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Cancer Biology and Translational Studies

Molecular Cancer Therapeutics

Acquired JHDM1D–BRAF Fusion Confers Resistance to FGFR Inhibition in *FGFR2*-Amplified Gastric Cancer

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Abstract

FGFR2 gene is frequently amplified in gastric cancer. Recently, targeting FGFR2 has drawn attention as a form of gastric cancer therapy, and FGFR-selective inhibitors have shown promising efficacy in clinical studies. Because overcoming acquired resistance is a common problem with molecular targeting drugs, we investigated a resistant mechanism of FGFR inhibitors using the gastric cancer cell line SNU-16, which harbors *FGFR2* amplification. We established single-cell clones of FGFR inhibitor–resistant SNU-16 (AZD-R) by continuous exposure to AZD4547, a selective FGFR inhibitor. To screen the genetic alterations acquired in AZD-R, we ran a comparative genomic hybridization assay and found an amplification of Chr7q34 region. The chromosomal breakpoints were located between the 12th and the 13th exon of jumonji C domain containing histone demethylase 1 homolog D (*JHDM1D*) and between the 3rd and the 4th exon of *BRAF*. We sequenced cDNA of the AZD-R clones and found fusion kinase *JHDM1D-BRAF*, which has previously been identified in primary ovarian cancer. Because JHDM1D–BRAF fusion lacks a RAS-binding domain, the dimerization of JHDM1D–BRAF was enhanced. A cell growth inhibition assay using MEK inhibitors and RAF-dimer inhibitors indicated the dependence of AZD-R clones for growth on the MAPK pathway. Our data provide a clinical rationale for using a MEK or RAF dimer inhibitor to treat *FGFR2*-amplified gastric cancer patients who have acquired resistance through the JHDN1D–BRAF fusion. *Mol Cancer Ther; 17(10); 2217–25. ©2018 AACR.*

Introduction

FGFRs form a family of receptor tyrosine kinases that consists of four highly conserved family members (FGFR1-4). Binding of FGF ligands to FGFR triggers activation of FGFR, causing diverse signals to be transmitted downstream, such as in the Ras/MAPK, PI3K/Akt, PLCγ/PKC, and STAT pathways (1–3). Although FGFR signaling is thus essential for biological processes and homeostasis, aberration of FGFR signaling is emerging as a potent oncogenic driver. To date, many kinds of *FGFR* genetic alterations associated with tumor growth and malignancy have been reported (4–6). *FGFR2* amplification is found in 10% of patients with gastric cancer, mainly in the diffuse type (7, 8), and it is mutually exclusive with gene amplifications of erb-b2 receptor tyrosine kinase 2 (*ERBB2*) or the *MET* proto-oncogene, receptor tyrosine kinase (*MET*; ref. 8). The *FGFR2* amplification in gastric cancer is also associated with poor prognosis and lymphatic invasion (8, 9). Preclinical studies demonstrate that a knockdown of FGFR2 protein in *FGFR2*-amplified gastric cancer cell lines leads to cell growth inhibition, and the cell lines are sensitive to FGFR-selective inhibitors not only *in vitro* but also *in vivo* (10–13). These lines of evidence support the development of FGFR inhibitors for gastric cancers harboring *FGFR2* amplification.

Currently, several FGFR-selective inhibitors, such as AZD4547 (11), NVP-BGJ398 (12), and Debio 1347/CH5183284 (13, 14), are being used in clinical trials with patients who have *FGFR* genetic alterations in several tumor types. In one study for gastric cancer, AZD4547 demonstrated promising efficacy in patients harboring *FGFR2* amplification (15), with three out of six *FGFR2*-amplified patients obtaining partial response on AZD4547 monotherapy. Notably, the response was seen only in patients with a high level of *FGFR2* amplification. At present, very few preclinical studies investigate acquired resistance to FGFR inhibitors, and no report about resistance in patients with *FGFR2*-amplified gastric cancer has been published. Recently, however, the first genetic mechanism of acquired resistance to FGFR inhibition in patients was reported in *FGFR2* fusion intrahepatic cholangiocarcinoma (ICC; ref. 16). In patients with FGFR2 fusion–positive ICC in a phase II trial, FGFR inhibitor NVP-BGJ398 displayed promising efficacy, but when several patients became resistant to NVP-BGJ398, a genomic analysis of cell-free circulating tumor DNA revealed that the patients had acquired a mutation in the FGFR2 kinase domain that conferred resistance to FGFR inhibition. Moreover, several preclinical studies suggested multiple resistant mechanisms, such as MET/ERBB receptor kinase activation (17–19), PI3K pathway activation (20, 21), PKC-mediated inhibition of GSK3β (22), epithelial–mesenchymal transition (23), acquired *FGFR2* rearrangement (24), and so on. Therefore, acquired resistance to FGFR inhibition

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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is a growing issue, and understanding its mechanism would provide a novel therapeutic option for overcoming it.

In this study, we generated an FGFR inhibitor-resistant *FGFR2*-amplified gastric cancer cell line and identified a clinically relevant and actionable BRAF fusion kinase. Here, we characterize the fusion kinase and propose a new therapeutic option for FGFR inhibitor–resistant patients harboring the BRAF fusion kinase.

