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Preclinical Development

Molecular Cancer Therapeutics

Comprehensive Predictive Biomarker Analysis for MEK Inhibitor GSK1120212

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Abstract

The MEK1 and MEK2 inhibitor GSK1120212 is currently in phase II/III clinical development. To identify predictive biomarkers, sensitivity to GSK1120212 was profiled for 218 solid tumor cell lines and 81 hematologic malignancy cell lines. For solid tumors, *RAF/RAS* mutation was a strong predictor of sensitivity. Among *RAF/RAS* mutant lines, co-occurring *PIK3CA/PTEN* mutations conferred a cytostatic response instead of a cytotoxic response for colon cancer cells that have the biggest representation of the comutations. Among *KRAS* mutant cell lines, transcriptomics analysis showed that cell lines with an expression pattern suggestive of epithelial-to-mesenchymal transition were less sensitive to GSK1120212. In addition, a proportion of cell lines from certain tissue types not known to carry frequent *RAF/RAS* mutations also seemed to be sensitive to GSK1120212. Among these were breast cancer cell lines, with triple negative breast cancer cell lines being more sensitive than cell lines from other breast cancer subtypes. We identified a single gene *DUSP6*, whose expression was associated with sensitivity to GSK1120212 and lack of expression associated with resistance irrelevant of *RAF/RAS* status. Among hematologic cell lines, acute myeloid leukemia and chronic myeloid leukemia cell lines were particularly sensitive. Overall, this comprehensive predictive biomarker analysis identified additional efficacy biomarkers for GSK1120212 in *RAF/RAS* mutant solid tumors and expanded the indication for GSK1120212 to patients who could benefit from this therapy despite the *RAF/RAS* wild-type status of their tumors. *Mol Cancer Ther; ©2011 AACR.*

Introduction

The RAS-RAF-MEK-ERK [mitogen-activated protein kinase (MAPK)] signaling pathway is one of the most activated and also most extensively studied pathways in cancer (1). Genetic alterations causing aberrant activation of members of the MAPK pathway are commonly observed in cancer. For instance, receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), HER2, or c-MET are frequently activated as a result of DNA amplification or gain-of-function mutations (2–4). Activating mutations in RAS family genes, most often in *KRAS*, are found in about 30% of cancers (1). *BRAF* is mutated in more than 40% of melanomas and is also mutated at lower frequencies in other tumor types such as colon cancer and ovarian cancer. Therefore, although mutations in *MEK1* or *MEK2* are rare, pathway activation caused by mutations in the *RTKs*, *RAS*, or *RAF* are mediated through MEK (MAP/ERK kinase) kinases, making inhibition of MEK an attractive cancer therapeutic strategy (1).

Targeted therapies rely on the phenomenon of oncogene addiction to attempt to inactivate a mutated oncogenic pathway, critical to survival of cancer cells while sparing normal cells, which do not carry the mutation and are not similarly addicted to the pathway (5, 6). Thus, predictive markers identifying the appropriate genetic background are crucial to the success of targeted therapies. Examples include translocations of *BCR-ABL* with imatinib and *ERBB2* DNA amplification with trastuzumab and lapatinib (7–9). Various reports suggest that activating mutations in the *RAS* or *BRAF* genes represent the most important tumor predictive biomarkers for sensitivity to MEK inhibitors (10, 11). However, there is clear variance in sensitivity even among the limited set of *RAF/RAS* mutant cell lines that have been profiled. Furthermore, early clinical data indicates that although *RAF/RAS* mutant tumors (e.g., melanoma) seem to be more sensitive, tumors with these mutations are not uniformly

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responsive (12, 13). In some cases, co-occurring genetic alterations presumably provide mechanisms of resistance. A more comprehensive profile of the genetic determinants of sensitivity and resistance may further increase response rates to MEK inhibitors and provide rational combination strategies. Furthermore, it may assist in identifying those tumors that are dependent on MAPK pathway activation but do not harbor a *RAF*/*RAS* mutation. Identifying *RAF*/*RAS* wild-type tumors that might be responsive to MEK inhibitor would enable more cancer patients to benefit from these inhibitors.

GSK1120212 is a potent and specific allosteric inhibitor of MEK1/2 that is currently in phase II/III clinical development (14). It has shown clinical activity in tumors with activating mutations in *BRAF* (ASCO 2010). In this study, we conducted a comprehensive predictive biomarker study for GSK1120212 using 218 solid tumor cell lines covering different tissue types and subtypes. A comprehensive genetic characterization has been done in these cell lines. Our data confirm the association of *RAF*/*RAS* mutations with sensitivity to GSK1120212 treatment. In addition, we observe that among *RAF*/*RAS* mutant cell lines, co-occurring *PIK3CA* and *PTEN* mutations seem to promote a cytostatic rather than a cytotoxic response. Furthermore, among *KRAS* mutant, *PIK3CA*/*PTEN* wild-type cell lines, those with expression markers characteristic of having undergone transition to a more mesenchymal phenotype tend to be less sensitive. Among *RAF*/*RAS* wild-type breast cancer lines, we observed that basal breast cancer cell lines tend to be more sensitive than luminal cell lines. For the majority of the tumor types with or without *RAF*/*RAS* mutations, the expression of *DUSP6* gene is associated with sensitivity and lack of the expression is associated with resistance. Sensitivity to GSK1120212 was also profiled for 81 hematologic malignancy cell lines, and we observed that a majority of acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) cell lines were sensitive to GSK1120212. This article provides a comprehensive analysis of the markers contributing to both sensitivity and resistance to GSK1120212, a selective MEK1/2 inhibitor.

