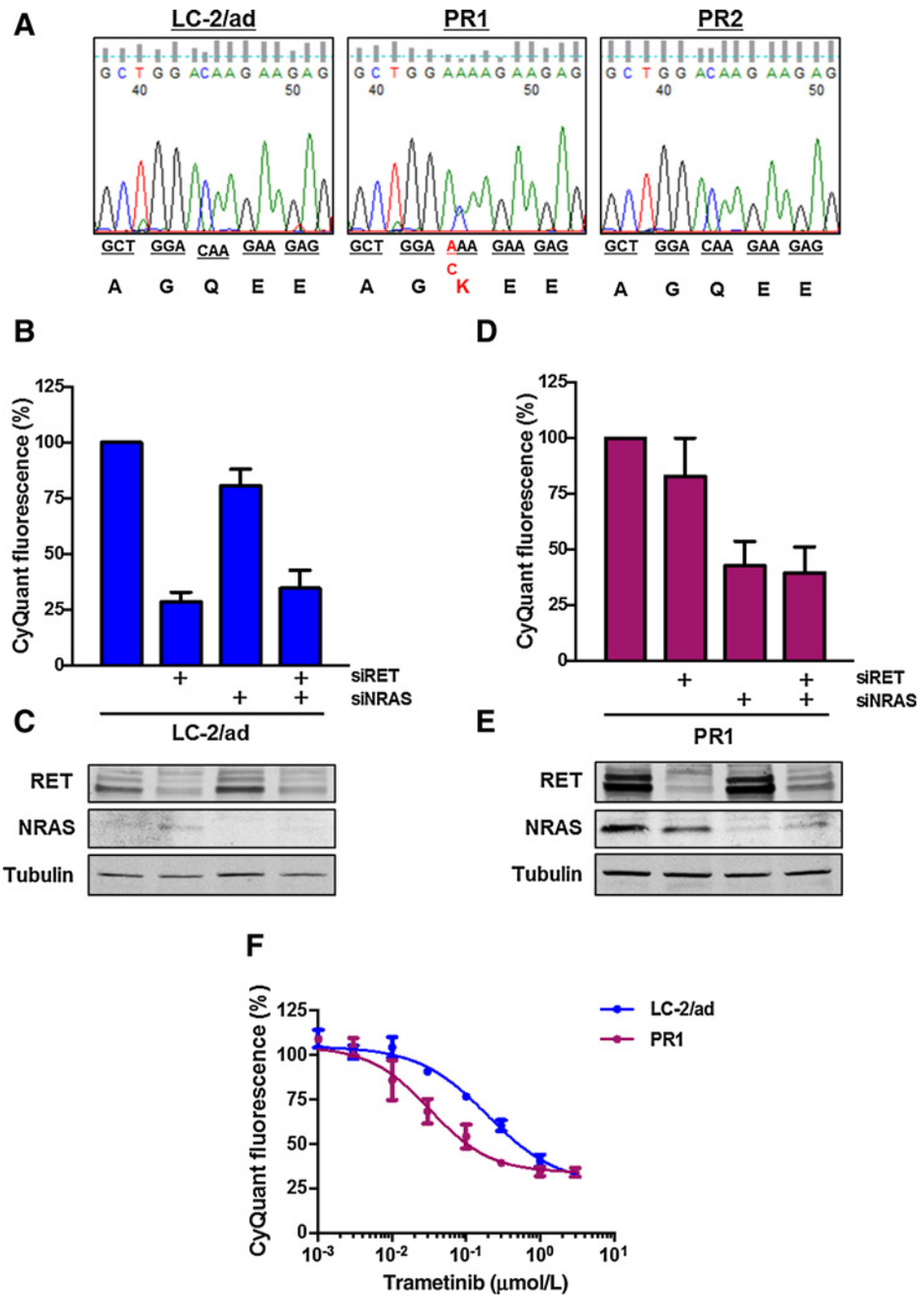

Figure 3.
NRAS Q61K drives resistance in the PR1 cell line. **A,** DNA sequencing of NRAS revealed the PR1 cell line is heterozygous for an A to C single base pair substitution responsible for the NRAS p.Q61K mutation. This mutation was not found in either LC-2/ad cells or PR2 cells. **B,** LC-2/ad cells were transfected with siRNA targeting RET, NRAS, RET and NRAS, or a nontargeted control. Cell viability was measured after 7 days using the CyQuant Direct Cell Proliferation Assay and normalized to the nontargeting control. Error bars, mean ± SEM for three triplicate experiments ($n = 9$). **C,** Western blot analysis of LC-2/ad cells transfected under the same conditions as **B**. Lysates were collected 72 hours after transfection and analyzed via western blotting with the antibodies indicated. **D,** PR1 cells were transfected with siRNA targeting RET, NRAS, RET, and NRAS, or a nontargeted control. Cell viability was measured after 7 days using the CyQuant Direct Cell Proliferation Assay and normalized to the nontargeting control. Error bars, mean ± SEM for three triplicate experiments ($n = 9$). **E,** Western blot analysis of PR1 cells transfected under the same conditions as **D**. Lysates were collected 72 hours after transfection and analyzed via western blotting with the antibodies indicated. **F,** Cell viability of LC-2/ad (blue) and PR1 (pink) cells (which had been removed from ponatinib for 24 hours) treated with increasing doses of trametinib for 72 hours at which point cell viability was measured using the CyQuant Direct Cell Proliferation Assay. Error bars, mean ± SEM for three triplicate experiments ($n = 9$).