Materials and Methods

Reagents

AZD4547 (11), AZD2171 (25), CH4987655 (26), PD0325901 (27), crizotinib (28), and GDC-0941 (29) were synthesized at Chugai Pharmaceutical Co., Ltd., according to published literature. NVP-BGJ398 (30) was purchased from Active Biochem, PD173074 (31) from Sigma-Aldrich, BGB659 (32) from Tocris Bioscience, and erlotinib, lapatinib, OSI-906 (33), trametinib, LY3009120 (34), AZ628 (35), vemurafenib, PLX4720 (36), and MK-2206 (37) from Selleck. The following antibodies were used: anti–phospho-FGF receptor (Tyr653/654), anti–phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-p44/42 MAPK (Erk1/2), anti–phospho-Akt (Ser473), anti-Akt, anti–phospho-MEK1/2 (Ser217/221), and anti-MEK1/2 were from Cell Signaling Technology; anti-FGFR2 and anti-FLAG were from Sigma-Aldrich; anti-BRAF (F-7) for detection of exon 1 to 3 of BRAF, anti-Myc, and anti-GAPDH (FL-335) were from Santa Cruz Biotechnology; and anti-BRAF (N2C1) for detection of exon 6 to 11 of BRAF was from GeneTex.

Cells and cell culture

SNU-16 and COS-7 cells obtained from ATCC were cultured according to the supplier's instructions. SNU-16 and COS-7 cells were obtained more than one year prior to the experiments and were propagated for less than 6 months after thawing. AZD4547-resistant SNU-16 clones were cultured in RPMI1640 supplemented with 10% FBS and 2 μmol/L AZD4547. Infection of *Mycoplasma* was not observed in cell lines we used by routinely performed PCR-based mycoplasma detection method.

Establishment of AZD4547-resistant SNU-16 clones

Parental SNU-16 cells were cultured with AZD4547. The concentration of AZD4547 was gradually increased to a level at which the resistant cells had growth kinetics similar to untreated parental cells, and was raised from 20 nmol/L to 2 μmol/L over several months. AZD4547-resistant SNU-16 clones were obtained by the limiting dilution method.

Cell viability assay

Cells were cultured at 37°C in 96-well plates with compounds that were diluted 4-fold from 20 μmol/L to 0.31 nmol/L or 2 μmol/L to 0.031 nmol/L (trametinib). After 96 hours of incubation, cell viability was measured with the Cell Counting Kit-8 (Dojindo) or the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega). The antiproliferative activity was calculated by the formula $(1 - T/C) \times 100\%$, where T represents the signal intensity of cells with drugs and C represents that of untreated control cells. The IC_{50} values were calculated with nonlinear regression analysis by using GraphPad.

Western blot analysis

Cells were lysed with Cell Lysis Buffer (Cell Signaling Technology) containing protease inhibitor Complete (Roche) and phosphatase inhibitor PhosSTOP (Roche). Lysates were subjected to SDS-PAGE, followed by semidry transfer to PVDF membrane using Trans-Blot Turbo Transfer system (Bio-Rad Laboratories). Nonspecific binding of proteins to the membrane was blocked by incubation in Blocking One (Nacalai Tesque). Membranes were incubated with antibodies diluted in Can Get Signal (Toyobo). Immunodetection was performed with Chemi-lumi One Super (Nacalai Tesque) and LAS-4000 (Fuji Film).

Comparative genomic hybridization

Sample DNA and control DNA were separately labeled with Cy5-dUTP and Cy3-dUTP, respectively. Then, they were mixed and hybridized to human genome 180K standard microarrays (Agilent Technologies) according to the manufacturer's instructions. The hybridization signals were detected with Agilent DNA Microarray Scanner and visualized with DNA Analytics Workstation (Agilent Technologies), where signal ratios relative to the control DNA were plotted in $\log_2$ scale against the sequence position.

RT-PCR and Sanger sequencing

RT-PCR was performed with the Transcriptor Universal cDNA Master (Roche) and PCR (30 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 68°C) was performed with the Tks Gflex DNA Polymerase (TaKaRa Bio). The primers for *JHDM1D* exon 5 and *BRAF* exon 8 were 5′-AGGCAGACAGCAAAATGACAC-3′ and 5′-TTTATATGCACATTGGGAGCTG-3, those for *JHDM1D* exon 1 and *BRAF* exon 10 were 5′-CCCGTGTACTGTGTGTGCC-3′ and 5′-CTTCCTTTCTCGCTGAGGTC-3, those for *JHDM1D* exon 8/9 and *BRAF* exon 5 were 5′-CAGCTCAGGTGTTATGAGATG-3′ and 5′-AGTTGTGTGTTGTAAGTGGAAC-3′ and those for GAPDH were 5′- ACCAGGGCTGCTTTTAACTC-3′ and 5′- TCAGGTCCAC-CACTGACACG-3′. PCR products were sequenced with the following primers: 5′-CTTTTCAAATTCCCTTTCTTTG-3′ and 5′-AGCGGAAACCCTGGAAAAG-3′.

mRNA knockdown by siRNA

BRAF individual siRNA targeting exon 2/3 (5′-CACCAUCAAU-AUAUCUGGAGGCCUA-3′), *BRAF* individual siRNA targeting exon 8 (5′-CAUCAGCUCCCAAUGUGCAUAUAAA-3′), *JHDM1D* individual siRNA targeting exon 6 (5′-CCCAAGCCAUUUGUU-CAGAAAUAUU-3′), and nontargeting scrambled control siRNA (Stealth RNAi siRNA negative control at medium GC level) were purchased from Thermo Fisher Scientific. Reverse transfections were conducted with Lipofectamine RNAiMAX (Thermo Fisher Scientific) with 50 nmol/L of siRNA. Forty-eight hours after the transfection, cell lysates were collected.

Immunoprecipitation assay

cDNAs encoding *BRAF* WT, *BRAF* V600E mutant, or *JHDM1D-BRAF* fusion were inserted into the pCXND3 vector (Kaketsuken) and used to transfect COS-7 cells. COS-7 cells were transfected with the FLAG-tagged and Myc-tagged expression construct alone or in combination, using the lipofectamine 2000 reagent (Thermo Fisher Scientific). At 24 hours posttransfection, cells were lysed in Cell Lysis Buffer (Cell Signaling Technology) and immunoprecipitation was performed with anti-FLAG M2 Affinity Gel (Sigma-Aldrich). Precipitates were washed three times with Cell Lysis Buffer and eluted at 95°C for 5 minutes with Reducing Reagent for SDS-PAGE (Thermo Fisher Scientific).