Materials and Methods

Cancer cell lines

Cell lines were purchased from the American Type Culture Collection (ATCC) and the German Resource Centre for Biological Material [DSMZ]. The majority of the cell lines were used within 6 months of acquisition and no reauthentication was carried out. For the DSMZ cell bank short-tandem repeat DNA typing was done for authentication, and numerous authentication tests were done at the ATCC cell bank (short-tandem repeat, sequencing, single nucleotide polymorphism fingerprinting). DNA copy number profiling and transcriptomics profiling on these cell lines were described previously (15). Briefly, unless otherwise recommended, cell lines were cultured in RPMI-1640 supplemented with a final concentration of 10% FBS, 2 mmol/L GlutaMAX, and 1 mmol/L sodium pyruvate. Genomic DNA was extracted from each line using Mini DNeasy Kit (Qiagen, Inc.) and copy number data was profiled using Affymatrix 500K SNP Chip. RNA was isolated from exponentially growing cells by replacing the media with TRIzol and purifying the RNA using Qiagen RNeasy spin columns. The transcriptomics data from these cell lines were collected using the Affymetrix U133 Plus2 Gene Chips in triplicate. All the genomic data on these cell lines have been deposited in https://cabig.nci.nih.gov/caArray_GSKdata/. Mutation data of these cell lines for BRAF, KRAS, NRAS, HRAS, PIK3CA, and PTEN were mostly obtained from COSMIC database (v49 release, Wellcome Trust Sanger Institute, United Kingdom).

Cell line proliferation assay

Each cell line was seeded into 384-well microtiter plates at high and low cell densities, ranging from 300 to 3,600 cells per well, depending on cell line doubling rate. Cells were plated in triplicate and incubated at 37°C in 5% CO_2 for 24 hours. Compound was added at 10 concentrations, ranging from 0.16 nmol/L to 5 μmol/L along with a dimethyl sulfoxide (DMSO) control. Cell numbers were measured using 4′,6-diamidino-2-phenylindole (DAPI) nuclei staining and quantitated with an InCell1000 High Content Analyzer (GE Biosciences). A zero-time value (D_0) was measured for each cell line, at each seeding density, immediately after the addition of DMSO control. Cell numbers at day 3 (D_3) were also measured using DAPI staining. A drug response curve was generated using model 205 of XLfit in Microsoft Excel. GI_{50} was defined as the drug concentration at which cell growth was inhibited at 50% compared with DMSO control. Compound cytotoxic effect was reached if the number of cells after 3-day drug treatment (D_3) is smaller than that measured at D_0 (i.e., cell number at D_3 < cell number at D_0) and compound cytostatic effect was reached if the number of cells did not decrease below the number measured at D_0 following up to 5 μmol/L drug treatment. For hematologic lines, a 3-day proliferation assays was conducted using CellTiter-Glo (Promega).

Western blot analysis

After media was aspirated from the dishes, cells were rinsed once with cold PBS and were then scraped off plates in cold PBS and spun down. Cell pellets were lysed in M-PER lysis buffer (Thermo Scientific) supplemented with Protease inhibitors (Roche Diagnostics) and phosphotase inhibitors (Upstate). Cell lysates boiled in loading buffer were run on 4% to 12% gels (NuPage), transferred to polyvinylidene difluoride membrane, and probed with antibodies. The antibodies used in the studies are anti-phospho-ERK at 1:1,000 dilution (catalog no. 4376; Cell Signaling), anti-phospho-AKT at 1:1,000 dilution (catalog no. 9271; Cell Signaling), anti-cleaved PARP at 1:1,000 dilution (catalog no. 9541; Cell Signaling), anti-total ERK at 1:500 dilution (catalog no. sc-93; Santa Cruz),

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anti-total AKT at 1:1,000 dilution (catalog no. 2967; Cell Signaling), anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 1:5,000 dilution (catalog no. G8795; Sigma-Aldrich), anti-DUSP6 at 1:500 dilution (catalog no. sc-8599; Santa Cruz).

Transcriptomics analysis for epithelial/mesenchymal features and breast cancer cell line characterization

On the basis of Affymetrix U133_2 transcriptomics dat from the cell lines (described previously), transcript abundance was estimated by normalizing all probe signal intensities to a value of 150 using the MAS5 algorithm in the Affymetrix MicroArray Analysis Suite 5.0. For subsequent analysis, the average probe intensity was used for sample triplicates. For determining epithelial or mesenchymal profile, 4 epithelial markers (*CDH1*, *OCLN*, *DSP*, and *CLDN1*) and 6 mesenchymal markers (*S100A4*, *CDH11*, *VIM*, *CDH2*, *ACTA2*, and *FN1*) were selected for analysis. The Affymetrix probes assaying each were interrogated. The probe with the highest expression values across the cell lines was selected, in which more than one probe existed for a gene, and the probes were further checked for specificity by BLASTing against the human genome. The selected probes identifiers were 201131_s_at, 227492_at, 200606_at, 222549_at, 2031869_s_at, 207173_x_at, 201426_s_at, 203440_at, 200974_at, 212464_at. The intensity values (averaged across the triplicates) were Z-score normalized across all the cell lines. An average of the epithelial markers and mesenchymal markers were taken. If the epithelial average was higher than the mesenchymal average, the cell line was classified as epithelial and if the mesenchymal average was higher, the cell line was classified as mesenchymal.