Activation of wild-type EGFR and AXL signaling mediates acquired resistance to ponatinib in PR2 cells

In the absence of any known, detectable oncogenic mutations, we hypothesized that resistance in the PR2 cell line may be mediated by increased expression or activation of a wild-type bypass signaling mechanism. Previously, our laboratory has shown that wild-type EGFR signaling can mediate acquired resistance to ROS1 inhibition in the HCC78 NSCLC cell line (18), and we hypothesized that increased expression and activation of a wild-type RTK in the PR2 cell line may be contributing to the resistance phenotype in a similar manner. Candidate RTKs were identified via the RNA sequencing data. Both AXL and EGFR, RTKs previously implicated in acquired resistance in NSCLC (18, 36), were upregulated in the PR2 cells (Supplementary Table S1). Furthermore, mRNA expression of the AXL ligand GAS6 and the HER RTK family ligands HB-EGF and NRG1 were also upregulated (Supplementary Table S2), suggesting that AXL and EGFR may be actively signaling in the PR2 cell line. Given that PR2 cells are resistant to cabozantinib (Supplementary Fig. S1), which also inhibits AXL, we hypothesized that EGFR was the primary driver of resistance in the PR2 cell line. To test this, LC-2/ad and PR2 cells were treated with 10 nmol/L ponatinib, 500 nmol/L afatinib (a second-generation EGFR TKI; ref. 37), or a combination of both ponatinib

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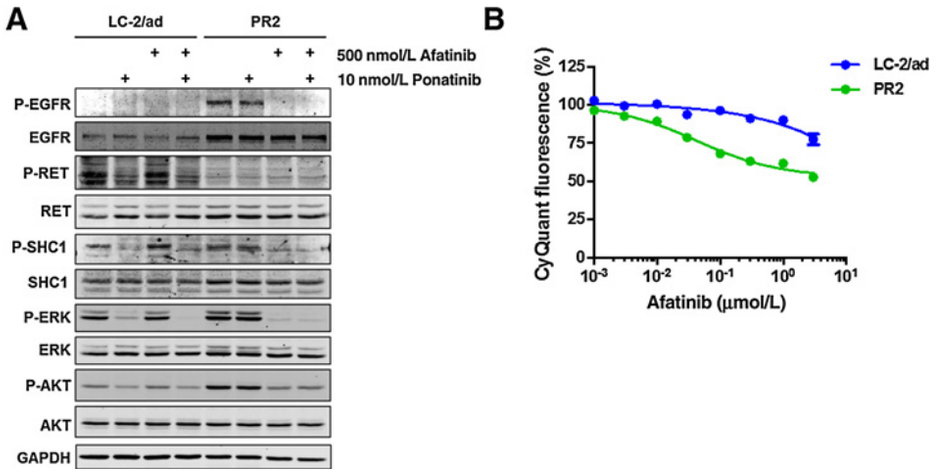