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Spheroid assay

Cells were cultured at 37°C in PrimeSurface96U (Sumitomo Bakelite) for three-dimensional culture with compounds that were diluted 4-fold from 20 μmol/L to 0.31 nmol/L or 2 μmol/L to 0.031 nmol/L (trametinib). After 14 days of incubation, spheroids were scanned and their areas were quantified with Cell^3 iMager CC-5000 (Screen).

Results

Establishment of an FGFR inhibitor–resistant strain of gastric cancer cell line SNU-16 harboring *FGFR2* amplification

To understand the mechanisms of resistance to FGFR inhibition in gastric cancer harboring *FGFR2* amplification, we generated a model that was resistant to the FGFR-selective inhibitor AZD4547 using the *FGFR2*-amplified SNU-16 gastric cancer cell line. AZD4547-resistant SNU-16 (AZD-R) cells were selected by culturing in gradually increasing concentrations of AZD4547 until cells were able to grow at 2 μmol/L. Then, four single-cell clones were isolated (Fig. 1A). We evaluated the resistance of AZD-R cells to AZD4547 in an antiproliferative assay. The IC_{50} value for cell viability of each clone was about 1,000-fold higher than that of parental cells (Fig. 1B; Supplementary Table S1A). In addition, AZD-R cells showed cross-resistance to other FGFR inhibitors, NVP-BGJ398, PD173074 (38), and AZD2171 (Fig. 1C; Supplementary Table S1A; ref. 39). Consistently, 2 μmol/L AZD4547 did not suppress ERK phosphorylation and AKT phosphorylation in AZD-R clones (Fig. 1D). Interestingly, regardless of AZD4547 exposure, FGFR2 phosphorylation in AZD-R cells disappeared and was accompanied by a substantial

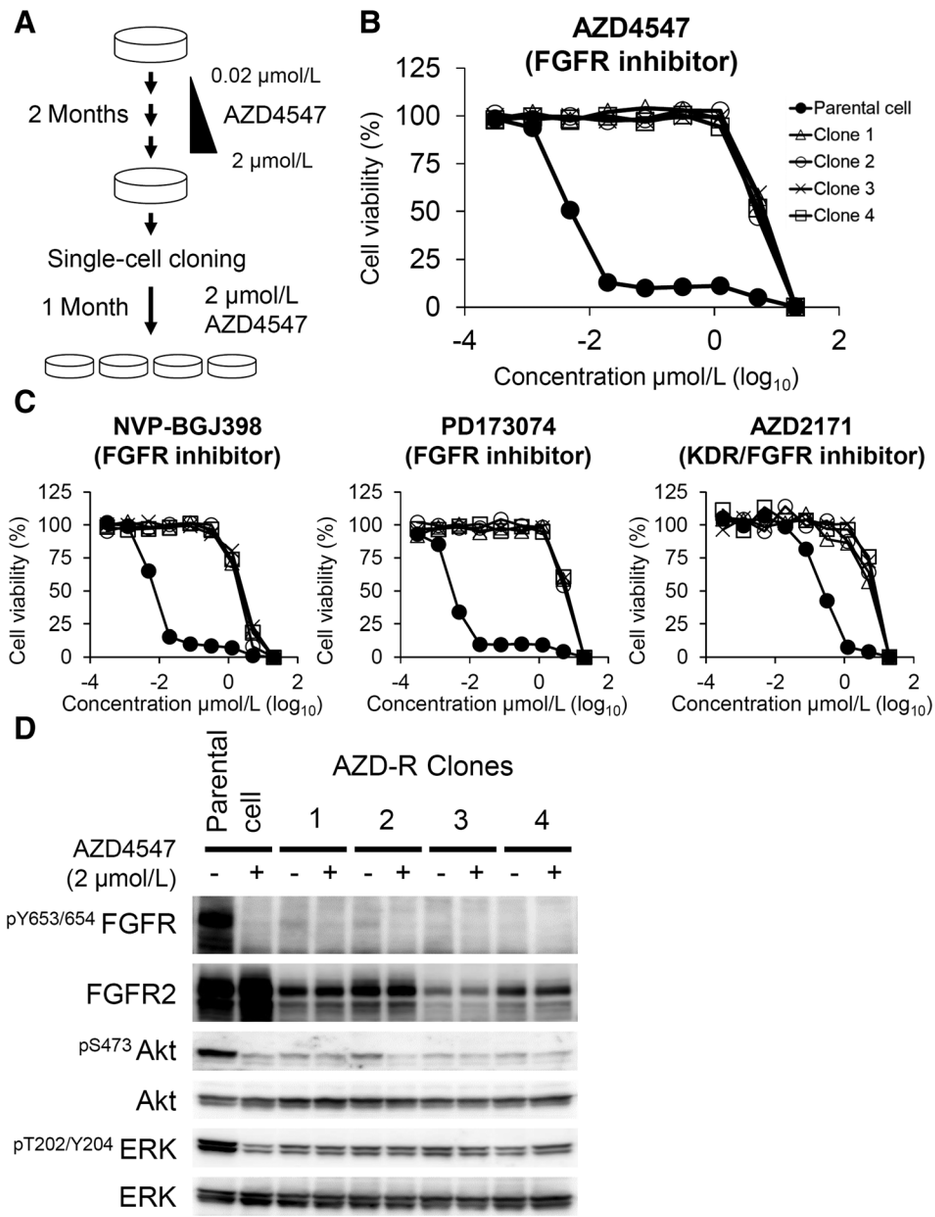

Figure 1.

