IRS2 Copy Number Gain, KRAS and BRAF Mutation Status as Predictive Biomarkers for Response to the IGF-1R/IR Inhibitor BMS-754807 in Colorectal Cancer Cell Lines

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Abstract

Insulin-like growth factor receptor 1 (IGF-1R)–targeting therapies are currently at an important crossroad given the low clinical response rates seen in unselected patients. Predictive biomarkers for patient selection are critical for improving clinical benefit. Coupling in vitro sensitivity testing of BMS-754807, a dual IGF-1R/IR inhibitor, with genomic interrogations in 60 human colorectal cancer cell lines, we identified biomarkers correlated with response to BMS-754807. The results showed that cell lines with BRAF<sup>V600E</sup> or KRAS<sup>G13D</sup> mutation were resistant, whereas cell lines with wild-type of both KRAS and BRAF were particularly sensitive to BMS-754807 if they have either higher RNA expression levels of IR-A or lower levels of IGFBP6. In addition, the cell lines with KRAS mutations, those with either insulin receptor substrate 2 (IRS2) copy number gain (CNG) or higher IGF-1R expression levels, were more sensitive to the drug. Furthermore, cell lines with IRS2 CNG had higher levels of ligand-stimulated activation of IGF-1R and AKT, suggesting that these cell lines with IGF-1R signaling pathways more actively coupled to AKT signaling are more responsive to IGF-1R/IR inhibition. IRS2 siRNA knockdown reduced IRS2 protein expression levels and decreased sensitivity to BMS-754807, providing evidence for the functional involvement of IRS2 in mediating the drug response. The prevalence of IRS2 CNG in colorectal cancer tumors as measured by qPCR-CNV is approximately 35%. In summary, we identified IRS2 CNG, IGF-1R, IR-A, and IGFBP6 RNA expression levels, and KRAS and BRAF mutational status as candidate predictive biomarkers for response to BMS-754807. This work proposed clinical development opportunities for BMS-754807 in colorectal cancer with patient selection to improve clinical benefit. Mol Cancer Ther; 14(2); 620–30. ©2014 AACR.

Introduction

Colorectal cancer is the third leading cause of cancer-related mortality in the United States (1). Activation of insulin-like growth factor receptor 1 (IGF-1R) and insulin receptor (IR) signaling contributes to proliferation, survival, angiogenesis, metastasis, and resistance to anticancer therapies in many human malignancies, including colorectal cancer, supporting the IGF axis as an attractive therapeutic target (2–6). Three major approaches have been taken to target this receptor family, including antibodies specifically targeting IGF-1R, small-molecule tyrosine kinase inhibitors (TKI) targeting both IGF-1R and IR kinases, and antibodies targeting IGFI and IGFII ligands, all of these approaches are currently being evaluated in clinical trials (7, 8).

Although several early-phase clinical trials of IGF-1R–specific monoclonal antibodies were promising, phase III trials in unselected patients so far have been negative (9). One possibility could be that inhibition of IGF-1R alone via IGF-1R–specific monoclonal antibodies may not be sufficient because IR also plays a role in cancer, especially in tumors having an IR-A-IGFII autocrine loop (10). Extensive cross-talk between downstream components in the IGF-1R and IR pathways occurs (5, 10), and IR may contribute to resistance to IGF-1R inhibition through a compensatory mechanism allowing for tumor survival (11–13). This suggests that IGF-1R/IR TKIs may offer an advantage in the treatment of cancers such as colorectal cancer that are dependent on both pathways for growth and survival (14). Another possibility could be that IGF-1R–specific targeted agents only work in a subset of patients with cancer, whose tumor growth is driven by IGF-1R pathway activation. It may be critical to select the subset of patients most likely to benefit from this class of drugs by identifying reliable predictive biomarkers. Efforts have been taken to identify potential predictive biomarkers by applying systematic genomic approaches and by examining IGF-1R pathway components, including receptor expression and circulating ligands (15–19). Moving forward, future trials may differ by incorporating predictive biomarkers; this approach remains to be proven clinically (9, 20, 21).

