Mechanisms of Primary Drug Resistance in FGFR1-Amplified Lung Cancer

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Abstract

Purpose: The 8p12-p11 locus is frequently amplified in squamous cell lung cancer (SQLC); the receptor tyrosine kinase fibroblast growth factor receptor 1 (FGFR1) being one of the most prominent targets of this amplification. Thus, small molecules inhibiting FGFRs have been employed to treat FGFR1-amplified SQLC. However, only about 11% of such FGFR1-amplified tumors respond to single-agent FGFR inhibition and several tumors exhibited insufficient tumor shrinkage, compatible with the existence of drug-resistant tumor cells.

Experimental Design: To investigate possible mechanisms of resistance to FGFR inhibition, we studied the lung cancer cell lines DMS114 and H1581. Both cell lines are highly sensitive to three different FGFR inhibitors, but exhibit sustained residual cellular viability under treatment, indicating a subpopulation of existing drug-resistant cells. We isolated these subpopulations by treating the cells with constant high doses of FGFR inhibitors.

Results: The FGFR inhibitor–resistant cells were cross-resistant and characterized by sustained MAPK pathway activation. In drug-resistant H1581 cells, we identified NRAS amplification and DUSP6 deletion, leading to MAPK pathway reactivation. Furthermore, we detected subclonal NRAS amplifications in 3 of 20 (15%) primary human FGFR1-amplified SQLC specimens. In contrast, drug-resistant DMS114 cells exhibited transcriptional upregulation of MET that drove MAPK pathway reactivation. As a consequence, we demonstrate that rational combination therapies re sensitize resistant cells to treatment with FGFR inhibitors.

Conclusions: We provide evidence for the existence of diverse mechanisms of primary drug resistance in FGFR1-amplified lung cancer and provide a rational strategy to improve FGFR inhibitor therapies by combination treatment.

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Introduction

Genetically activated kinases have emerged as drug targets in lung adenocarcinoma with unprecedented therapeutic efficacy (1). Examples include mutant EGFR that can be effectively inhibited by EGFR inhibitors or rearranged ALK and ROS1 that are susceptible to ALK and ROS inhibition, respectively (2–4).

In contrast, such genetically activated therapeutic targets had been lacking in squamous cell lung cancer (SQLC; ref. 5). We and others have discovered FGFR1 amplifications in SQLC that were associated with sensitivity to FGFR inhibition in preclinical models (6–8).

In a phase I clinical trial, 11% of patients with FGFR1-amplified SQLC treated with a highly selective and potent FGFR inhibitor, BGJ398, experienced a partial response (9). Additional clinical trials using the FGFR inhibitors, AZD4547 or JNJ-42756493 to treat solid tumors bearing FGFR1, 2, or 3 alterations yielded similar results. All FGFR inhibitors were associated with moderate toxicity including hyperphosphatemia, decreased appetite, constipation, fatigue, dry mouth, and nail toxicity (1, 10, 11). Of interest, FGFR2-amplified gastric cancers and FGFR2/3-rearranged urethral/bladder cancers appear to be the more sensitive to FGFR inhibition.

While these observations support the notion that FGFR1 amplification associates with FGFR dependency in some cases, they question the overall generalizable conclusion that FGFR1 amplification always causes response to FGFR inhibition. Of note, additional patients exhibited tumor shrinkage, but less than required for a partial response, thus suggesting insufficient tumor cell killing by FGFR inhibition alone (9, 11, 12). We therefore sought to identify mechanisms that underlie primary drug resistance in FGFR1-amplified lung cancer.
Materials and Methods

Cell culture and reagents

H1581 (from p.99 to p.108) and DMS114 (from p.75 to p.83) cell lines were purchased from ATCC and cultured using RPMI, supplemented with 10% FCS. Cell line authentication was done by short tandem repeat analysis (Supplementary Fig. S1). Adherenent cells were routinely passaged by washing with PBS buffer and subsequent incubation in Trypsin/EDTA. Trypsin was inactivated by addition of culture medium and cells were plated or 1/6 diluted. Suspension cell lines were passaged by suitable dilution of the cell suspension. All cells were cultured at 37°C and 5% CO₂.