Establishment of FGFR inhibitor–resistant gastric cancer cell line SNU-16 harboring *FGFR2* amplification. **A,** Schema of process to establish AZD4547-resistant SNU-16 cells. **B,** Cell growth inhibition by AZD4547 in SNU-16 parental cell and four AZD-R clones. Cells were incubated with AZD4547 for 4 days, and cell viability was measured. **C,** Cell growth inhibition by FGFR inhibitors NVP-BGJ398, PD173074, and AZD2171 in SNU-16 parental cell and four AZD-R clones. Cells were incubated with compound for 4 days, and cell viability was measured. **D,** Phosphorylation in SNU-16 parental cells and four AZD-R clones. After 4-hour incubation of 2 μmol/L AZD4547, SNU-16 parental cell and four AZD-R clones were lysed and analyzed by Western blot analysis.

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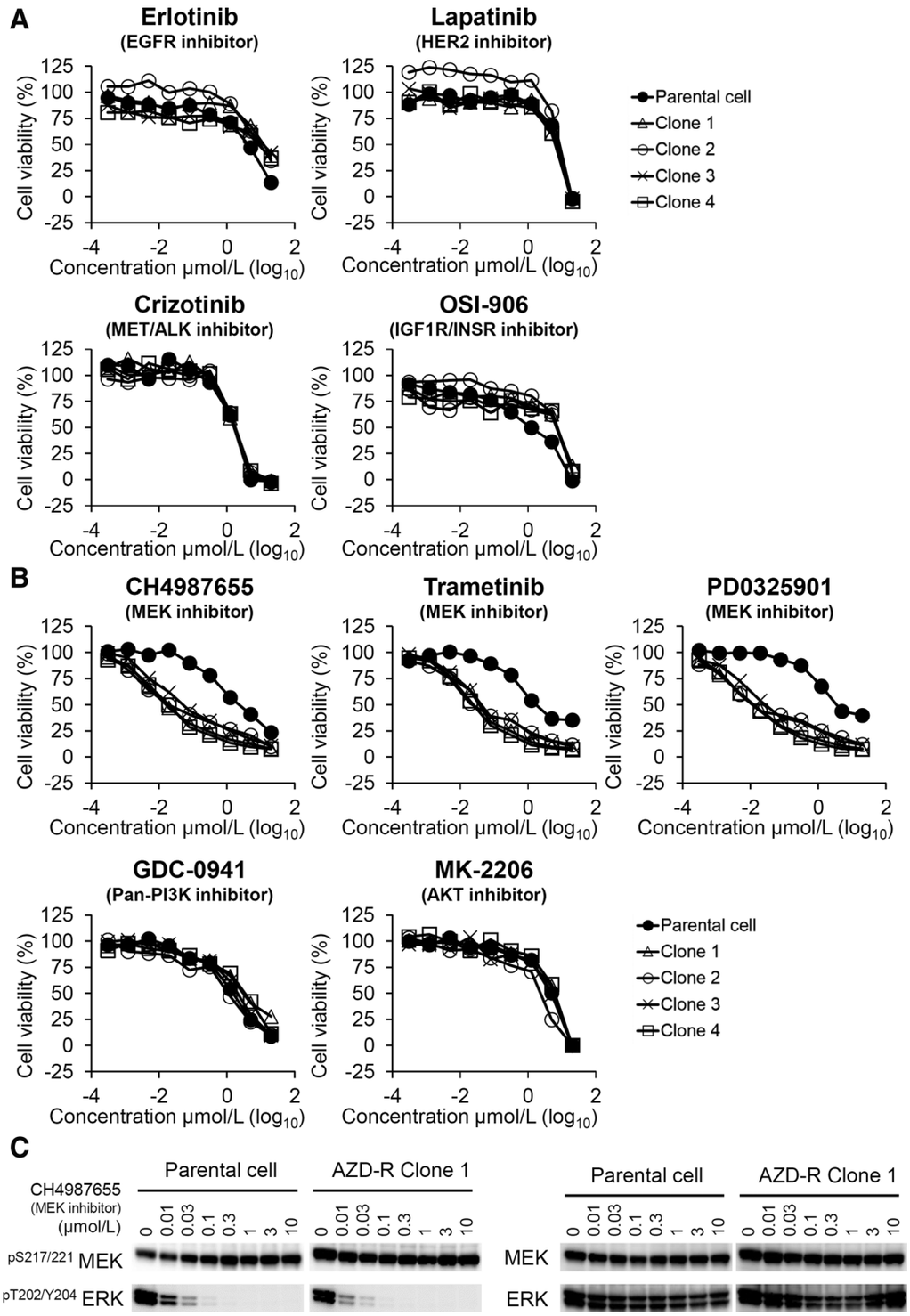

Figure 2.

Sensitivity of SNU-16 parental cell and AZD-R clones to an array of compounds. **A,** Cell growth inhibition by receptor tyrosine kinase inhibitors: erlotinib (an EGFR inhibitor), lapatinib (a HER2 inhibitor), crizotinib (a MET/ALK inhibitor), and OSI906 (an IGF1R/INSR inhibitor) in SNU-16 parental cells and four AZD-R clones. Cells were incubated with each compound for 4 days, and cell viability was measured. **B,** Cell growth inhibition in SNU-16 parental cells and four AZD-R clones by MAPK or PI3K pathway inhibitors: CH4987655, trametinib, PD0325901 (all MEK inhibitors), GDC-0941 (a pan-PI3K inhibitor), and MK-2206 (an AKT inhibitor). Cells were incubated with each compound for 4 days, and cell viability was measured. **C,** Signal engagement by MEK inhibitor. Cells were treated with a serial concentration of CH4987655 and incubated for 2 hours before harvesting. Cells were lysed and analyzed by Western blot analysis.