BMS-754807 is a potent and reversible small-molecule TKI (see ref. 22 for chemical structure) with equipotent activity against...
both IGF-1R and IR. The compound has demonstrated growth inhibition both in vitro and in vivo in multiple tumor types, including colorectal cancer, in which a subset of colorectal cancer cell lines is very sensitive to the drug (22). This behavior suggests that colorectal cancer may be a promising indication for IGF-1R/IR TKIs and provides an opportunity for predictive biomarker discovery. In this study, we took a comprehensive genomic approach, including evaluation of gene mutation, DNA copy number, and gene/protein expression, to molecularly characterize a panel of 60 human colorectal cancer cell lines. By linking these data with the response to BMS-754807, we identified candidate predictive biomarkers and proposed hypotheses to be tested in future clinical development of this drug.

Materials and Methods

Cell lines and in vitro cellular proliferation assays

Supplementary Table S1 lists the sources of all 60 colorectal cancer cell lines that were tested by SNP 6.0 array for authentication. Cells were seeded into 96-well tissue culture plates, BMS-754807 (chemical structure was previously published in ref. 22) in different dilutions were added 24 hours after seeding, and plates were further incubated at 37°C for 72 hours and cell proliferation was evaluated by MTS assay as described previously (23).

Mutational analysis

KRAS, BRAF, PIK3CA, IGF-1R, and IR mutational status of the cell lines was determined from the COSMIC database (24), supplemented with custom sequencing. Primers for PCR amplification and sequencing of each exon for the above mentioned genes are described in the Supplementary Information.

Whole-genome copy number variation analysis

The sources of SNP 6.0 array data of 60 colorectal cancer lines are listed in Supplementary Table S1. They were either generated according to the Affymetrix protocols or downloaded from two public resources: the Cancer Cell Line Encyclopedia project (http://www.broadinstitute.org/ccle/home) and the Cancer Cell Line Project (http://www.sanger.ac.uk/genetics/CGP/CopyNumberMapping/Affy_SNP6.shtml). The Cell files processing, normalized raw copy number data segmentation, and copy number gain (CNG; or loss) of a gene were performed as described previously (25).

FISH

Approximately 2 × 10⁷ cells from each cell line were fixed in 5 mL of 10% neutral buffered formalin at room temperature for 24 hours to make paraffin-embedded blocks and 3- to 4-μm thickness sections were cut. IRS2 copy number was tested by FISH assay as developed by LapCorp using Repeat-Free Poseidon probe (Kreatech). Details are described in the Supplementary Information.

IRS2 qPCR-CNV analysis

The primers and probes for IRS2 and RNaseP were purchased from Applied Biosystems (Foster City; cat. nos. 4400291 and 4403326), and copy number was detected using the ABI PRISM 7900HT Sequence Detection System according to manufacture protocols and calculated from quadruplet reactions using ABI CopyCaller software, whereby the cycle threshold (Cₘ) of IRS2 was normalized against the Cₘ of RNaseP reference assay. Details are described in the Supplementary Information.

Western blot analyses and MesoScale Discovery multiplex plate-based assays

Cell lysates and Western blot analyses were carried out as previously described (22). Antibodies for pIGF-IR/pIR, pAKT, p-p44/42 MAPK, and IRS2 were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, β-actin sourced from Millipore. Protein signals from Western blot analyses were visualized using Odyssey Imaging (Li-Cor Biosciences). Measurement of phospho- and total IGF-1R, IR, IRS-1, AKT, and MAPK was also determined by commercially available multiplex plate-based assays (MSD). The assays were performed according to the manufacturer’s protocol. Measurement of IRS2 was determined using customized assays utilizing MesoScale Discovery (MSD) technology.

siRNA

Cell transfections were carried out using siRNA to human IRS2 (Santa Cruz Biotechnology) with DharmaFECT transfection reagents (GE Dharmacon) and Opti-MEM medium (Life Technologies) according to the DharmaFECT General Transfection Protocol. Nontargeting siRNA was used as the negative control. Cells were then incubated for 72 hours. After transfection, the cells were treated with BMS-754807 for 72 hours followed by MTS assay (CellTiter 96 AQueous, Promega) or probed for IRS2 knockdown by Western blot analysis. More details can be found in the Supplementary Information.