For breast cancer cell lines, basal and luminal cell lines were identified on the basis of hierarchical clustering. Basal breast cancer cell lines were differentiated from luminal cell lines on the basis of previously published signatures (16).

Results

Sensitivity profiling of MEK1/2 inhibitor, GSK1120212, in solid cancer cell lines

To identify predictive biomarkers for GSK1120212, 218 solid cancer cell lines encompassing different tissue of origins were profiled for sensitivity using a 3-day proliferation assay (Supplementary Table S1). Cell lines were defined as sensitive if GI50 was below 50 nmol/L and resistant if above 1 μmol/L, whereas if between 50 nmol/L and 1 μmol/L the sensitivity of the cell lines was considered intermediate. As shown for other MEK inhibitors, GSK1120212 showed increased activity against *RAS* or *RAF* mutant cell lines compared with cell lines wild type for these genes (Fig. 1A). When segregated into tissue types, tumor types known to carry high frequency of *RAF*/*RAS* mutations, such as pancreatic cancer, colon cancer, and melanomas showed generally increased sensitivity to MEK inhibitor GSK1120212 (Fig. 1B)

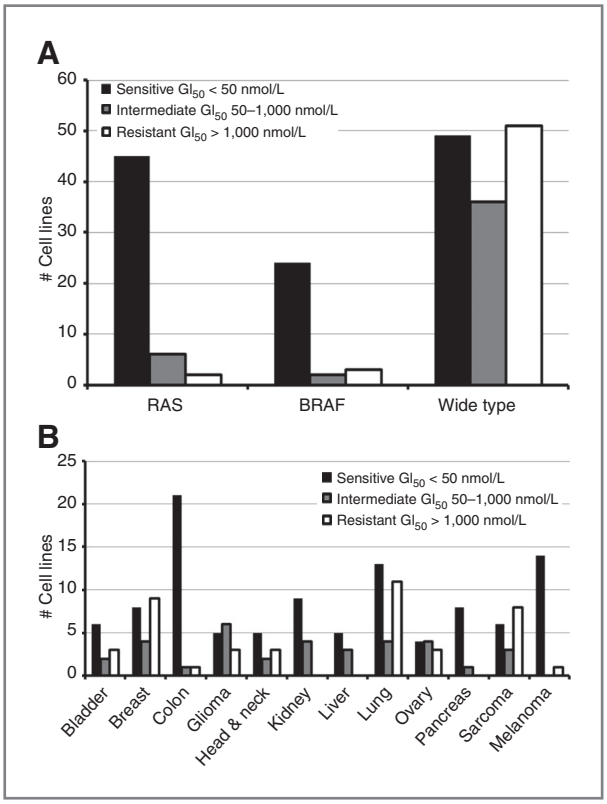

Figure 1. The response to GSK1120212 was profiled in a panel of 218 solid cancer cell lines using a 3-day proliferation assay. The cell lines were defined as sensitive if GI_{50} was below 50 nmol/L and resistant if GI_{50} was above 1 μmol/L. GI_{50} values between 50 nmol/L and 1 μmol/L were considered having an intermediate response. A, cell line response to GSK1120212 in relation to *BRAF* or *RAS* (i.e., *KRAS*, *NRAS*, and *HRAS*) mutation status. B, cell line response to GSK1120212 in relation to different tissues of origin.

However, for certain tissue types known to carry low frequency of *RAF*/*RAS* mutations, a significant proportion of cell lines showed sensitivity to GSK1120212. Among these were breast, glioma, head and neck, kidney, and ovarian cancer. The biological determinants of the sensitivity of these *RAF*/*RAF* wild-type cell lines were further analyzed and discussed in subsequent sections.

GSK1120212 caused a cytostatic, rather than cytotoxic, response on *RAF*/*RAS* mutant tumors encoding co-occurring *PI3K* or *PTEN* mutations

On the basis of GI_{50}, more than 80% of *RAF*/*RAS* mutant lines would be considered sensitive to MEK1/2 inhibitor GSK1120212 (GI_{50} < 50 nmol/L). However, because drug sensitivity in preclinical studies commonly overpredicts the observed clinical response (17), we attempted to further refine the sensitivity determinant for tumors harboring *RAS* or *RAF* mutations. To that end, an additional criteria to GI_{50} was added in which cell line response to GSK112212 was categorized as cell killing (cytotoxic = cell number at $D_3 < D_0$) or cell growth inhibition (cytostatic

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= cell number at $D_3 > D_0$). Because mutation in the phosphoinositide 3-kinase (PI3K) pathway was reported to be associated with resistance to MEK inhibitors (18), *PIK3CA* and *PTEN* mutation status was collected for all cell lines tested (Supplementary Table S1). Among *RAF/RAS* mutant cell lines, only colon cancer cell lines were significantly represented carrying co-occurring *PIK3CA/PTEN* and *RAF/RAS* mutations. For the colon cell lines with concomitant *RAF/RAS* and *PIK3CA* or *PTEN* mutations, the response to GSK1120212 was primarily cytostatic (9 of 10) instead of cytotoxic (1 of 10). Accordingly, 4 of 5 *RAF/RAS* mutant colon cancer cell lines with $GI_{50} < 50$ nmol/L and responded in a cytotoxic manner to GSK1120212 were wild type for *PIK3CA/PTEN* (Fig. 2A). Taken together, these data suggested that in a *RAF/RAS* mutant background, *PIK3CA* or *PTEN* mutations are predictive of cytostatic, rather than cytotoxic, response to GSK1120212.