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decrease in FGFR2 protein expression (Fig. 1D). These data suggested that AZD-R cells lost their dependence on *FGFR2* gene amplification and acquired dependence on an alternative pathway.

Enhanced sensitivity of AZD-R cells to MEK inhibition

To identify which pathway the AZD-R cell growth was dependent upon, we determined sensitivity of these lines to an array of 11 compounds that inhibit diverse signaling pathways other than FGFR. The IC_{50} values for cell viability of receptor tyrosine kinase inhibitors, EGFR inhibitor erlotinib, HER2 inhibitor lapatinib, MET/ALK inhibitor crizotinib, and IGF1R/INSR inhibitor OSI-906 did not change significantly (Fig. 2A; Supplementary Table S1A). Then, we checked the sensitivity to intracellular signal inhibitors. AZD-R cells displayed enhanced sensitivity to three MEK inhibitors (CH4987655, trametinib, and PD0325901) but not to the PI3K inhibitor GDC-0941 or the AKT inhibitor MK-2206 (Fig. 2B; Supplementary Table S1A). Also, chemotherapy agents 5-FU and oxaliplatin showed the same antiproliferative activity against parental cells and AZD-R clones (Supplementary Fig. S1; Supplementary Table S1A). Although the suppression of phospho-ERK by CH4987655 in SNU-16 parental cells was accompanied with upregulation of phospho-MEK, in AZD-R clone 1 cells the compound could suppress phospho-ERK without any upregulation of phospho-MEK (Fig. 2C). In addition, CH4987655 did not reactivate phospho-FGFR in AZD-R cells (Supplementary Fig. S2). A MEK inhibitor induces MEK phosphorylation in cancers with *RAS* mutation or activated receptor tyrosine kinase, but not in BRAF-activated cancers, such as *BRAF* V600E cancer (40). Therefore, we hypothesized that AZD-R cells acquired a dependency on the MAPK pathway by activating RAF and lost FGFR dependency.

Identification of a clinically relevant and actionable BRAF fusion kinase, JHDM1D–BRAF, in AZD-R cells

To identify the genetic alterations that had been acquired in AZD-R clones, we screened point mutations in major oncogenes with qBiomarker Somatic Mutation PCR Array (Qiagen) but did not find any acquired mutation in the assay, including *BRAF* V600E (Supplementary Fig. S3). Then, we performed a comparative genomic hybridization (CGH) assay to identify copy number changes in AZD-R cells. CGH data revealed that the Chr7q34 region was dramatically amplified in the genome of AZD-R clones. The CGH data of AZD-R Clones are shown (Fig. 3A; Supplementary Fig. S4). In parental cell, the focal amplification in the region was not observed in in-house analysis and Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle). The amplified Chr7q34 region contained 10 genes, and the chromosomal breakpoints were located between the 12th and the 13th exon of jumonji C domain containing histone demethylase 1 homolog D (*JHDM1D*) and between the third and the fourth exon of *BRAF*. (Fig. 3B). Because the copy numbers of *BRAF* and *JHDM1D* in AZD-R cells were similar (Supplementary Table S1B), we hypothesized that AZD-R cells possessed *JHDM1D–BRAF* fusion gene. The putative structure of JHDM1D–BRAF fusion is illustrated (Fig. 4A). The JHDM1D–BRAF fusion protein was seen to possess a kinase domain but to lack part of the RAS-binding domain (RBD). We performed RT-PCR with mRNA of AZD-R cells and amplified PCR products with primers that are located on exon 5 of *JHDM1D* and exon 8 of *BRAF*, on exon 1 of *JHDM1D* and exon 10 of *BRAF*, or on exon 8/9 of *JHDM1D* and

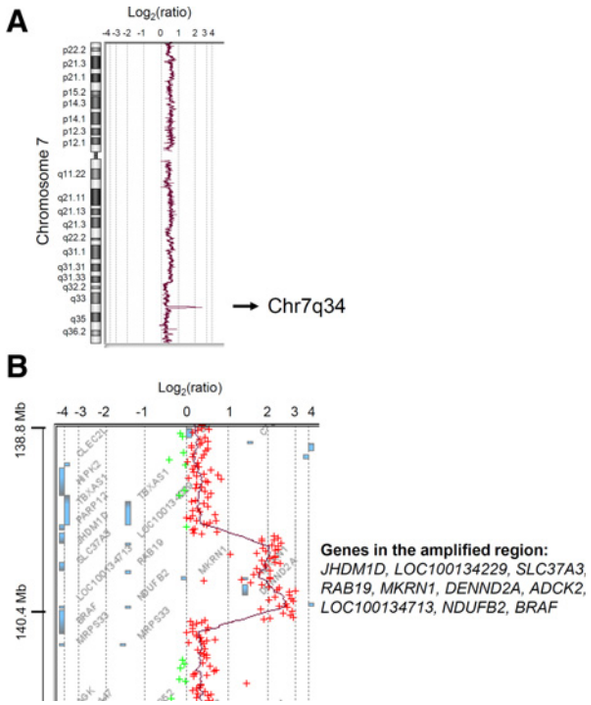

Figure 3.