Statistical analysis

Categorical data were analyzed by the Fisher exact test. Continuous data were analyzed using the Student t test. Pearson correlation was used for assessing the correlation between DNA CNV, RNA and protein expression levels, and IC₅₀ values. All differences were considered to be statistically significant for P values < 0.05.

Results

Association of gene mutations, DNA copy number alteration with in vitro sensitivity to BMS-754807

Antiproliferative effects of BMS-754807 were tested in a panel of 60 colorectal cancer cell lines using a MTS assay, and a broad range of sensitivity was observed as defined by IC₅₀ values, ranging from 3 to 5500 nmol/L (Supplementary Table S1). Twenty-one cell lines with IC₅₀ ≤ 50 nmol/L were defined as sensitive and all other lines with IC₅₀ > 50 nmol/L were defined as resistant (Fig. 1A). Although the demarcation for sensitivity is arbitrary, PK data from phase I solid tumor clinical trials showed that 50 nmol/L is clinically relevant and achievable (26, 27). The results indicated that approximately 30% of the colorectal cancer lines tested were sensitive to BMS-754807, providing an opportunity for predictive biomarker discovery.

To determine the relationship between KRAS, BRAF, and PIK3CA and the sensitivity of BMS-754807, we characterized mutational status of these genes and assessed the association by the Fisher exact tests (Supplementary Table S2). Figure 1B demonstrated that the association between BMS-754807 sensitivity and BRAF mutational status was significant, all cell lines harboring BRAF militant mutations were resistant; although the association
was not statistically significant for mutational status of KRAS or PIK3CA, when mutations at different amino acid positions were assessed, we found that all 10 cell lines with KRAS<sup>G13D</sup> mutations were resistant to the drug, whereas mutations on codon 12 did not correlate with drug sensitivity (P = 0.27). In addition, nine of ten cell lines with PIK3CA activating mutations within exon 20 were resistant. Furthermore, 10 of the 16 cell lines wild-type (WT) for both KRAS and BRAF were sensitive to BMS-754807 (P = 0.013).

Sequencing the drug target genes IGF-1R and IR in a subset of cell lines did not uncover any mutational hot spots, and the detected mutations in these two genes did not reveal significant association with the drug sensitivity (Supplementary Table S1), which is consistent with a previous report that IGF-1R mutations in colorectal cancer had no apparent association with the sensitivity to IGF-1R antibody, figitumumab (28).

To determine whether copy number variation (CNV) of any gene was associated with in vitro sensitivity to BMS-754807, whole-genome CNV analysis was performed by SNP array. Statistical analyses identified CNV of 197 genes located on chromosome 13 significantly associated with the drug sensitivity (P < 0.005; Supplementary Table S3). Cell lines with chromosome 13 gene CNG were enriched in the sensitive group (Fig. 1C). To test whether the CNG of genes on chromosome 13 observation is an artifact of cell-line models, we evaluated and compared the CNV profiles of colorectal cancer cell lines to those of a published dataset of colorectal cancer primary tumor samples (29), and found that both were very similar (Supplementary Fig. S1), confirming that CNG on segments of chromosome 13 is a frequent event in colorectal cancer tumors.

Among those genes with CNG, IRS2 encodes for insulin receptor substrate 2 (IRS2), a downstream substrate of both IGF-1R and IR signaling pathways. As indicated in Fig. 1C, seven of the 10 cell lines with IRS2 CNG were sensitive, whereas three lines were resistant to BMS-754807 (P = 0.025). To confirm the IRS2 CNG results from SNP analysis, FISH assay was performed on 35 lines,
results of representative cell lines were shown (Fig. 1D). The concordance of IRS2 amplification status was 94% (Supplementary Table S1).