To evaluate whether cell lines that harbor concomitant mutations in the PI3K and MAPK pathways depend on both activated pathways, we conducted studies with GSK1120212 and a PI3K inhibitor GSK1059615 (19), or a combination of the two drugs. Treatment of a colon cell line RKO (*BRAF* and *PI3K* mutant) with GSK1120212 or GSK1059615 alone significantly reduced p-ERK or p-AKT levels, respectively, indicative of pathway inhibition. However, neither compound alone caused substantial apoptotic cell death as evidenced by nondetectable cleaved PARP (c-PARP). In contrast, when cells were treated with both compounds (200 nmol/L GSK1120212 and 5 μmol/L of GSK1059615), p-ERK and p-AKT were reduced to nondetectable levels, and c-PARP was readily detected, suggesting cell death (Fig. 2B). These results suggested that the presence of concomitant activating mutations can permit alternative redundant growth or survival signals that may reduce sensitivity to a therapy targeting a single pathway, although cotreatment with inhibitors of each pathway cause cell death.

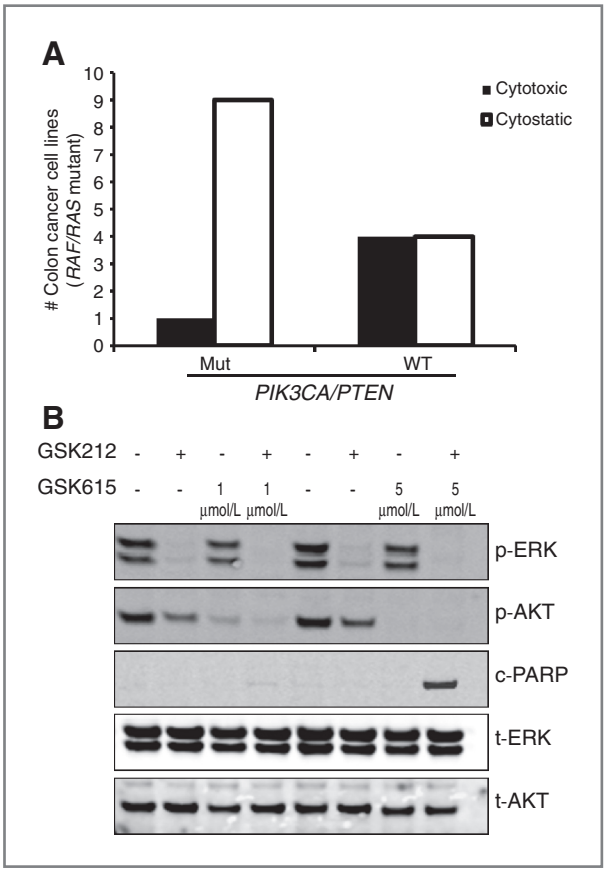

Figure 2. *RAF/RAS* mutant colon cancer cell lines with co-occurring *PIK3CA/PTEN* mutations gave more frequent cytostatic instead of cytotoxic responses. A, for the 18 *RAF/RAS* mutant colon cell lines, cytotoxic response was defined as cell number at $D_3 < D_0$ and cytostatic response was defined as cell number at $D_3 > D_0$. Cell lines were grouped based on *PIK3CA* and *PTEN* mutation status; Mut, cell lines encoding *PIK3CA* or *PTEN* mutations; WT, cell lines wild type for both genes. For the colon cell lines with concomitant *RAF/RAS* and *PIK3CA* or *PTEN* mutations, the response to GSK1120212 was primarily cytostatic (9 of 10) instead of cytotoxic (1 of 10). B, a colon cancer cell line RKO (BRAF and PIK3CA mutant) was treated with either GSK1120212 (GSK212) at 200 nmol/L or a PI3K inhibitor GSK1059615 (GSK615) at 1 or 5 μmol/L as single agents or in combination. After 30 hours of drug treatment, cell lysates were collected and Western blots were conducted for p-ERK for MAPK pathway activation, p-AKT for PI3K pathway activation, or c-PARP for cell death. Treatment of cells with GSK1120212 or GSK1059615 alone significantly reduced p-ERK or p-AKT levels, respectively, with total proteins unchanged. However, substantial apoptotic cell death was only observed when cells were treated with both compounds.