Comparative genomic hybridization assay of SNU-16 parental cell and AZD-R clones. **A,** Vertical array CGH profile of chromosome 7 of AZD-R clone 1. Normalized $\log_2$ signal ratios were plotted using DNA Analytics Workstation. **B,** Magnified view of the vertical array CGH profile around Chr7q34 region. Ten genes are included in the amplified area: *JHDM1D, LOC100134229, SLC37A3, RAB19, MKRN1, DENND2A, ADCK2, LOC100134713, NDUFB2*, and *BRAF*.

exon 5 of *BRAF*. PCR products were consistent in size with those of *JHDM1D-BRAF* cDNA (Fig. 4B), and amplified fragments were sequenced by Sanger sequencing. The sequence waveform of AZD-R clone 1 is shown as a representative (Fig. 4C), and the other three clones had the same sequence (Supplementary Fig. S5). Then, we examined JHDM1D–BRAF protein expression in AZD-R cells. Western blot analysis using an antibody that recognizes a region within exon 6 and exon 11 of BRAF (Antibody C) suggested that AZD-R cells mainly expressed two sizes of BRAF protein, 84 and 129 kDa (Fig. 4D). According to amino acid sequences, 84 kDa was BRAF WT protein and 129 kDa was JHDM1D–BRAF fusion protein. Consistently, an antibody recognizing a region within exon 1 and exon 3 of BRAF detected just WT protein (Fig. 4D). To confirm that these bands were BRAF proteins, we treated parental SNU-16 cells or AZD-R clone 1 cells with nontargeting siRNA, *BRAF* siRNA targeting 5′-region, *BRAF* siRNA targeting 3′-region, and *JHDM1D* siRNA targeting 5′-region and detected BRAF protein expression with Antibody C. As a result, although *BRAF* siRNA targeting 3′-region suppressed both 84 and 129 kDa proteins, *BRAF* siRNA targeting 5′-region suppressed only 84 kDa protein, and *JHDM1D* siRNA targeting 5′-region suppressed only 129 kDa protein (Fig. 4E), which suggests that AZD-R cells expressed BRAF WT protein and JHDM1D–BRAF fusion protein. More importantly, the *JHDM1D–BRAF* fusion has recently been identified in patients with primary ovarian cancer (41) and is therefore a clinically relevant fusion.

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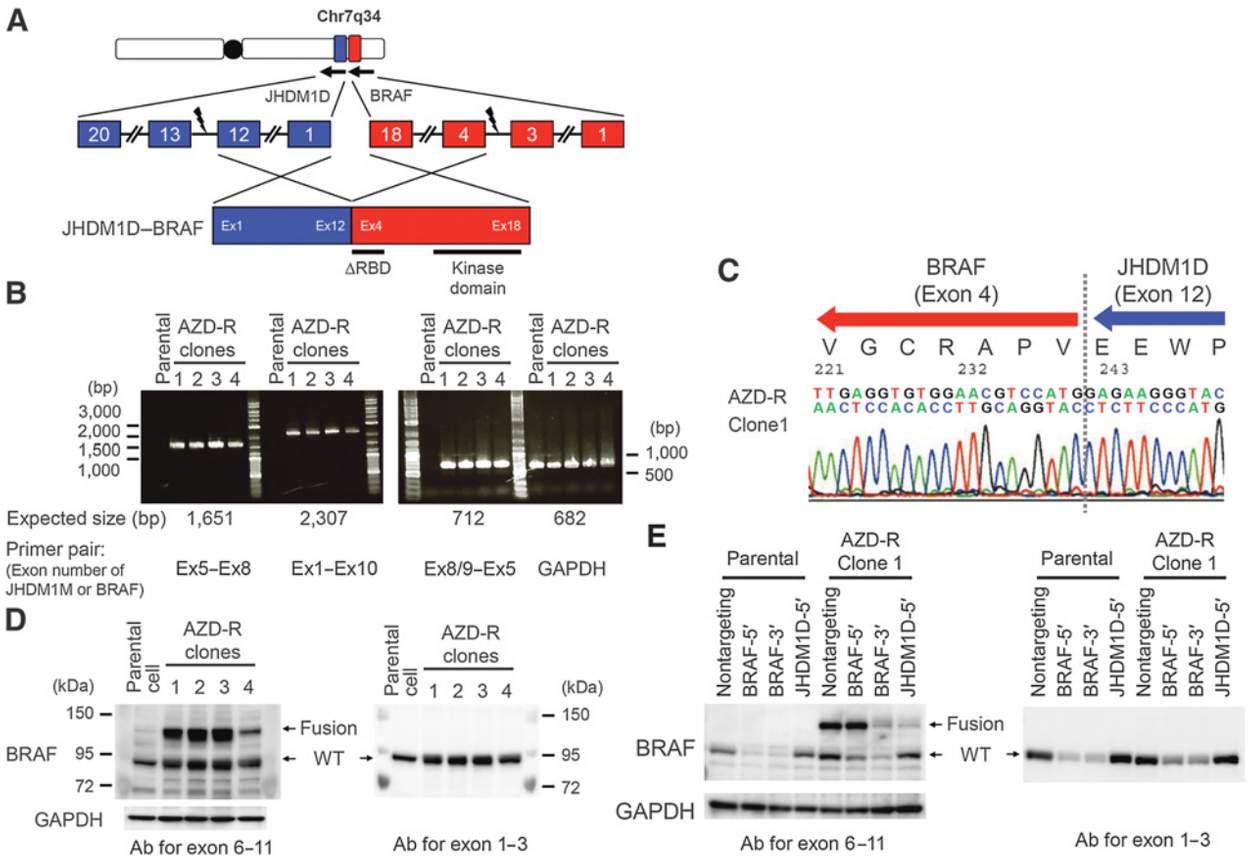

Figure 4.