Cell lines with IRS2 CNG are more sensitive to BMS-754807 than IRS2 nonamplified cells in KRAS mutants

Our results showed that cell lines with either KRAS<sup>G13D</sup> or BRAF<sup>V600E</sup> mutations are not sensitive to BMS-754807; however, a subset of KRAS mutations at other positions or in KRAS/BRAF-WT subpopulation were likely to respond to the drug (Fig. 2A). As IRS2 CNG is enriched in the sensitive cell lines (Fig. 1C), we next explored IRS2 CNG in relation to KRAS mutational status and found that IRS2 CNG was more significantly correlated with the drug sensitivity in KRAS-mutated colorectal cancer lines, that is, five of the six lines with IRS2 CNG were sensitive to BMS-754807, and interestingly, none of KRAS<sup>G13D</sup>-mutant lines had IRS2 CNG (Fig. 2B), whereas in KRAS-WT, there was no apparent correlation observed between IRS2 CNG status and drug sensitivity: among four lines with IRS2 CNG, two lines with BRAF-WT were sensitive, and two with BRAF mutation were resistant to BMS-754807 (Fig. 2C).

Differential expression patterns of IGF-1R, IR-A, and IGFBP6, and their relation to BMS-754807 sensitivity in subpopulations defined by KRAS and BRAF mutational status

KRAS and BRAF mutational status divides this panel of colorectal cancer cell lines into three subpopulations: KRAS mutant, BRAF mutant and KRAS/BRAF-WT. Because cell lines with BRAF mutation were not sensitive to BMS-754807, we evaluated the IGF pathway components by comparing RNA expression levels of receptors, ligands, and IGFBPs between sensitive and resistant cell lines in KRAS-mutant and KRAS/BRAF-WT subpopulations.
Figure 3.
The mean (average) RNA expression levels comparison between the sensitive and resistant groups in all cell lines, KRAS mutants, KRAS/BRAF-WT/WT subpopulations for IGF-1R (A), IR (B), IR-A isoform (C), and IGFBP6 (D). The P values are from t tests.

Comparing with resistant lines, the sensitive cell lines had significantly higher levels of IGF-1R RNA expression only in KRAS-mutated subpopulation, not in KRAS/BRAF-WT subpopulations (Fig. 3A). Although no significant difference in the levels of total IR (Fig. 3B) or IR-B isoform (Supplementary Fig. S2A) was apparent, significant higher RNA levels of IR-A isoform were observed in KRAS/BRAF-WT only, not in KRAS mutants or in the whole population (Fig. 3C). No significant differences in the levels of IGF1 or IGF2 ligands or IRS1 were seen between sensitive and resistant lines in either subpopulation, although they had good dynamic range of expression seen between sensitive and resistant lines in either subpopulation (Fig. 3A). Although no significant differences in IGF signaling pathways in response to ligand stimulation, and were inhibited by BMS-754807 treatment in a dose-dependent manner (Fig. 5A). Similar results were observed for LS513 and SW-403 cell lines, which are also KRAS mutant and IRS2 CNG (Supplementary Fig. S5). On the contrary, DLD-1 with normal IRS2 copy number and low to undetectable levels of IRS2 protein expression, showed a limited response to IGFII or insulin stimulation for pIGF-1R and pAKT activation, and was not significantly inhibited by BMS-754807 (Fig. 5B).

Cell lines with IRS2 CNG are more responsive to stimulation by IGF-1R ligands and are more sensitive to BMS-754807 inhibition

Next, we performed cell signaling studies to determine differences in IGF signaling pathways in response to ligand stimulation, in relation to BMS-754807 sensitivity and to IRS2 copy numbers. All 60 colorectal cancer cell lines were either stimulated with IGF1, IGFII, insulin, or nonstimulated. The levels of phospho- and total IGF-1R, IR, IRS1, IRS2, AKT, and MAPK were evaluated by both Western blot and MSD analyses. The results showed that the levels of ligand-stimulated activation of IGF-1R (Fig. 4A) and AKT (Fig. 4B), which are determined as the ratio of the pIGF-1R/IGF-1R or pAKT/AKT value in specific ligand-stimulated cells versus the ratio in the nonstimulated cells, are positively correlated with IRS2 copy number. In addition, significantly lower basal levels of pMAPK/MAPK were seen in cell lines with IRS2 CNG than cell lines with normal copy number (P = 0.002); and similar results were observed in the sensitive cell lines compared with resistant lines (Supplementary Fig. S4), suggesting that cell lines with normal copy number of IRS2 or resistant cell lines had higher activation of the MAPK pathway at basal level.