DNA/RNA extraction and next-generation sequencing

DNA was isolated using the Gentra Puregene DNA extraction kit (Qiagen) following the manufacturers’ protocol. Isolated DNA was hydrated in TE buffer and stored at −80°C. RNA was isolated using the Qiagen RNAeasy Mini Kit according to manufacturers’ protocol and stored at −80°C. One microgram of RNA was transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, #18064) for qPCR use. Whole-exome sequencing was performed with the SureSelectXT All Exon kit (Agilent) following manufacturers’ protocol. Exon-enriched libraries were subjected to paired-end sequencing with a read-length of 2 x 100 bp. Libraries were sequenced to a minimum coverage of 60x. RNA-sequencing (RNA-seq) was performed using cDNA libraries prepared from poly(A)-selected RNA (Illumina TruSeq protocol for mRNA). The libraries were then sequenced with a 2 x 100 bp paired-end protocol to a minimum mean coverage of 30x. Data were processed and analyzed as described previously (16, 17). All data have been deposited at the European Genome-phenome Archive under the accession code EGAS00001002491.

Quantitative real-time PCR

Quantitative real-time PCR was performed using a 7300 Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems) following the manufacturers’ protocol. ΔΔCt values were determined using the 7300 System Software (Applied Biosystems) using GADPH as reference control. Gene expression was calculated by ΔΔCt method.

Immunoprecipitation

One day prior treatment, cells were seeded to 70% confluence on 6-cm dishes. The next day, cells were treated for 24 hours with distinct inhibitor concentrations or DMSO. Cells were washed with cold PBS and lysed in lysis buffer (Cell Signaling Technology) supplemented with protease (Roche) and phosphatase inhibitor (Calbiochem) cocktails. After 20 minutes of incubation on ice, lysates were centrifuged at 18,000 × g for 25 minutes. Protein concentration in supernatants was measured using BCA Protein Assay (Thermo Fisher Scientific). Equivalent amounts of protein (30–60 μg) were denatured at 95°C and separated on 4%–12% SDS-PAGE followed by blotting on nitrocellulose membranes (Amersham Hybond-C Extra). The following antibodies were used for immunoblotting: Anti-RAS (ab108602, Abcam); GST-HRP (Sc-459, Santa Cruz Biotechnology); phospho-AKT (#9271), total AKT (#9272), phospho-ERK (#9106), total ERK (#9102), and phosphor-FRS2 (#3221) all from Cell Signaling Technology.

Viability assays and compound activity prediction

Cell lines were plated as triplicates into sterile 96-well plates at 1,500 cells/well density as described previously (13). After 24 hours of incubation, compounds were added at increasing dosages, ranging from 10 μmol/L to 0.002 μmol/L together with a separate DMSO control. To predict synergy, we added the second inhibitor in constant doses of 20, 50, 100, or 500 nmol/L. After 96-hour incubation at 37°C, relative cell viability was determined by comparing the ATP content of each well, assessed by CellTiter Glo Assay (Promega), to the content of the DMSO control. Finally, half-maximal growth-inhibitory concentrations (GL50) were calculated by the package “ic50” (R programming language; ref. 14). Combination index (CI) was calculated using CI = c1/GL50 I1 + c2/GL50 I2 (15).

Active RAS pull-down assay

Ras pull-down activation assay was conducted according to the protocol of Cytoskeleton (cat #BK0008). Two-hundred micrograms of cell lysate proteins were incubated with 5 μL of Ral-RBD.
beads for 1 hour at 4°C on a rotator. Afterwards, the beads were pelleted by centrifugation at 5000 × g at 4°C for 1 minute. Supernatant was carefully removed and beads were washed with 500 μL of wash buffer (25 mmol/L Tris pH 7.5, 30 mmol/L MgCl2, 40 mmol/L NaCl) followed by centrifugation at 5,000 × g at 4°C for 3 minutes. Supernatant was removed and 4 μL of 5 × Laemmli sample buffer was added to the pellet. After a 10-minute boiling step at 95°C, samples were ready for immunoprecipitation.