KRAS mutant cell lines with a mesenchymal expression pattern are less sensitive to GSK1120212

Analysis of GSK1120212 sensitivity of cell lines with MAPK pathway mutations showed that although most *BRAF* mutant cell lines respond with single digit nmol/L GI_{50} values, *KRAS* mutant cell lines exhibited a wider range of sensitivity (Fig. 3A). To further define the determinants of *KRAS* mutant cell line sensitivity to GSK1120212, additional genetic and transcriptomic analysis were carried out. At the genetic level, we observed a trend for *KRAS* mutant cells with co-occurring *PIK3CA* or *PTEN* mutations to be less sensitive to GSK1120212 (Fig. 3A). However this is not statistical significant (*t* test). Epithelial-to-mesenchymal transition (EMT) has been implicated in resistance to multiple cancer therapies including EGFR inhibitors (20). Furthermore, Singh and colleagues (21) showed that *KRAS* mutant cell lines sensitive to RNA interference (RNAi)-mediated KRAS depletion had higher levels of E-cadherin protein, an epithelial state marker, than *KRAS* mutant lines insensitive to KRAS RNAi depletion. The process of EMT was studied using transcriptomics analysis for the *KRAS* mutant cell lines. A 10-gene transcript signature designed to assay epithelial and mesenchymal status was derived and used to correlate with response of *KRAS* mutant cell lines to GSK1120212. Using this gene transcript signature, we showed that cell lines with increased mesenchymal features showed higher GI_{50} values (less sensitive) than *KRAS* mutant

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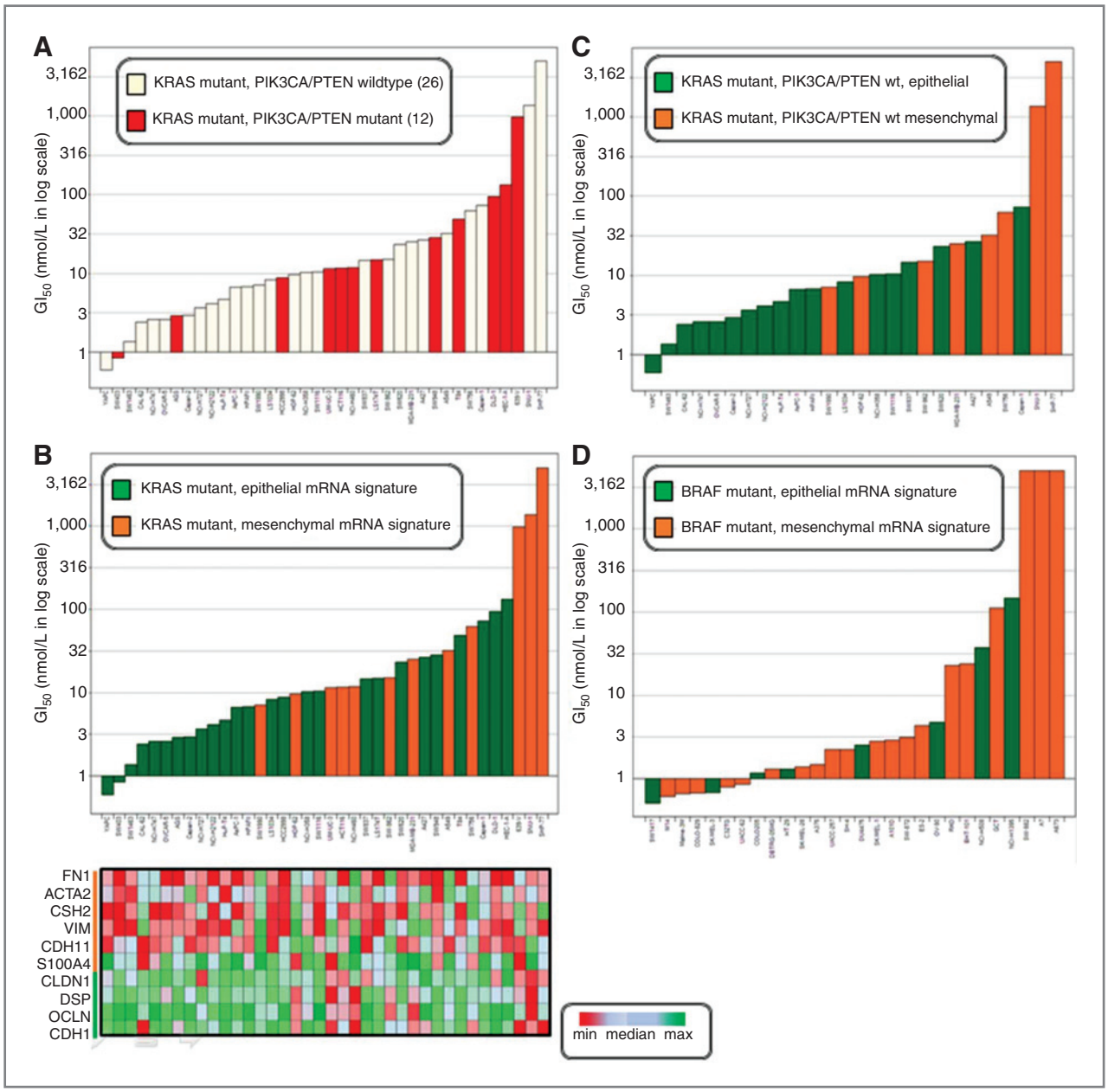