To further explore the mechanisms of differential response to BMS-754807, ligand-stimulated activation of pIGF-1R–pIR and pAKT was evaluated between KRAS-mutated cell lines with IRS2 CNG and those with normal copy numbers. SK-CO-1 cells with IRS2 CNG had higher expression levels of IRS2 protein; pIGF-1R–pIR and pAKT levels increased in response to individual ligand stimulation, and were inhibited by BMS-754807 treatment (Fig. 6B). These results provided evidence that IRS2 has a functional role in mediating sensitivity to BMS-754807.

Modulation of IRS2 level alters the sensitivity to BMS-754807

To investigate the role of IRS2 in response to BMS-754807, we utilized siRNA studies to knockdown the IRS2 level in three cell lines that were sensitive to BMS-754807 and either KRAS-WT or mutant. As shown in Fig. 6A, IRS2 siRNA significantly decreased the expression level of IRS2 protein compared with nontargeting control siRNA and nontransfected cells. Knockdown of IRS2 in all three cell lines resulted in a shift of increased IC50 values compared with the nontargeted control siRNA, indicating reduction of sensitivity to BMS-754807 (Fig. 6B). These results provided evidence that IRS2 has a functional role in mediating sensitivity to IGF-1R/IR inhibitor BMS-754807.
IRS2 CNG is more prevalent in colorectal cancer than in other tumor types

As IRS2 copy number status is associated with sensitivity of BMS-754807, and modulation of IRS2 expression level altered the response to the drug, IRS2 CNG could be used as a potential predictive biomarker for patient selection. To estimate the size of the targeted population, we next assessed the prevalence of IRS2 amplification in different cancer types. By mining publically available SNP array data on tumors (Supplementary Table S4), the percentage of IRS2 CNG in colorectal cancer ranged from 8% to 26% in a total 648 samples from four datasets, which is higher than in any other tumor types (0%–2.9%). For examples, the prevalence of IRS2 CNG in breast, ovary, lung, and liver cancers is 2.9% (20/699), 2.6% (16/608), 1.8% (16/911), and 1.9% (3/154), respectively. IRS2 CNG was not seen in prostate (0/165), renal cancers (0/593), and acute lymphoblastic leukemia (0/378).

To further stratify the prevalence of IRS2 CNG by KRAS mutational status, we subsequently analyzed 94 colorectal cancer specimens either from primary or metastatic tumors for IRS2 copy number and KRAS mutational status. The results indicated that the prevalence of IRS2 CNG is approximately 35% by qPCR-CN assay, with no significant differences observed between primary (35.7%) and metastatic colorectal cancer tumors (33%) or between KRAS-WT (33.8%) and mutated (38.3%) populations (Table 1).

Discussion

Linking genomic data, including gene expression, DNA copy number, and sequencing data with in vitro sensitivity to anticancer agents on large panel of cancer cell lines provided highly useful, large-scale resources for the generation and testing of hypotheses related to the overall goal of personalizing cancer medicine (30, 31). In this study, we elucidated potential predictive markers of response to the IGF-1R/IR TKI, BMS-754807, by testing drug sensitivity in a panel of 60 colorectal cancer cell lines coupled
with systematic genomic analysis. As illustrated in Fig. 7A, we discovered that (i) in KRAS-mutated cell lines, KRASG13D is not sensitive to BMS-754807, whereas IRS2 CNG and/or higher IGF-1R RNA expression levels are associated with increased drug sensitivity; (ii) in KRAS WT cell lines, BRAF V600E-mutated lines are not sensitive to the drug; and the ones having higher

Figure 5.
Western blot analyses for IRS2, pIGF-1R-pIR, pAKT, and actin in the SK-CO-1 cell line (A; KRASG12V, IRS2 DNA copy number = 3, IC50 = 0.003 μmol/L) and in the DLD-1 cell line (B; KRASG13D, IRS2 DNA copy number = 2.2, IC50 = 0.666 μmol/L). Cells were cultured in medium containing 10% FBS overnight, then untreated or treated with 10 or 100 nmol/L of BMS-754807 for 1 hour, followed by 50 ng/mL IGF, IGFII, or insulin stimulation for 10 minutes.