Figure 4.
EGFR regulates MAPK and PI3K signaling and proliferation in the PR2 cell line. **A,** Western blot analysis of LC-2/ad and PR2 cells treated with a DMSO control, 10 nmol/L ponatinib, 500 nmol/L afatinib, or 10 nmol/L ponatinib, and 500 nmol/L ponatinib for 4 hours. Lysates were analyzed via western blotting with the antibodies indicated, with P-TYR used to assess P-RET. **B,** Cell viability of LC-2/ad (blue) and PR2 (green) cells treated with increasing doses of afatinib for 72 hours. Error bars, mean ± SEM for three triplicate experiments ($n = 9$).

and afatinib for 4 hours. Consistent with previous experiments, ponatinib decreased phosphorylation of RET, ERK1/2, and AKT in the parental LC-2/ad cells but not the PR2 cells (Fig. 4A). The opposite was true of afatinib, which robustly decreased phospho-ERK1/2 and phospho-AKT in PR2 cells, but had no effect on phospho-ERK1/2 or phospho-AKT in the LC-2/ad cells (Fig. 4A). We did detect low levels of total EGFR protein in the LC-2/ad cells, although phospho-EGFR was virtually absent, in contrast to the robust phospho-EGFR signal detected in PR2 cells (Fig. 4A). A similar effect was observed in PR2 cells treated with gefitinib, an EGFR TKI with a distinct mechanism of inhibition (Supplementary Fig. S6A), minimizing the possibility that this observation was due to off-target effects of either TKI.

Given that control of oncogenic signaling appears to have switched from RET to EGFR in the PR2 cell line, we posited that PR2 cell proliferation would be more sensitive to EGFR inhibition than that of the parental LC-2/ad cells. As expected, PR2 cell proliferation was more sensitive to afatinib than proliferation of the parental LC-2/ad cells (Fig. 4B). However, addition of 10 nmol/L ponatinib did not further sensitize the PR2 cells to afatinib, nor did afatinib restore PR2 sensitivity to ponatinib (Supplementary Fig. S6bB and S6C), suggesting that EGFR-driven PR2 cell proliferation is independent of RET.

We hypothesized that AXL signaling may contribute to the EGFR-mediated resistance phenotype we observed in the PR2 cells given that AXL has been shown to diversify signaling and mitigate resistance to EGFR TKIs (38). Inhibition of AXL with the TKIs cabozantinib or foretinib (39) enhanced the degree to which afatinib inhibited cell proliferation in the PR2 cells (Fig. 5A). Consistent with earlier data, cabozantinib had no effect on cell proliferation as a single agent (Supplementary Fig. S1B). Furthermore, siRNA knockdown of AXL also specifically enhanced the degree to which cell proliferation was inhibited by afatinib in the PR2 cell line (Fig. 5B) to a similar degree to that observed with cabozantinib, indicating that this effect is AXL-specific. Interestingly, EGF stimulation increased phosphorylation of ERK1/2 and AKT in the PR2 cells, which was blocked by afatinib, while GAS6 stimulation only increased phosphorylation of AKT in a manner that was blocked by the AXL inhibitor foretinib (Fig. 5C and D), suggesting that AXL signals primarily via the prosurvival PI3K/AKT pathway. GAS6 failed to increase phospho-AKT in the parental LC-2/ad cells, perhaps due to the low expression of AXL in these cells and foretinib (which is also a RET inhibitor) inhibited phosphorylation of both AKT and ERK (Fig. 5D), but EGF treatment did increase phospho-ERK1/2 (Fig. 5C), indicating that the RET-driven LC-2/ad cells are primed to utilize EGFR to drive MAPK signaling. These findings were replicated in an additional fusion kinase inhibition resistance model, SLC34A2-ROS1 lung adenocarcinoma cells with acquired resistance to the ROS1 inhibitor TAE-684 (HCC78-TR; ref. 18). Cabozantinib and foretinib dramatically sensitized HCC78-TR cells to afatinib and GAS6 stimulation also preferentially activated AKT signaling (Supplementary Fig. S7), suggesting that AXL contributes to wild-type EGFR-driven cell proliferation, regardless of a cell's original molecular driver.