Crystal violet staining

A total of 3 × 10^6 cells per well were seeded in 6-well plates and incubated overnight. Cells were exposed to DMSO and indicated inhibitor for 96 hours at 37°C. After treatment, cells were washed twice with 1-mL PBS per well and fixed with 500-μL 4 % paraformaldehyde (PFA) for 15 minutes at room temperature. After two washing steps with PBS, cells were stained with crystal violet (Sigma). Stock crystal violet solution was prepared with 0.5 g in 100 mL distilled water. Stock solution was diluted 1:5 in 10% ethanol. After removing the crystal violet and conducting two washing steps with PBS, plates were ready for scanning (HP Scanjet G4050).

Human phospho-receptor tyrosine kinase array

The human phosphor-receptor tyrosine kinase (pRTK) Array Kit from R&D Systems was used. Cells were seeded into 10-cm dishes with 1 × 10^6 cells per dish. After 24-hour incubation at 37°C, cells were washed twice with 3-mL cold PBS. Lysis buffer (150–200 μL) was added, scraped, and incubated on ice for 30 minutes. Samples were centrifuged at 14,000 rpm for 5 minutes and supernatants were transferred into a new cold 1.5-mL reaction tube. pRTK arrays were blocked for 1 hour at room temperature. Two-hundred micrograms protein per sample was diluted and incubated on a platform shaker together with the array overnight at 4°C following the manufacturers’ protocol. After incubation, the array was washed three times in 20-mL washing buffer. Diluted anti-phospho-tyrosine-HP-RP detection antibody was added to the array and incubated for 2 hours at room temperature on a rocking platform shaker. After three additional washing steps, the Chemi Reagent Mix was pipetted onto the array and placed between transparency film and a light-sensitive film (GE Healthcare) to detect the signals.

NRAS FISH

H1581 and H1581_BGJr cells were trypsinized, washed, fixed overnight in 4% formalin and embedded in paraffin (FFPE). Two-micron thick cuts of the FFPE blocks and additional tumor microarrays with 163 different SQLC tumors plus available corresponding tumor-normal were transferred on SuperFrost plus slides. According to the hybridization protocol, NRAS-FISH was performed using the Agilent Life Sciences SureFISH 1p13.2 NRAS RD probe (G100205SR). Probe signals of 60 different cells were evaluated by two different experimenters and copy number as well as signal ratio was calculated. A sample was referred to be subclonal NRAS amplified if 5% of the cells had a copy number of 6 or more observed by both experimenters.

Results

A subclonal population of FGFR inhibitor–resistant cells

We first determined half-maximal inhibitory concentrations (GI50 values) of the FGFR1-amplified, FGFR inhibitor–sensitive cell lines H1581 and DMS114 against three selective and potent FGFR inhibitors, AZD4542, BGJ398, and JNJ-42756493 (5, 18–20). Confirming earlier observations, both cell lines were highly sensitive to FGFR inhibition with GI50 values in the range of 2 to 20 nmol/L and 50 to 300 nmol/L for the H1581 and DMS114 cell lines, respectively (Fig. 1A). However, in both cell lines, we predicted the presence of a subpopulation of cells with primary resistance to FGFR inhibition because of the apparent inability of all FGFR inhibitors to fully reduce cellular viability. (Fig. 1A). After 96 hours of treatment with 0.1 to 3 μmol/L of the FGFR inhibitors, the cells exhibited only subtle changes in viability causing a plateau in the dose–response curves (Fig. 1A and B). We therefore treated H1581 and DMS114 cells with cycles of 1 μmol/L of each FGFR inhibitor to isolate these resistant cell populations (Fig. 1C; ref. 21). After 8–12 weeks of culture, the cells were entirely resistant to all FGFR inhibitors tested (Fig. 1D and E). Short tandem repeat analysis confirmed that the resistant cells originate from the parental cell line (Supplementary Fig. S1). As expected, FGFR inhibitor treatment led to decreased ERK phosphorylation in parental cells (2–4, 6–8). Of interest, all resistant cells exhibited sustained ERK phosphorylation under 1 μmol/L of FGFR inhibitor treatment (Fig. 1E). Furthermore, resistant DMS114 cells showed slightly increased AKT phosphorylation, whereas H1581 cells exhibited no detectable levels of phosphorylated AKT (Fig. 1E). Mutations in the open reading frame of FGFR1 that might have caused resistance were not found. All FGFR inhibitor–resistant cells displayed sustained ERK activation in the presence of drug thereby indicating a possible relevance of MAPK pathway activation in mediating resistance to FGFR inhibition.