Identification of JHDM1D–BRAF fusion kinase. **A,** Schematic representation of *JHDM1D-BRAF* fusion gene. **B,** Agarose gel separation of RT-PCR amplicons specific to *JHDM1D-BRAF* fusion. Three pairs of primers were designed. Exon 5 of *JHDM1D* and exon 8 of *BRAF*, exon 1 of *JHDM1D* and exon 10 of *BRAF*, and exon 8/9 of *JHDM1D* and exon 5 of *BRAF*. Expected band sizes were 1,651, 2,307, and 712 bp, respectively. **C,** Amplified fragments were sequenced by Sanger sequencing. Representative sequence waveforms are shown. **D,** BRAF protein detection by Western blot analysis in SNU-16 parental cells and AZD-R clones. Two primary antibodies that recognize exon 6-11 of BRAF or exon 1-3 of BRAF were used. **E,** siRNA effects on BRAF protein expression. Cells were seeded and treated with indicated siRNAs for 48 hours before cells were lysed and analyzed by Western blot analysis. *BRAF*-5′ or *BRAF*-3′ siRNA recognizes *BRAF* exon 2/3 or *BRAF* exon 8, respectively, and *JHDM1D*-5′ siRNA recognizes *JHDM1D* exon 6. Two primary antibodies that recognize exon 6 to 11 of BRAF or exon 1 to 3 of BRAF were used.

Contribution of constitutively dimerized JHDM1D–BRAF to proliferation of AZD-R cells

BRAF fusions have been reported in several tumor types (41). The lack of N-terminal region of RAF in BRAF fusions negatively regulates RAF activity and induces RAS-independent RAF dimerization and kinase activation (42). Because JHDM1D–BRAF lacks part of the RBD in RAF, we evaluated the dimerization activity of JHDM1D–BRAF fusion protein. We expressed flag or myc-tagged BRAF WT, BRAF V600E, or JHDM1D-BRAF in COS-7 cells and immunoprecipitated the flag-tagged protein. We observed more dimer formation in JHDM1D–BRAF than in BRAF WT or BRAF V600E (Fig. 5A). To elucidate whether the JHDM1D–BRAF dimer or the monomer contributes to the proliferation of AZD-R cells, we determined the sensitivity of AZD-R cells to BRAF monomer inhibitors, vemurafenib and PLX4720, and to BRAF dimer inhibitors, LY3009120, AZ628, and BGB659 (32, 43, 44). As a result, AZD-R clone 1 was more sensitive to BRAF dimer inhibitors than BRAF monomer inhibitors (Fig. 5B; Supplementary Table S1A), and other clones showed similar results (Supplementary Fig. S6; Supplementary Table S1A). We also observed similar results in an anchorage-independent assay (Supplementary Fig. S7). Consistent with these data, BRAF dimer inhibitors suppressed phospho-MEK and phospho-ERK in AZD-R cells (Fig. 5C), but BRAF monomer inhibitors did not (Fig. 5D). Taken together, the findings suggest that JHDM1D-BRAF induced RAS-independent dimerization and contributed to the proliferation of AZD-R cells, thus conferring resistance to FGFR inhibitors.

Discussion

In this study, we found that JHDM1D–BRAF played a crucial role in the resistance to FGFR inhibition in an *FGFR2*-amplified gastric cancer model. This is the first report of a BRAF fusion kinase conferring resistance to FGFR inhibition, and the first time a mechanism of downstream signal activation has been clarified. We obtained four independent clones, all of which harbored the same isoform of JHDM1D–BRAF (Fig. 4B). Although it was possible that these clones derived from one

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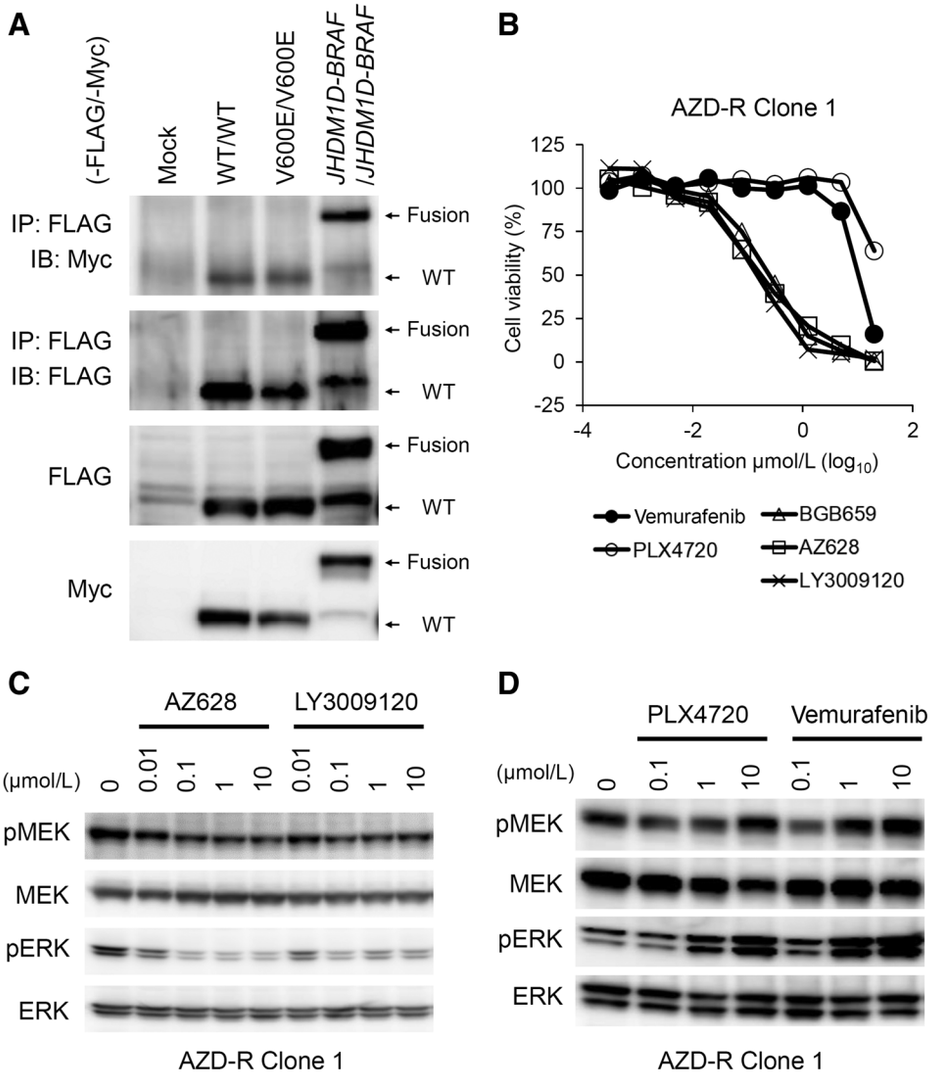