In the absence of chronic RET inhibition, PR2 cells are codependent upon EGFR and RET

Having demonstrated the permanence of the oncogene switch in the PR1 cell line, we sought to determine whether the same was true of EGFR signaling in the PR2 cells. PR2 cells were removed from ponatinib-containing media for six weeks (PR2-OP), resulting in partial restoration of ponatinib sensitivity (Supplementary Fig. S8A) and a concomitant loss of sensitivity to afatinib (Supplementary Fig. S8B). We hypothesized that in acquiring resistance to RET inhibition the PR2 cells had gained a degree of plasticity with regards to the source of oncogenic signaling; in the presence of a single-agent RET or EGFR inhibitor, the PR2-OP cells can rapidly adapt to rely upon signaling through the remaining uninhibited pathway. Therefore, it is not surprising that maximal inhibition of PR2-OP cell proliferation is observed when both RET and EGFR signaling is inhibited (Supplementary Fig. S8C). This observation is further supported by what was observed at the protein level: maximal inhibition of phospho-ERK and phospho-AKT is only observed in PR2-OP cells treated with combination ponatinib plus afatinib when RET and EGFR are both fully inhibited (Supplementary Fig. S8D).

EGFR and AXL are increased and capable of driving resistance in LC-2/ad cells following RET inhibition

We hypothesized that the EGFR-driven resistance phenotype observed in the PR2 cells may reflect an outgrowth of a population of LC-2/ad cells capable of engaging early adaptive

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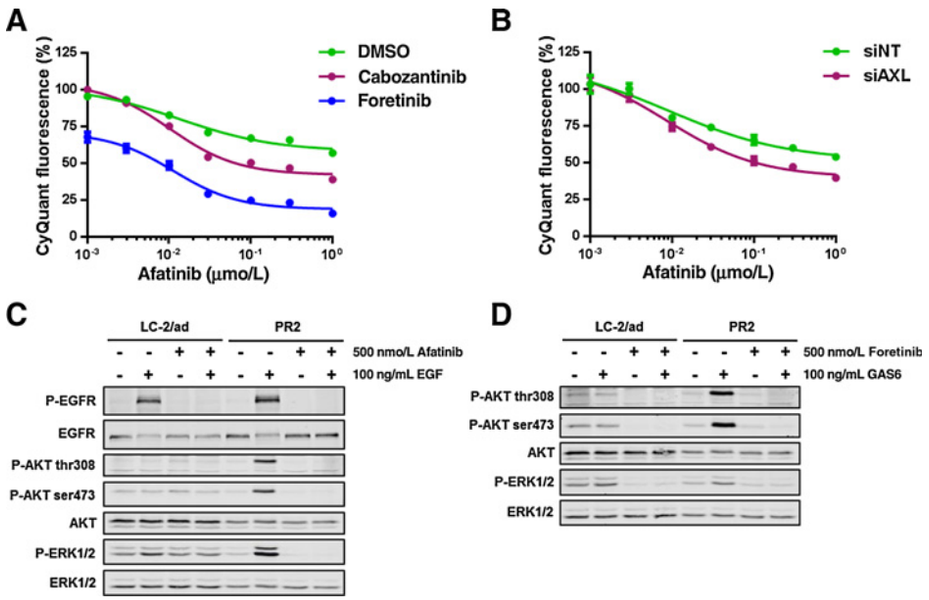

Figure 5.