NRAS amplification induces resistance to FGFR inhibition in H1581 cells

As a next step, we performed whole-exome and whole-transcriptome sequencing of the parental and the BGJ398-resistant cells. We analyzed the sequencing data as previously described, matching the sequencing results of the BGJ398-resistant cells to the data of the parental cells (5, 9, 16, 22). In the case of resistant H1581 cells (H1581_BGJr), we detected a focal (6 kbp) amplification with a copy number of 20 on chromosome 1p12 including NRAS and a chromosomal arm level loss on 1p21 including DUSP6 (Fig. 2A; Supplementary Fig. S2). The amplification on chromosome 1q12 led to 19-fold transcriptional upregulation of NRAS (Fig. 2B; Supplementary Fig. S2A). Furthermore, the arm level loss on chromosome 12p resulted in significant transcriptional downregulation of DUSP6 (Supplementary Fig. S2B), a negative regulator of the MAPK signaling pathway (6–8, 23, 24). We therefore hypothesized that NRAS amplification caused resistance to FGFR inhibition through MAPK pathway activation, possibly in concert with loss of DUSP6. In support of this hypothesis, we observed that H1581_BGJr cells displayed strong enrichment of activated GTP-bound RAS (Fig. 2C). We next treated H1581 and H1581_BGJr cells with the FGFR inhibitor, BGJ398, or the MEK inhibitor, trametinib, or with a combination of both (Fig. 2D). As expected, H1581 cells were highly sensitive to inhibition with 50 nmol/L BGJ398 while the H1581_BGJr cells remained unaffected by such treatment (Fig. 2D). The viability of H1581 and H1581_BGJr cells was largely unaffected by treatment with 50 nmol/L of trametinib alone. In contrast, the combination of both inhibitors led
to a significant reduction of cell growth and viability in both cell lines, as assessed in viability as well as in clonogenic assays (Fig. 2D and E). However, while H1581 cells exhibited only a moderate benefit of the combination treatment ($P = 0.004$), the H1581_BGj cells were highly sensitive ($P = 2 \times 10^{-11}$).

We stably transduced H1581 cells with wild-type NRAS to test the hypothesis that NRAS overexpression caused MAPK activation and drug resistance. Empty vector and KRAS G12V constructs served as controls (Fig. 2F; refs. 9, 25). As predicted, RAS-transduced H1581 cells demonstrated a severalfold increased GI50 value under treatment with FGFR inhibitors, compared with the empty vector control (Fig. 2G). Furthermore, combined treatment of H1581 cells ectopically expressing NRAS or KRAS G12V with BGJ398 and 20 nmol/L of trametinib led to downregulation of the MAPK signaling pathway and cytotoxicity (Fig. 2F–H).

To obtain an estimate of the abundance of the drug-resistant cell population in the original parental cell line, we performed a limited dilution assay and identified three different populations of cells (Fig. 2I). As expected, the majority of H1581 colony populations (82%) were sensitive to the inhibitor. However, 16% of the colonies exhibited a moderate increase of viability under 500 nmol/L FGFR inhibitor treatment and might be "persistent" cells (i.e., cells that survive drug treatment, but do not proliferate in the presence of drug). Of interest, nearly 2% of the colonies kept proliferating in the presence of drug (25 of 1621 colonies). These colonies reached an average of 4-fold viability increase during treatment. Thus, FGFR1-amplified H1581 cells harbor subpopulations of cells with primary resistance to FGFR inhibition.

In summary, treatment of H1581 cells with FGFR inhibitors triggers the outgrowth of a preexisting drug-resistant subpopulation of cells. The resistant subpopulation demonstrated high-level amplification of NRAS, RAS, and MAPK activation as well as resistance to FGFR inhibition. Of note, resistant cells can be effectively killed by combined FGFR and MEK inhibition.