Figure 6.
Modulation of IRS2 expression levels changes the sensitivity to BMS-754807 in three colorectal cancer cell lines. After siRNA transfection, the cells were exposed to BMS-754807 at different concentrations for 72 hours and the sensitivity profiles were assessed by cell proliferation MTS assay. A, IRS2 siRNA in colorectal cancer cell lines significantly reduces the expression levels of IRS2 as shown by Western blot analysis compared with nontargeting siRNA control and untransfected cells. B, knockdown IRS2 caused a shift of the IC50 curves to the right (data are graphed as mean percent of control with SD), indicating decreased sensitivity to BMS-754807.
IR-A and/or lower IGFBP6 RNA expression levels, are more sensitive to BMS-754807. Utilizing KRAS and BRAF mutational status, IRS2 CNG, IGF-1R, IR-A, and IGFBP6 RNA expression level, we were able to correctly classify the responsiveness to BMS-754807 in 90% (54/60) of colorectal cancer cell lines.

Colorectal cancer is a heterogeneous disease defined by different activating mutations or loss-of-function mutations in KRAS/BRAF/PI3K/PTEN intracellular pathways that impact the efficacy of targeted therapies (32, 33). KRAS has the ability to activate multiple downstream signaling pathways, including PI3K/AKT

![Figure 7](image_url)

**Figure 7.**
A, diagram depicts the predictive classification of responsiveness to BMS-754807 in KRAS mutants and WT subpopulations. The number in each box refers the predictive score. The true sensitivity class is based on IC50 and the predicted class is based on the sum scores of three biomarkers. If a cell line with the sum of scores ≥ 2, it is classified as sensitive; if the sum of score <2, it is classified as resistant.

The average value of expression level for IGF-1R, IR-A, or IGFBP6 across all 60 colorectal cancer cell lines is used to define higher expression levels for making predictions on each of these biomarkers. B, schematic illustration of possible mechanisms for sensitivity and resistance to IGF-1R/IR TKI. C, illustration of how the biomarkers KRAS and BRAF mutations, IRS2 copy number, IGF-1R, IR-A, and IGFBP6 RNA expression levels could be used for personalized treatment of colorectal cancer patients with IGF-1R/IR inhibitors.

### Table 1. The prevalence of IRS2 amplification stratified by KRAS status in colorectal cancer tumor samples

<table>
<thead>
<tr>
<th>Colorectal Cancer Tumor Sample</th>
<th>Total</th>
<th>KRAS-WT</th>
<th>KRAS-Mut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumors</td>
<td>70</td>
<td>54</td>
<td>16</td>
</tr>
<tr>
<td>Metastatic tumors</td>
<td>24</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>68</td>
<td>26</td>
</tr>
</tbody>
</table>