AXL inhibition enhances sensitivity to EGFR inhibition in the PR2 cell line. **A,** Cell viability of PR2 cells treated with increasing doses of afatinib plus DMSO (green), 500 nmol/L cabozantinib (pink), or 500 nmol/L foretinib (blue) for 72 hours. Error bars, mean ± SEM for three triplicate experiments ($n = 9$). **B,** Cell viability of PR2 cells transfected with siRNA targeting AXL (siAXL) or a nontargeting control (siNT) for 24 hours and subsequently treated with increasing doses afatinib for 72 hours. Error bars, mean ± SEM for three triplicate experiments ($n = 9$). **C,** Western blot analysis of LC-2/ad and PR2 cells serum starved for 2 hours with DMSO (-) or 500 nmol/L afatinib (+) b and then treated with vehicle (-) or 100 ng/ml EGF (+) for 15 minutes. Lysates were analyzed via western blotting with the antibodies indicated. **D,** Western blot analysis of LC-2/ad and PR2 cells serum starved for 2 hours with DMSO (-) or 500 nmol/L foretinib (+) and then treated with vehicle (-) or 100 ng/mL GAS6 (+) for 30 minutes. Lysates were analyzed via western blotting with the antibodies indicated.

resistance mechanisms to increase expression of EGFR and/or AXL in order to promote cell proliferation and survival when challenged with RET inhibition. Interestingly, after only 24 hours of ponatinib treatment, expression of AXL was increased in parental LC-2/ad cells (Fig. 6A). Furthermore, treatment with EGF induced resistance to ponatinib in the LC-2/ad cells (Fig. 6B), demonstrating that EGFR signaling may represent an early adaptive resistance mechanism capable of promoting cell proliferation. Stimulation of AXL with GAS6 was not able to induce resistance to ponatinib, nor did it enhance the resistance phenotype observed with EGF (Fig. 6B). Therefore, AXL signaling is likely not part of the immediate early adaptive response, but can contribute to acquired drug resistance after longer term cellular reprogramming.

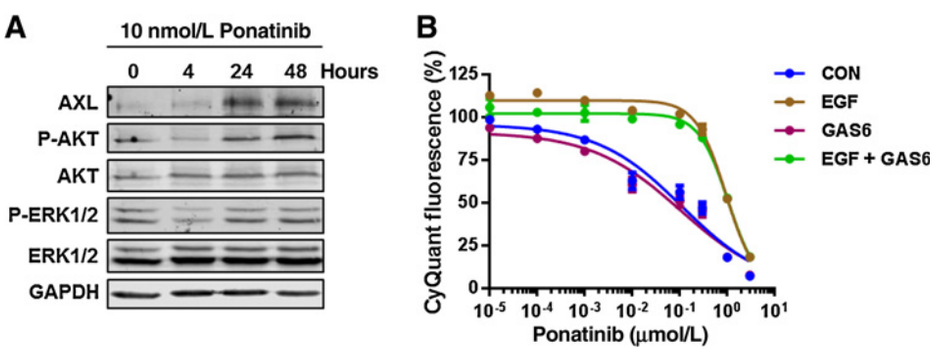

Figure 6.

LC-2/ad cells are primed to utilize AXL and EGFR signaling when RET is inhibited. **A,** LC-2/ad cells were treated with 10 nmol/L ponatinib for the 0, 4, 24, and 48 hours. Lysates were analyzed via Western blotting with the antibodies indicated. **B,** Cell viability of LC-2/ad cells treated with increasing doses of ponatinib plus vehicle control (blue), 100 ng/mL EGF (gold), 100 ng/mL GAS6 (pink), or 100 ng/mL EGF + 100 ng/mL GAS6 (green) for 72 hours. Error bars, mean ± SEM for three triplicate experiments ($n = 9$).

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Discussion

Drug resistance is universal in NSCLC patients treated with targeted therapies; thus, understanding the molecular mechanisms that contribute to resistance, and subsequent TKI failure, will facilitate the development of improved pharmacologic strategies and treatment options for these patients. In particular, RET fusion–positive NSCLC patients do not appear to benefit as much from TKI therapies as patients with other fusion-driven NSCLCs. While the objective response rates (ORR) of patients with advanced ALK or ROS1 fusion–positive NSCLC treated with crizotinib were 65% and 72%, respectively (40). RET fusion–positive patients with advanced disease treated with cabozantinib experienced an ORR of only 28% (41). This underscores the need to better understand the basic biology of RET fusions in NSCLC and the mechanisms of intrinsic and acquired resistance that limit patient responses.