**MET overexpression and activation induce FGFR inhibitor resistance in DMS114 cells**

We analyzed whole-exome sequencing data of the original DMS114 and the isolated resistant subpopulation (DMS114_BGj), but could not identify a genomic alteration that might explain resistance of DMS114_BGj cells (Supplementary Fig. S3). However, transcriptome sequencing revealed an at least 11-fold transcriptional upregulation of MET (Fig. 3A), which was one of the most upregulated transcripts in DMS114_BGj cells (Supplementary Fig. S4). We also found a strong increase of MET phosphorylation in DMS114_BGj cells suggesting that MET activation might cause resistance to FGFR inhibition, similar to other settings of drug resistance (e.g., resistance to EGFR activation; refs. 1, 10, 11, 26; Fig. 3B). In support of this notion, combined treatment of...

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**Figure 1.** Generation of FGFR inhibitor-resistant cells. A, FGFR dependency evaluation in FGFR1-amplified cell lines H1581 (red) and DMS114 (green) using the FGFR inhibitors AZD4547, BGJ398, and JNJ-42756493 by measuring cellular ATP content after 96 hours. Viability data were individually pooled for the three different FGFR inhibitors. B, Residual cell viability between 0.1 to 3 μmol/L after 96 hours treatment of AZD4547, BGJ398, and JNJ-42756493 was quantified and merged for the cell lines H1581 (red) and DMS114 (green). C, Schematic overview to generate FGFR-resistant cells. Cells were treated with constant dose of 1 μmol/L AZD4547, BGJ398, or JNJ-42756493. After 48–72 hours the inhibitor was removed and fresh medium without inhibitor was added until cells recovered. The procedure was repeated until the resistant cells showed similar growth kinetics under treatment as untreated cells. D, FGFR dependency was evaluated in the parental cell lines H1581 (red), DMS114 (green), and resistant cell lines DMS114_BGj (black), DMS114_JNJr (brown), H1581_BGj (grey), H1581_AZDr (purple), and H1581_JNJr (orange) using the FGFR inhibitors BGJ398 by measuring cellular ATP content after 96 hours. E, Continuous ERK phosphorylation in FGFR inhibitor resistant cells under 24-hour treatment with 1 μmol/L BGJ398 assessed by immunoblotting.
Figure 2.
NRAS amplification as a resistance factor in H1581 cells. A, H1581 and H1581_BGJr whole exome sequencing data were matched and analyzed for copy number changes. Copy number gain (red) and copy number loss (blue) are illustrated below the indicated human chromosomes 1 to 22 (top). The focal amplified region on chromosome 1 and the broad deleted region on chromosome 12 is zoomed in (bottom). Dashed lines indicate the genomic position of NRAS and DUSP6. B, H1581 and H1581_BGJr expression values generated from RNA sequencing data are plotted as FPKM values. NRAS FPKM values are highlighted (red). C, Active RAS pull-down assay from BGJ398 treated and untreated H1581, H1581_BGJr, and NIH3T3 KRAS G12C cells (positive control) followed by immunoprecipitation. Blots were stained with total KRAS antibody and GST-HRP antibody as loading control. D, Crystal violet clonogenic assay of H1581 and H1581_BGJr cells. Cells were plated on 6-well plates and treated 96 hours with DMSO, 50 nmol/L of BGJ398, trametinib, or in combination. E, Quantification of clonogenic assay (crystal violet, black bars) and ATP based viability assay (gray bars) of H1581 and H1581_BGJr cells after 96-hour treatment with DMSO, 50 nmol/L of BGJ398, trametinib, or in combination. P values were calculated using the two-tailed Student's t test and significant results (P < 0.05) are indicated with *, highly significant results (P < 0.0005) are indicated with **. F, FGFR dependency evaluation in parental (H1581, blue), empty vector (H1581 e.V., red), NRAS (H1581 NRAS wt, green), and KRAS (H1581 KRAS G12V, purple) retroviral transduced H1581 cells using the FGFR inhibitors BGJ398 (left) alone or in combination with 20 nmol/L trametinib (right) by measuring cellular ATP content after 96 hours. G, Quantification of G32 values in H1581 e.V. (red), H1581 NRAS wt (green), and H1581 KRAS G12V (purple) cells. Cells were treated with BGJ398 alone or in combination with 20 nmol/L trametinib. Viability was assessed after 96 hours by ATP-based viability assay. H, Immunoprecipitation of H1581, H1581 e.V., H1581 NRAS wt, and H1581 KRAS G12V cells using the FGFR inhibitors BGJ398 (left) alone or in combination with 20 nmol/L trametinib (right) by measuring cellular ATP content after 96 hours. I, Limited dilution assay of H1581 cells. H1581 cells were seeded in 96-well plates at a concentration of 0.75 cells per well and treated with 500 nmol/L of BGJ398 for 72 hours. The ratio between 72-hour treated and 0-hour untreated H1581 single-cell colonies was calculated. Highly significant results (P < 0.0005) are indicated by ** and were calculated by two-tailed Student's t test.