Figure 3. GI_{50} values for GSK1120212 are represented on the y-axis and individual cell lines on the x-axis. A, *KRAS* mutant, *PIK3CA*/*PTEN* mutant lines are colored red, showing a slight tendency for these lines to have higher GI_{50} values than *KRAS* mutant, *PIK3CA*/*PTEN* wild-type lines (not statistically significant, *t* test). B, *KRAS* mutant lines are colored for their epithelial/mesenchymal transcriptional signature; green lines denote higher average expression of the epithelial marker genes *CLDN1*, *DSP*, *OCLN*, and *CDH1* than the mesenchymal marker genes, *FN1*, *ACTA2*, *CSH2*, *VIM*, *CDH11*, and *S100A4*. Orange lines denote higher average expression of the mesenchymal marker genes compared with the epithelial marker genes. Lines with a mesenchymal signature tend to have higher GI_{50} values ($P = 0.00162$). Below the bar chart is a heatmap showing the Z-score–normalized expression values of the markers genes in a heatmap. Higher expression is denoted by green, lower expression by red, and the median by blue. C, *KRAS* mutant, *PIK3CA*/*PTEN* wild-type lines are colored for their epithelial/mesenchymal transcriptional signature. Lines with a mesenchymal signature tend to have higher GI_{50} values ($P = 0.000902$). D, *BRAF* mutant lines are colored for their epithelial/mesenchymal transcriptional signature. Sensitive lines have both epithelial and mesenchymal signatures. The 3 most resistant lines have mesenchymal signatures.

cell lines with increased epithelial features (*t* test $P = 0.00162$; Fig. 3B). The gene expression levels from which the EMT prediction was derived are represented in the heatmap at bottom of Fig. 3B. Exclusion of *PIK3CA*/*PTEN* mutant cell lines from this analysis improved the significance of this association ($P = 0.000902$; Fig. 3C), suggesting that epithelial-like (by transcriptional analysis) *KRAS* mutant cell lines wild-type for PIK3CA/PTEN are more sensitive to GSK1120212. Interestingly, despite the high sensitivity of most *BRAF* mutant lines

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regardless of *PIK3CA/PTEN* mutation or mesenchymal-like features (Supplementary Table S3; Fig. 3D), 3 *BRAF (V600E)* mutant sarcoma cell lines (GCT, A673, and SW-982) were not sensitive. Sarcomas typically arise from transformed mesenchymal cells, and the 3 cell lines have a mesenchymal-like gene signature.

Basal or triple negative breast cancer cell lines showed increased sensitivity to GSK1120212 compared with other breast cancer subtypes

Although breast cancer cell lines carry low frequency of *RAF/RAS* mutations, a significant number were sensitive to GSK1120212 (Fig. 1B). To better understand the determinants of breast cancer cell line sensitivity to GSK1120212, we correlated the HER2, ER, and PR status as well as basal or luminal status defined by published transcriptomics signature (16) to sensitivity to GSK1120212, as shown in Fig. 4. A total of 9 of 12 (75%) of more sensitive cell lines were of the basal subtype, whereas only 2 of 9 (22%) resistant lines were basal ($P = 0.029$). Notably, 3 of 4 (75%) of *ERBB2*-amplified cell lines were less sensitive, and 9 of 11 (82%) of triple negative (HER2, ER, and PR negative) cell lines were more sensitive, suggesting the triple negative subtype may preferentially respond to GSK1120212. Although basal breast cancers are not equivalent to triple negative breast cancers, they largely overlap in the clinic (22).

DUSP6 expression is a predictive biomarker of MEK inhibitor sensitivity

Although multiple gene signatures have been derived to predict MAPK pathway activation or response to MEK inhibitor (10, 23, 24), the technical difficulty to clinically measure these multiple transcripts has hindered their utility and usage. Our search to narrow the number of transcripts to predict cellular response to GSK1120212 led to identification of DUSP6 as single transcriptional marker of MEK inhibitor sensitivity. Of special interest is the fact that presence or absence of DUSP6 expression seems to be associated with MEK inhibitor sensitivity, not just degree of expression. DUSP6 is a cytoplasmic phosphatase inactivating pERK2 and is transcriptionally upregulated following activation of the MAPK pathway (25). Transcriptomics profiling of DUSP6 expression revealed that its expression was associated with sensitivity to GSK1120212 (Sensitivity: 79%; specificity: 82%: $P = 0.0027$; Fig. 5A).

Notably, among head and neck (H&N) cancer cell lines, none of the 5 sensitive cell lines carry *RAF/RAS* mutations. However, they all express high levels of DUSP6 (Supplementary Table S1 and Supplementary Table S2), and the only H&N cancer cell line not expressing DUSP6 was resistant to GSK1120212. Moreover, among the 7 tested cervical cancer cell lines, the 4 cell lines that do not express DUSP6 were all resistant to GSK1120212, and the remaining 3 cell lines expressing DUSP6 showed an intermediate response to GSK1120212. Among ovarian cancer cell lines, *RAF/RAS* mutation status alone would correctly predict 3 of 4 sensitive cell lines. A2780 had a sensitive response to GSK1120212 with GI_{50} of 28 nmol/L, and although it is wild type for *RAF/RAS*, it has a high expression of DUSP6. None of the 3 resistant ovarian cell lines express DUSP6 (MAS5 signal below background of 100; Supplementary Table S2; Fig. 5B). Western blot against DUSP6 was done on cell extracts from 7 ovarian cancer cell lines and showed that levels of DUSP6 protein correlated with that of DUSP6 mRNA levels and sensitivity to MEK GSK1120212 inhibitor (Fig. 5C). In addition, treatment of a sensitive cell line OVCAR5 with GSK1120212 led to dose- and time-dependant protein expression decrease of DUSP6 (Fig. 5D), suggesting that DUSP6 has the potential to be simultaneously a predictive (high endogenous level) and pharmacodynamic (decreased expression upon drug treatment) marker of GSK1120212 sensitivity.