Here, we demonstrate that the multi-kinase inhibitor ponatinib is active in the LC-2/ad cell line, a preclinical patient-derived model of RET-driven NSCLC, and report on the development of two ponatinib-resistant derivatives with distinct mechanisms of resistance. Our decision to specifically study resistance to the RET-TKI ponatinib was guided by the low nanomolar affinity with which ponatinib inhibits the RET kinase domain, its approval for use in patients with CML, as well as the ongoing clinical studies assessing its efficacy in RET-positive NSCLC. Furthermore, a recent study in KIF5B-RET–dependent BaF3 cells demonstrated that the vandetanib-resistant RET G810A mutation exhibited increased sensitivity to ponatinib, concluding that ponatinib is the current "drug of choice" for targeted inhibition of RET in the clinic (42).

The scarcity of RET-driven lung cancer cell model systems represents a significant limitation. Currently, the LC-2/ad cell line is the only publicly available patient-derived RET fusion–driven NSCLC cell line. The TPC1 PTC cell line, also utilized here, represents the only other patient-derived cancer cell line harboring a RET fusion. Ba/F3 and NIH-3T3 cells engineered to express RET fusions provide adequate proof-of-concept model systems to examine the oncogenicity and pharmacologic inhibition of the fusion protein; however, they do not adequately recapitulate the biology of a human lung cancer cell, which is critical for studies on drug resistance. The derivation of additional patient-derived RET fusion models is critical to further our understanding of resistance to RET inhibition and remains an ongoing priority for our laboratory.

Despite this limitation, the findings presented here illuminate our understanding of resistance to RET TKIs in the clinic. We demonstrated that ponatinib is a RET inhibitor that inhibits cell proliferation in the LC-2/ad cell line with similar potency as previously reported in RET-dependent thyroid cancer models (28). We described the development of two ponatinib-resistant LC-2/ad derivatives, PR1 and PR2, which both maintain expression of the CCDC6-RET protein. Interestingly, RET phosphorylation was undetectable in either PR1 or PR2 cell lines, remaining absent even when cells were removed from ponatinib for 24 hours. This finding reinforced the lack of requirement for RET signaling and suggested that chronic RET inhibition can suppress phosphorylation independent of ponatinib's ability to bind to the RET kinase domain, perhaps through increased expression of phosphatases or increased turnover of phosphorylated RET. We are not the first to report this phenomenon: dovitinib-resistant LC-2/ad cells with Src activation were also reported to have lost phosphorylation of the RET fusion protein (43). We identified several tyrosine phosphatases that were upregulated in both the PR1 and PR2 cell lines compared with the LC-2/ad control cells: PTPRG, PTPN21, PTPN14, PTPN9, and CDC14A (Supplementary Table S3). While none of these have been previously shown to regulate RET phosphorylation, we cannot rule out the possibility that they are functionally regulating RET phosphorylation in this context. Notably, expression of the known RET phosphatases, SHP1 and SHP2, was not changed in either the PR1 or PR2 cell line; however, this does not preclude the possibility that SHP1 or SHP2 activity is increased in either resistant LC-2/ad derivative. This finding brings into question our fundamental understanding of fusion kinase signaling. It is assumed that the dimerization domain containing 5′-fusion partner drives constitutive expression, homodimerization, and activation of the fusion protein. However, our data suggest that expression alone is not sufficient for dimerization-mediated activation and that, perhaps, cellular reprogramming that suppresses fusion kinase activity (for example, by the upregulation of phosphatases or increased turnover of the fusion protein) may regulate the oncogenicity of an expressed fusion kinase.