Figure 3.
MET activation as a resistance factor in DMS114 cells. A, DMS114 and DMS114_BGJr expression values generated from RNA sequencing data are plotted as FPKM values. MET FPKM values are highlighted (red). B, Lysates of the DMS114 (top) and DMS114 BGJ398-resistant cells (bottom) were analyzed by human phospho-receptor tyrosine kinase array. The arrow indicates the phosphorylated MET (pMET) signal. C, FGFR dependency evaluation in DMS114 BGJ398 resistant (top) and PC9 cells (control, bottom) using the FGFR inhibitors BGJ398 or the MET inhibitor crizotinib alone or in combination. The combination was screened using increasing concentrations of BGJ398 (blue) or crizotinib (red) combined with constant concentrations of crizotinib (50 nmol/L, yellow, 100 nmol/L black) or BGJ398 (50 nmol/L green, 100 nmol/L purple). D, Crystal violet clonogenic assay of DMS114_BGJr and PC9 cells. Cells were plated on 6-well plates and treated for 96 hours with DMSO, 500 nmol/L of BGJ398, crizotinib, or in combination. E, Quantification of clonogenic assay (crystal violet, black bars) and ATP-based viability assay (gray bars) of DMS114_BGJr and PC9 cells after 96-hour treatment with DMSO, 500 nmol/L of BGJ398, crizotinib, or in combination. P values were calculated using the two-tailed Student’s t test and highly significant results (P < 0.0005) are indicated with **. F, Immunoprecipitation of DMS114_BGJr and PC9 cells. Cells were treated for 24 hours with DMSO, 500 nmol/L of BGJ398, crizotinib, or in combination. G, Calculated combination index (CI) values are plotted as a boxplot. The background (light purple to purple) indicates antagonistic/additivity (CI > 0.9) effects or slight (CI = 0.7–0.9), moderate (CI = 0.5–0.7), and strong synergy (CI < 0.5). In three independent experiments, the cell lines HCC827GR, HCC827, DMS114, DMS114_BGJr, and PC9 cells (annotated top) were screened using the FGFR inhibitors BGJ398, the MET inhibitors crizotinib or EMD1214063 alone or in combination. The combinations were screened using increasing concentrations of the inhibitors BGJ398, crizotinib or EMD1214063 combined with constant concentrations (50 and 100 nmol/L) of crizotinib/EMD1214063 or BGJ398 (annotated bottom). GI50 values for each single experiment were calculated followed by calculation of the combination index (CI = cI1/IC50 I1 + cI2/IC50 I2). P values were calculated using the two-tailed Student’s t test and highly significant results (P < 0.0005) are indicated with ** and not significant results are indicated with n.s. H, CT before (top left, baseline) and after 12 weeks (top right, partial response), 44 weeks (bottom left, partial response), and 76 weeks (bottom right, progressive disease) of BGJ398 therapy. Arrows highlight target lesion for evaluation of tumor response. I, Pathologic examination of a rebiopsy taken after 76 weeks of BGJ398 therapy. IHC HE stain (top) and phosphorylated MET stain (middle) and MET dual-color FISH with indicated copy number (bottom).
DMS114_BGJr cells with BGI398 and the MET inhibitor, crizotinib, led to depletion of pERK and effective cytotoxicity (Fig. 3C–F). We next calculated synergistic interactions using the FGFR inhibitor BGI398 in combination with the MET inhibitors, crizotinib or EMD1214063, a potent and selective c-Met inhibitor (9, 11, 12, 27). To this end, we measured the effects of several different FGFR/MET inhibitor combinations in comparison with the single drug and quantified the combination index (CI) and the isobologram derived from Loewe additivity models (13, 15, 28). As a positive control, we used EGFR-mutant HCC827 cells that are sensitive to EGFR inhibition and a derivative cell line, HCC827GR, which is resistant to erlotinib because of a MET amplification (14, 29). We treated the cells with the EGFR inhibitor, erlotinib, or the MET inhibitor, EMD1214063, alone or with a combination of both. For the HCC827 and HCC827GR cell lines, we calculated average CI = 0.78 and CI = 0.09 (P = 2.195e–06), respectively, confirming strong synergistic effects of the EGF/MET inhibitor combination in HCC827GR cells (Fig. 3G). In the case of DMS114 cells, we calculated an average CI = 1.24 indicating no benefit for a FGFR/MET inhibitor combination. However, we detected robust synergistic FGFR/MET inhibitor interactions in DMS114_BGJr cells (Fig. 3G). For the BGI398/crizotinib combination, we calculated a CI = 0.45 and for the BGI398/EMD1214063 combination a CI = 0.22 (P = 2.3e–04; Fig. 3G). Thus, combined FGFR and MET inhibition was highly effective in DMS114_BGJr cells, supporting a role of MET in causing resistance to FGFR inhibition. As expected, EGFR-mutant PC9 cells were unaffected by the combination treatment (CI > 1).