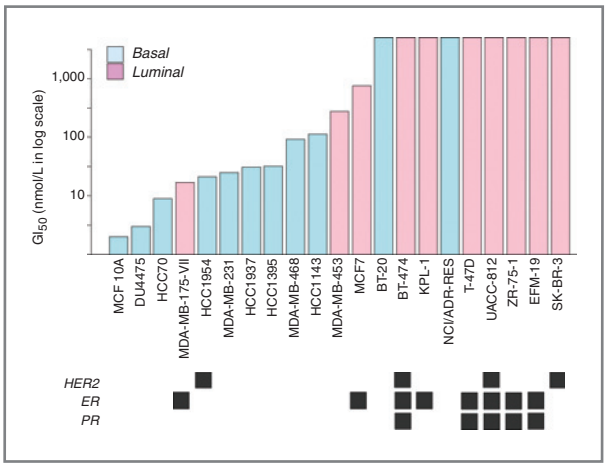

Figure 4. Proliferation response, measured by GI_{50}, for GSK1120212 in 21 breast cancer cell lines indicates that basal breast cancer cells are more responsive than luminal cell lines (depicted in blue and pink bars, respectively). A total of 9 of 12 (75%) of more sensitive cell lines were of the basal subtype, whereas only 2 of 9 (22%) resistant lines were basal ($P = 0.029$). *ERBB2* amplification, ER positivity, and PR positivity (by RNA levels) are denoted for each cell line. Notably, 3 of 4 (75%) of *ERBB2*-amplified cell lines were less sensitive, and 9 of 11 (82%) of triple negative (HER2, ER, and PR negative) cell lines were more sensitive.

Among hematopoietic malignancies cancer cell lines from AML and CML showed increased sensitivity to GSK1120212

GSK1120212 was profiled against 81 cancer cell lines from hematologic malignancies (Fig. 6). As with the solid tumor lines, activating mutations in *RAF/RAS* were predictors of sensitivity to GSK1120212, with 12 of 22 sensitive cell lines encoding mutations in *NRAS*, *KRAS*, *HRAS*, or *BRAF* (Supplementary Table S4). Interestingly, majority of AML lines tested showed sensitivity to GSK1120212 (11 of 12 cell lines), which can only be partly explained by *RAF/RAS* activating mutations (6 of 12 lines with *RAF/RAS* mutation). Similarly, the majority (4 of 6) of CML lines tested were sensitive to GSK1120212, but none encoded activating mutations in *RAF/RAS*. In both cases,

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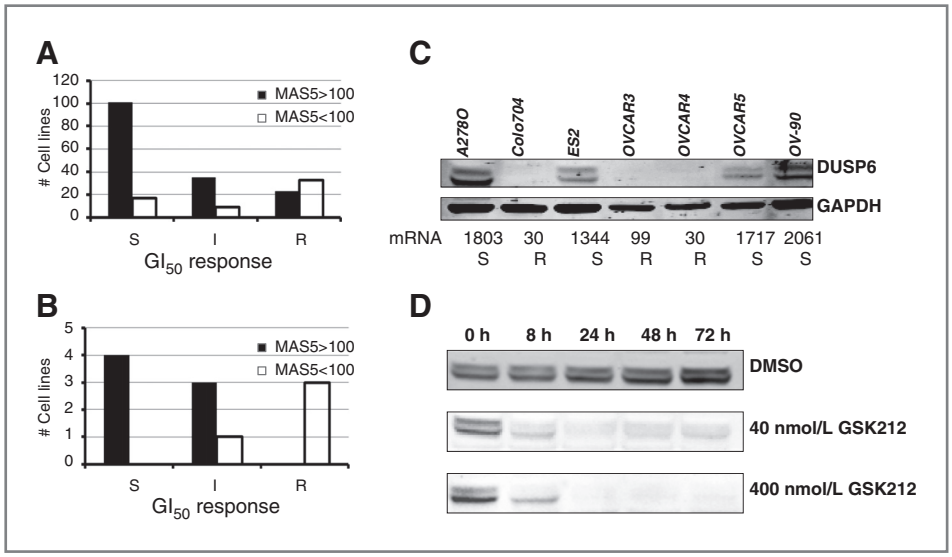