Using next-generation RNA sequencing we identified an oncogenic NRAS p.Q61K mutation which constitutively activates MAPK and PI3K/AKT signaling, freeing the cell from RET-dependent proliferation and survival (44). While we were not able to detect this mutation in parental LC-2/ad or PR2 cells, we did not use single-cell sequencing or other highly sensitive approaches to rule out the existence of a preexisting population of parental LC-2/ad cells harboring this mutation. We confirmed that PR1 cell proliferation is dependent upon NRAS p.Q61K expression by knocking down NRAS, and demonstrated that PR1 cells are more sensitive to the MEK inhibitor trametinib than parental LC-2/ad cells. Furthermore, NRAS p.Q61K remains the dominant oncogene driving PR1 cell proliferation and survival even in the absence of chronic RET inhibition. While we were able to induce ponatinib resistance with exogenous expression of NRAS p.Q61K in TPC1 cells, we were unable to achieve expression of this oncogene in LC-2/ad cells. Oncogenic RAS can promote apoptosis and senescence through a variety of downstream effector mechanisms including the activation of the MAPK pathway and JNK signaling (45, 46), possibly explaining our failure to induce ponatinib-resistance via expression of NRAS p.Q61K in the LC-2/ad cell line.

The emergence of oncogenic NRAS as the mechanism of resistance in the PR1 cell line buttresses a recent study in which NRAS was shown to induce resistance to ROS1 inhibition (47). It is interesting that mutations in NRAS, and not KRAS, are increasingly being identified as mechanisms of resistance to TKI therapy in NSCLC, despite the fact that KRAS is a far more common primary oncogenic driver of NSCLC (48). Assessment of NRAS mutational status in postprogression, drug-resistant patient tumor samples may provide insight into the clinical significance of this finding. The requirement for RET-inhibition in order for NRAS-expressing TPC1 cells to exhibit resistance to ponatinib suggests that oncogenic signaling in these cells is tightly regulated and that RET-driven cells must be forced to undergo an "oncogene switch" to transition to a RAS-driven state. This is not without precedent: pharmacologic inhibition of ROS1 was required for a novel activating KIT mutation to acquire control of oncogenic signaling in NSCLC cell lines HCC78 and CUTO-2 and induce

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resistance to crizotinib (49), and loss of EGFR T790M mutation has been shown to coincide with acquisition of MET amplification as a mechanism of resistance to second-generation EGFR inhibitors (50). Considering the current lack of pharmacologic RAS inhibitors, the emergence of a RAS-dependent resistance mechanism *in vitro* provides a rationale for the evaluation of upfront combination MEK and RET TKI therapy in RET-positive NSCLC patients to prevent RAS-dependent resistance. In fact, in both ALK- and EGFR-driven NSCLC, upfront combination ALK/MEK or EGFR/MEK inhibition has been shown to prevent the emergence of MAPK pathway–addicted resistance mechanisms (51, 52).

Although next-generation sequencing failed to identify any oncogenic mutations in the PR2 cell line, expression of EGFR and AXL as well as their ligands, HBEGF, NRG1, and GAS6, were increased. We demonstrated that PR2 cells are more sensitive to EGFR inhibition compared with LC-2/ad cells; and, while AXL inhibition did not decrease cell proliferation, it did enhance sensitivity to EGFR inhibition. While we did not rule out the possibility that other ERBB isoforms activated by NRG1 were contributing to resistance in the PR2 cells, we did determine that EGF-dependent EGFR signaling regulates MAPK pathway signaling in PR2 cells, suggesting that EGFR signaling is sufficient to promote proliferation in the PR2 cells. Conversely, AXL primarily signaled through the prosurvival PI3K/AKT pathway. Interestingly, stimulation of EGFR, but not AXL, signaling in LC-2/ad cells increased activation of downstream signaling, suggesting that RET-driven cells are primed to utilize EGFR to regulate oncogenic signaling. Here, we have shown that upregulation of both wild-type EGFR and wild-type AXL signaling contribute cooperatively to the resistance phenotype observed in the PR2 cell line. The differential sensitivities to single-agent EGFR or AXL inhibition may be due to the specific oncogenic signaling pathways they regulate. PR2 cell proliferation is sensitive to EGFR inhibition due to the loss of pro-proliferative signaling elicited by decreased MAPK pathway activation. Conversely, AXL appears to signal primarily through PI3K/AKT, which is a key regulator of cell survival and anti-apoptotic functions.