NOTE: Ninety-four formalin-fixed paraffin-embedded (FFPE) colorectal cancer specimens either from primary or metastatic tumors were analyzed for IRS2 copy number by qPCR CNV and KRAS mutational status by Sanger sequencing.
and MEK/MAPK. Both pathways have been implicated as independent drivers of tumorigenesis. Our study demonstrated that all cell lines harboring KRAS\textsubscript{G13D} mutation were resistant to BMS-754807, whereas KRAS mutations at other positions were not significantly correlated with sensitivity to the drug (Fig. 2A). These findings are interesting to note that this observation is opposite for response to EGFR antibody-targeted therapies such as cetuximab. It is generally accepted that the presence of KRAS mutations in metastatic colorectal cancer predicts lack of benefit for treatment with cetuximab (34), but response were seen in patients with colorectal cancer with KRAS\textsubscript{G13D} and KRAS\textsubscript{D204E} mutations (35). KRAS mutations in codon 12 and 13 have functional and molecular differences in the regulation of apoptosis, cell–cell contact inhibition, and predisposition to anchorage-independent growth by the differential regulation of KRAS downstream pathways (36). IGF-1R and EGFR pathways crosstalk and interact to drive tumor growth and survival. Each is activated reciprocally as an escape mechanism when inhibiting one or the other (37). It is unclear why KRAS\textsubscript{G13D} determines response to IGF-1R/IR and EGFR inhibitors differently. We found that KRAS\textsubscript{G13D} cell lines had no IRS2 CNCG (Fig. 2B), significantly lower levels of IRS2 protein, IGF-1R RNA expression, and more activation of MAPK at basal level compared with cell lines with other KRAS mutations (Supplementary Fig. S3). Less activated IGF-1R pathway may be one of reasons why KRAS\textsubscript{G13D} cell lines are less responsive to BMS-754807 than other KRAS mutants. It may be critical to assess the spectrum of activation of the MEK/MAPK signaling pathway. BRAF\textsubscript{V600E} is an activating mutation and results in constitutive activation of the MEK/MAPK signaling pathway. BRAF\textsubscript{V600E} is an activating mutation and results in constitutive activation of the MAPK pathway. In our study, colorectal cancer cell lines with BRAF\textsubscript{V600E} mutations were not sensitive to BMS-754807 (Fig. 2A), and the resistant lines appeared to have higher baseline levels of MAPK activation (Supplementary Fig. S4B), suggesting that MAPK activation is one of the resistance mechanisms to IGF-1R/IR TKIs (17). Cotargeting MEK and IGF-1R/IR in colorectal cancer has been shown to lead to a loss of AKT and ERK activity, marked growth suppression, and robust apoptosis compared with single-agent EGFR, MEK, or IGF-1R inhibitors or combined EGFR and IGF-1R inhibitors in human KRAS-mutant colorectal cancer in vitro and in vivo (38), these data supported clinical testing of combining MEK with IGF-1R/IR inhibitors.

Mutations in the PI3KCA gene occur in 12% to 30% of colorectal cancer (39). Most of them are located in hot spots in the helical (exon 9) or kinase domains (exon 20) leading to constitutive activation of the PI3K/AKT signaling pathway (40). PI3K is also an important mediator in the IGF-1R/IR pathway. Our results (Fig. 1B and C) showed that nine of ten cell lines with PI3KCA mutation in exon 20 were resistant to BMS-754807, and none of them with IRS2 CNCG; cell lines with PI3KCA mutation in exon 9 and with IRS2 CNCG were sensitive, whereas the lines without IRS2 CNCG were resistant to the drug. It may be important to assess PI3KCA mutations in clinical trials of IGF-1R/IR inhibitors in colorectal cancer and determine their association to clinical benefit. PI3K-initiated signaling is inhibited by PTEN, whose activity can be lost through various mechanisms including mutations and deletion. Only two of 60 cell lines had PTEN mutations; therefore, we were not able to assess its association with sensitivity to BMS-754807.

IRS2 CNCG and expression is associated with sensitivity to BMS-754807 in our study. We did not find the same association for other IRS family members such as IRS1. IRS2 as a candidate predictive biomarker is biologically plausible as it is a direct target of IGF-1R/IR and plays a key role in the transduction of IGF-1R/IR signaling to RAS/ERK and PI3K/AKT pathways, leading to cell proliferation and survival (2, 3). Interestingly, the association between IRS2 CNCG and sensitivity to BMS-754807 is more significant in KRAS-mutant (Fig. 2B) than in WT cell lines (Fig. 2C). This may be due to the fact that KRAS-mutated colorectal cancer tumors have increased IGF-1R/IR pathway activation and are possibly more dependent on IGF-1R pathways for growth. This hypothesis is supported by studies showing that in KRAS-mutant cell lines, PI3K pathway activity is dominant controlled by IGF-1R activity through interaction of PI3K and IRS1/IRS2 in colorectal cancer and non–small cell lung cancer (38, 41). Indeed, KRAS-mutated cell lines with higher IRS2 copy number tended to respond better to ligand-stimulated activation of IGF-1R and AKT, and were more responsive to BMS-754807 inhibition (Fig. 3A) compared with cell lines with normal copy number of IRS2 (Fig. 3B).