Thus, the drug-resistant subpopulation of DMS114 cells overexpress and activate MET, thus leading to FGFR inhibitor resistance. Furthermore, the resistant subpopulation can be effectively treated by combined FGFR and MET inhibition.

**MET activation in a patient relapsing after initial response to FGFR inhibition**

MET amplification is a known resistance mechanism in EGFR-mutant lung cancer (15, 29, 30) and our preclinical finding of transcriptional MET upregulation in DMS114_BGJr cells raised the possibility that MET may mediate resistance to FGFR inhibition as well. In a phase 1 clinical trial using the FGFR inhibitor, BGI398, a patient was diagnosed with a T4N2M0 squamous cell lung cancer tumor (9, 16, 17). The tumor exhibited an amplification of MET by FISH (copy number, 3.1 and a low-level amplification of MET by FISH (copy number, 3.7). Furthermore, we observed MET activation in approximately 50% of tumor cells as determined by IHC staining of pMET (Fig. 3I). These observations suggest that MET activation may drive tumor progression in FGFR inhibitor sensitive squamous cell lung cancer tumor patients, similar to EGFR-mutant lung cancer. However, we cannot exclude the possibility that MET was activated before the FGFR inhibitor therapy was initiated because of the lack of sufficient tumor specimen.

**Subclonal NRAS amplification in FGFR1-amplified primary squamous cell lung tumors**

We next sought to test whether the results observed in vitro are also of potential relevance clinically. We hypothesized that NRAS amplification might be a subclonal event in H1581 cells, inducing FGFR inhibitor resistance. We therefore labeled the NRAS locus in the H1581 and H1581_BGJr cells by FISH (Fig. 4A). Confirming our sequencing results, NRAS was highly amplified in the H1581_BGJr cells. We detected an NRAS copy number range of 4 to 29 signals with an average copy number of 14 (Fig. 4A). As hypothesized, we detected clear subclonal NRAS amplifications in the parental H1581 cells as well. We observed 2 to 8 NRAS signals in the H1581 population with an average of 3.4 signals per cell (Fig. 4A). However, the ratio of NRAS and centromere signals was 1 suggesting, compatible with a triploid genome.

We next tested whether subclonal NRAS amplification can also occur in primary FGFR1-amplified squamous cell lung cancer to explore whether NRAS amplification might in general be a potential resistance mechanism in this tumor entity. To this end, we performed NRAS FISH on 163 primary tumor specimens (all of squamous histology; refs. 21, 31). Twenty of these tumors were known to be FGFR1 amplified (CN > 4). We scored subclonal NRAS amplification if at least 5% of the cells exhibited six or more NRAS signals. We detected subclonal NRAS amplifications in 3 (15%) FGFR1-amplified squamous cell lung cancer tumors (Fig. 4B). Of note, 30% of the FGFR1-amplified primary tumors exhibited NRAS polysomy, suggesting that ploidy could be a driver of NRAS amplification in primary squamous cell lung cancer tumors with FGFR1 amplification.