Individually, both EGFR and AXL are well-documented mediators of acquired resistance in NSCLC. Activation of EGFR signaling is a common bypass signaling mechanism responsible for acquired resistance to targeted therapies in fusion-driven NSCLC (19, 53–56) and AXL mediates resistance via canonical upregulation of downstream signaling and by inducing EMT (36, 57–61). Here, we demonstrate that AXL and EGFR independently regulate downstream signaling through PI3K/AKT and MAPK, respectively, and together contribute to the acquired resistance phenotype seen in the PR2 cells.

The activation of wild-type signaling in the PR2 cells provides a glimpse into how RET-dependent cells may be primed for resistance to targeted therapies. Interestingly, PR1 cells also express increased EGFR and AXL (Supplementary Table S1), although to a lesser degree than the PR2 cells. One might therefore speculate that both PR1 and PR2 cells are evolved from an early persister state, with the PR1 cells subsequently acquiring the NRAS p.Q61K mutation and the PR2 resistance phenotype reflecting the outgrowth of dependence upon RTKs that support the maintenance of an early persister population following TKI exposure. Parental LC-2/ad cells express low levels of both EGFR and AXL, although they do not rely on either of these RTKs for regulation of oncogenic signaling. LC-2/ad cells with higher EGFR and/or AXL expression may be overrepresented among early persisters of RET inhibition and further, cells that are able to transition to EGFR- and/or AXL-driven oncogenic signaling are more likely to survive and proliferate amidst RET inhibition. The partial restoration of RET dependence in PR2-OP cells further supports the notion that RET-driven lung cancer cells can flexibly rely on different oncogenic drivers. From a clinical standpoint, this suggests that a brief hiatus from RET TKI therapy in patients with acquired resistance due to the activation of WT RTKs may result in the restoration of drug sensitivity.

Few studies have been performed with the aim of understanding RET biology in the context of NSCLC and it is possible that RET-driven NSCLC cells may be primed for particular modes of resistance to targeted therapies. Here, we demonstrate that LC-2/ad cells are capable of circumventing RET inhibition via reactivation of the RAS/MAPK pathway via the acquisition of oncogenic NRAS p.Q61K or upregulation of signaling through wild-type receptors expressed in ponatinib-naïve LC-2/ad cells. These studies also underlie the need for improved proteomic tools to detect signaling dependence on wild-type RTKs, rather than relying solely on the detection of acquired drug resistance through gene mutations. Assessing posttreatment biopsies in RET fusion–positive NSCLC patients treated with RET TKIs specifically for the emergence of the mechanisms of resistance to RET inhibitor therapy we have identified *in vitro* will aid in validating the clinical relevance of the findings presented here and ultimately lead to the development of improved combination therapy strategies that will improve patient outcomes by preventing or delaying drug resistance.

Disclosure of Potential Conflicts of Interest

RCD reports sponsored research agreements with Ignyta, Mirati Therapeutics, and Loxo Oncology; consulting or honoraria from Pfizer, Ariad, and AstraZeneca; and royalties and/or licensing fees from Abbott Molecular, Chugai, GVKbio, Loxo, Blueprint Medicines, Ariad. ATL reports royalties and/or licensing fees from Abott Molecular, Chugai, and Blueprint Medicines.

Authors' Contributions

Conception and design: S.K. Nelson-Taylor, A.T. Le, R.C. Doebele
Development of methodology: S.K. Nelson-Taylor, A.T. Le, A.-C. Tan, R.C. Doebele
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.K. Nelson-Taylor, A.T. Le, L. Schubert, K.M. Mishall, A. Doak, M. Varella-Garcia
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.K. Nelson-Taylor, A.T. Le, M. Yoo, L. Schubert, K.M. Mishall, M. Varella-Garcia, A.-C. Tan, R.C. Doebele
Writing, review, and/or revision of the manuscript: S.K. Nelson-Taylor, A.T. Le, L. Schubert, M. Varella-Garcia, A.-C. Tan, R.C. Doebele
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.K. Nelson-Taylor, A.T. Le, R.C. Doebele
Study supervision: R.C. Doebele

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Resistance to RET-Inhibition in RET-Rearranged NSCLC Is Mediated By Reactivation of RAS/MAPK Signaling

Sarah K. Nelson-Taylor, Anh T. Le, Minjae Yoo, et al.

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