Figure 5.
Contribution of constitutively dimerized JHDM1D–BRAF to proliferation of AZD-R cells. **A,** Comparison of dimerization activity. COS-7 cells were transfected with expression vectors encoding *BRAF* WT, *BRAF* V600E, or *JHDM1D–BRAF* and subsequently lysed. The lysates were immunoprecipitated with anti-FLAG M2 Affinity Gel and detected in Western blots using an anti-FLAG or anti-Myc antibody. Total cell lysates were also analyzed by Western blot analysis. **B,** Cell growth inhibition by RAF dimer inhibitors (LY3009120, AZ628, and BGB659), and by RAF monomer inhibitors (vemurafenib and PLX4720) in AZD-R clone 1. Cells were incubated with each compound for 4 days, and cell viability was measured. **C** and **D,** Signal engagement by RAF inhibitors. AZD-R clone 1 cells were treated with a serial concentration of an RAF dimer inhibitor (AZ628 or LY3009120; **C**) or an RAF monomer inhibitor (PLX4720 or vemurafenib; **D**) and incubated for 4 hours before harvesting. Cells were lysed and analyzed by Western blot analysis.

identical cell, these clones had different FGFR2 and JHDM1D–BRAF expression levels and copy numbers (Fig. 1D; Fig. 4D; Supplementary Table S1B) and must have derived from four different cells. Therefore, the results show that all independent clones inevitably acquired resistance through formation of JHDM1D–BRAF, so this fusion could be one of the major mechanisms of resistance to FGFR inhibition in gastric cancer harboring *FGFR2* amplification.

Although most of the BRAF fusions lose the whole RBD (41), JHDM1D–BRAF lacks a part of the RBD. Previous studies showed that Arg166 and Val168 of BRAF were essential to associate with RAS (45, 46), and these sites are located on BRAF exon 3, which JHDM1D–BRAF does not contain. This suggests that even partial loss of the RBD could induce RAS-independent dimerization and activation of JHDM1D–BRAF. Indeed, we confirmed that the dimerization activity of JHDM1D–BRAF was more potent than that of BRAF wild type (Fig. 5A). To confirm that the JHDM1D–BRAF dimer, not the monomer, was responsible for proliferation of AZD-R cells, we looked at the sensitivity of AZD-R cells to RAF monomer inhibitors and RAF dimer inhibitors. Previous studies showed that vemurafenib and PLX4720, which we used as monomer inhibitors, cannot bind the second site of the BRAF dimer when the first is occupied by drug (32, 43), and thus do not wholly suppress the activity of dimerized BRAF. In the case of JHDM1D–BRAF, the sensitivity of AZD-R clones to monomer inhibitors was quite low (Fig. 5B; Supplementary Fig. S6), and these inhibitors could not suppress the MAPK pathway (Fig. 5D). LY3009120, AZ628, and BGB659, which we used as dimer inhibitors, are able to bind the second site of the RAF dimer even when the first is occupied by drug (32, 43, 44), and in fact these dimer inhibitors were active in the AZD-R clones (Fig. 5B; Supplementary Fig. S6) and inhibited the MAPK pathway (Fig. 5C). Although the results show that RAF dimer inhibitors are much better than monomer inhibitors, the efficacy of RAF dimer inhibitors can be improved still further. Studies in RAS-mutant cancer cells have shown that current RAF dimer inhibitors paradoxically induce RAF dimerization in these cells, which show highly activated RAF (43, 44), so even in the presence of these drugs, RAF signaling would partially be leaked. Therefore, a novel RAF inhibitor that can bind to the second site of the RAF dimer and does not induce dimerization is

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warranted not only for the treatment of *JHDM1D–BRAF* cancer but also for *RAS*-mutant cancer.

Interestingly, FGFR2 phosphorylation in AZD-R cells disappeared and was accompanied by a substantial decrease in FGFR2 protein expression (Fig. 1D). We suggest that a switching addiction between FGFR2 and JHDM1D–BRAF occurred as a mechanism of resistance to FGFR inhibitor. The similar findings in preclinical study have been reported in an alectinib resistance between ALK and IGF1R or HER3 and in a lapatinib resistance between HER2 and FGFR2 (47, 48). Because AZD-R cells no longer depend on FGFR signaling and a MEK inhibitor did not reactivate FGFR (Supplementary Fig. S2), the combination therapy of a FGFR inhibitor and a MAPK inhibitor would not work.

In conclusion, our data indicated that constitutively dimerized JHDM1D–BRAF, which has been also identified in patients with ovarian cancer, provided the growth capability in SNU-16 cells instead of amplified *FGFR2* and thus conferred resistance to FGFR inhibition. RAF dimer inhibitors or MEK inhibitors may be a treatment option for patients with *FGFR2*-amplified gastric cancer who have acquired resistance to FGFR therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Sase, Y. Nakanishi, S. Aida, N. Akiyama
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Sase, Y. Nakanishi, S. Aida, T. Fujii, K. Sakata
Writing, review, and/or revision of the manuscript: H. Sase, Y. Nakanishi, T. Mio
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Nakanishi, S. Aida, K. Horiguchi-Takei, N. Akiyama, T. Fujii, K. Sakata
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Molecular Cancer Therapeutics

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