In summary, subclonal NRAS-amplified cells exist in the H1581 cell line and in primary FGFR1-amplified squamous cell lung cancers. Thus, NRAS amplification might underlie resistance to FGFR inhibition in FGFR1-amplified lung cancer.

**Discussion**

Early clinical trials testing FGFR inhibitors suggest that a subset of FGFR1-amplified lung tumors depend on FGFR signaling for their survival (9, 11). However, even in the amplified tumors response rates were low and several tumors exhibited shrinkage to a lesser extent than required for a response. Thus, molecular resistance mechanisms may exist before treatment that limits the overall efficacy of FGFR inhibitors in these patients. Here we show that subpopulations of FGFR1-amplified lung cancer cells exhibit primary resistance to FGFR inhibition. We demonstrate that distinct molecular mechanisms can underlie such primary resistance to FGFR inhibition that may cause insufficient tumor shrinkage in patients. Without harboring mutations in the FGFR1 gene, the cells examined in this study exhibited cross-resistance to all FGFR inhibitors tested. These results suggest that increasing FGFR inhibitor potency will not overcome FGFR inhibitor resistance.

Drug-resistant H1581 cells harbor amplified NRAS associated with transcriptional upregulation of NRAS and increased
GTP-bound RAS, thus causing MAPK activation. As a consequence, ectopic NRAS overexpression in H1581 cells induced resistance to FGFR inhibition. Accordingly, combined FGFR and MEK inhibition was still able to effectively kill drug-resistant H1581 cells. In contrast, resistant DMS114 cells exhibited sustained MAPK signaling through MET activation. In these cells, MET was transcriptionally upregulated and activated. In line with this observation, combined FGFR and MET inhibition was highly effective in these cells. Supporting a role for this mechanism in patients, we found amplification of MET in a patient with acquired resistance to the FGFR inhibitor, BGJ398. Furthermore, we observed subclonal copy number gains of NRAS in FGFR1-amplified primary human lung cancers. These results suggest that NRAS amplification might be a general mechanism of resistance to FGFR inhibition. Of note, both mechanisms described here involved sustained MAPK signaling.

Figure 4. Subclonal NRAS amplification in a subset of FGFR1-amplified SQLC tumors. A, Dual-color FISH (NRAS, green; control, red) of H1581 BGJ398-resistant cells (left) and parental H1581 cells (right). Pictures were taken with a 600-fold magnification and zoomed in for cells of interest. B, FISH analysis (NRAS, green; control, red) of three subclonal NRAS amplified (top) and three NRAS nonamplified samples (controls, bottom). Pictures were taken with a 600-fold magnification and zoomed in for cells of interest.
activation under treatment, thus reinforcing the notion that this pathway is critically involved in signaling downstream of activated FGFR1. Furthermore, adding MAPK pathway inhibitors to FGFR inhibitors may be therapeutically beneficial.

Our findings shed light on possible mechanisms of primary resistance in FGFR1-amplified lung cancer. Similar mechanisms may also induce acquired resistance in such tumors and it will be important to analyze tumor specimens after clinical relapse to test this hypothesis.

In summary, comprehensive molecular characterization of tumors and molecularly informed choice of combination drug regimens may help overcoming resistance to FGFR inhibition in FGFR1-amplified lung cancer, thereby improving therapeutic efficacy in this detrimental tumor type.

Disclosure of Potential Conflicts of Interest
F. Malchers is a consultant/advisory board member for NVO Neo Oncology GmbH. S. Michel is a consultant/advisory board member for Boehringer-Ingelheim, Novartis, and Roche. M.L. Sos reports receiving commercial research grants from Novartis. J. Wolf is a consultant/advisory board member for AstraZeneca, Bristol-Myers Squibb, Boehringer-Ingelheim, Clovis, Lilly, MSD, Novartis, Pfizer, and Roche. R.K. Thomas has ownership interests (including patents) at AstraZeneca, Bayer, Novartis, and Roche, is a consultant/advisory board member for AstraZeneca, Bayer, Boehringer-Ingelheim, Clovis, Daiichi-Sankyo, Johnson & Johnson, Lilly, Merck, MSD, New Oncology, Puma, Roche, and Sanofi-Aventis. No potential conflicts of interest were disclosed by the other authors.

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