IGF-1R and IGFBP6 levels have been reported to be associated with sensitivity to IGF-1R/IR inhibitors in several studies (15, 18–20), which support our observations that sensitive cell lines had higher levels of IGF-1R RNA expression, especially in colorectal cancer cell lines with KRAS mutations (Fig. 3A), whereas lower levels of IGFBP6 were seen in sensitive lines (Fig. 3D). IGFBPs are important members of the IGF axis; they regulate the IGF1 pathway and influence IGF signaling by modulating the biologic accessibility and activity of the IGFs. Cells with lower level of IGFBP6 may have higher IGF-1R pathway activation, therefore more susceptible to IGF-1R inhibition.

The role of IR-A in cancer has important implications for anticancer treatments. Activation of IR signaling or increased expression of the IR-A isoform was observed in cancer cell lines when treated with a selective anti-IGF-1R antibody supporting the notion that activation of the IR-A/IGFII autocrine loop represents a mechanism of resistance to IGF-1R antibody therapies (13, 42). Our results demonstrate that KRAS/BRAF-WT cell lines with higher expression of IR-A were more sensitive to BMS-754807 (Fig. 3C), suggesting that KRAS/BRAF-WT cells may be more dependent on IR-A than on IGF-1R, therefore, cotargeting IGF-1R and IR with a dual inhibitor such as BMS-754807 may have enhanced efficacy in biomarker-selected tumors compared with an IGF-1R mAb that targets only IGF-1R.

Taken together, we hypothesize (Fig. 7B) that sensitive cells with activated IGF-1R/IR pathway via IRS2 amplification, high expression of IGF-1R or IR-A, low expression of IGFBP6, are more dependent on IGF-1R/IR pathways as the predominant driver for activation of AKT and ERK, making them more susceptible to IGF-1R/IR TKI inhibition which leads to decreased downstream PI3K/AKT and RAS/RAF signaling and consequently decreases cell proliferation. Whereas resistant cells have less activated IGF-1R/IR pathways and dysregulation of ERK and AKT pathways due to KRAS, PI3KCA, or BRAF mutations, making them less dependent on IGF-1R/IR signaling for proliferation; although targeting IGF-1R/IR with a TKI still inhibits IGF-1R/IR activity, it does not sufficiently inhibit the activity of ERK and AKT pathways caused by these mutations downstream of IGF-1R/IR.

In summary, we have identified a panel of candidate biomarkers, including KRAS and BRAF mutations, IRS2 CNCG, IGF-1R, IR-A, and
IGFBP6 RNA expression levels that could potentially select patients with colorectal cancer to enrich response to IGF-1R/IR inhibitor BMS-754807, and different biomarkers could be used in different subpopulations as defined by KRAS and BRAF mutation status as depicted diagrammatically in Fig. 7C. It is particularly interesting that KRAS-mutated colorectal cancers with IRS2 CNG are more active to IGF-1/IR inhibitor, as KRAS-mutated colorectal cancer is a population with limited treatment option and was previously overlooked by this class of agents. Our results provide testable hypotheses that warrant clinical investigation of different biomarkers in different subpopulations of colorectal cancer.

Disclosure of Potential Conflicts of Interest
C. Fairchild has ownership interest in Bristol-Myers Squibb stock. F.G. Finckenstein has ownership interest (including patents) in Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: F. Huang, H. Chang, F.G. Finckenstein, J. Jackson, J.M. Carboni

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IRS2 Copy Number Gain, KRAS and BRAF Mutation Status as Predictive Biomarkers for Response to the IGF-1R/IR Inhibitor BMS-754807 in Colorectal Cancer Cell Lines

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