Figure 5. DUSP6 expression was associated with sensitivity to MEK inhibitor GSK1120212. A, the 218 solid cancer cell lines with GSK1120212 sensitivity data were grouped based on GI_{50} response, with S being sensitive ($GI_{50} < 50$ nmol/L); I being intermediate (GI_{50} between 50 nmol/L and 1 μmol/L) and R being resistant ($GI_{50} > 1$ μmol/L). DUSP6 mRNA expression was based on cell line transcriptomics data, with MAS5 signal above background value of 100 as being expressed (filled bar) and MAS5 signal below 100 as being lack of expression (empty bar). DUSP6 mRNA expression was significantly associated with sensitivity toward GSK1120212 ($P = 0.00266169$). B, the 11 ovarian cancer cell lines with GSK1120212 response data were grouped based on GI_{50} response. All the sensitive ovarian cancer cell lines expressed DUSP6 mRNA, and none of the resistant ovarian cancer cell lines expressed DUSP6 mRNA. C, the protein lysates from 4 sensitive and 3 resistant ovarian cancer cell lines were tested for DUSP6 protein expression by Western blot. Only the 4 sensitive ovarian cancer cell lines showed detectable levels of DUSP6 protein. The DUSP6 mRNA expression represented as MAS5 signal from transcriptomics data, as well as the sensitivity to GSK1120212 were also labeled for each cell line. D, a sensitive ovarian cancer cell line OVCAR5 was treated with 40 or 400 nmol/L of GSK1120212, and protein lysates were collected after different time points. Treatment with GSK1120212 led to dose- and time-dependent protein expression decrease of DUSP6.

sensitivity to GSK1120212 in the absence of *RAF*/*RAS* mutations may be explained by other commonly occurring oncogenic events (*BCR-ABL* translocation in CML and *FLT3* internal tandem duplications in AML) that have been previously shown to signal through the MAPK pathway (26, 27). Whereas AML and CML lines tested were predominantly sensitive to GSK1120212, the majority of B cell lymphoma lines [Burkitt, Hodgkin's, non-Hodgkin's lymphoma (NHL), and various other subtypes], acute lymphocytic leukemia (ALL; B cell or B cell precursor), multiple myeloma, T-cell derived ALL, and cutaneous T-cell leukemia (CTL) cell lines were resistant to GSK1120212. Somewhat surprisingly, although 4 of 7 T-cell derived ALL lines had activating mutations in *KRAS* or *NRAS*, only one line was sensitive to GSK1120212. Taken together, these data showed that AML and CML cancer cell lines are more sensitive to GSK1120212 than other hematopoietic cancer cell lines, in which sensitivity correlates weakly with *RAF*/*RAS* mutational status.

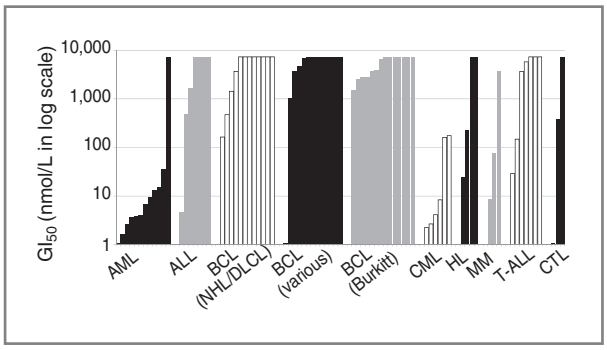

Figure 6. Growth inhibitory effect of GSK1120212 on various hematologic malignancy cell lines. A set of 81 cell lines from hematologic malignancies were treated with GSK1120212 in a 3-day growth assay. Although the majority of cell lines were insensitive ($GI_{50} > 1$ μmol/L) or intermediately sensitive (GI_{50}: 50–1,000 nmol/L), the majority of AML and CML lines showed GI_{50} below 20 nmol/L. BCL, B cell lymphoma; DLCL, diffuse large-cell lymphoma; various, lymphomas include follicular, mantle cell, pleural effusion, diffuse small-cell lymphoma; Burkitt, Burkitt's lymphoma; HL, Hodgkin's lymphoma; MM, multiple myeloma; T-ALL, T cell acute lymphocytic leukemia.

Discussion

Clinical response has been seen with GSK1120212 in *BRAF* and *RAS* mutant tumors (ASCO 2010). However, only a subset of *BRAF* and *RAS* mutant tumors responded to the MEK inhibitor GSK1120212 (ASCO 2010). These results are reminiscent of the clinical response to Herceptin, in which only a subpopulation of HER2-amplified breast cancers show benefit with treatment (28). On the basis of these data, it is imperative that additional biomarkers to *BRAF*/*RAS* mutation be identified to improve the clinical success rate and benefit to patients. On the other hand, it is also important that we discover predictive markers that can identify sensitive patients with

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wild-type *RAF*/*RAS* tumors, broadening the therapeutic benefit of MEK inhibitors.

With these goals in mind, we conducted a comprehensive predictive biomarker analysis using response data for GSK1120212 from approximately 300 cancer cell lines. As with previously characterized MEK inhibitors (10, 11), we observed that activating *RAF*/*RAS* mutations were the predominant predictors of sensitivity. Consequently, tumor types known to carry high frequency of *RAF*/*RAS* mutations (melanoma, pancreatic, and colon cancers), thus particularly depend on MEK pathway activation for growth, showed the highest rate of response (Fig. 1B). Among *RAF*/*RAS* mutant colon cancer cell lines, cell lines encoding both *RAF*/*RAS* and *PI3K*/*PTEN* mutations tend to have a cytostatic (inhibition of cell growth) rather than a cytotoxic (cell death) response (Fig. 2A). This observation was further confirmed with RKO colon cancer cell line (*BRAF*/*PI3K* dual mutant), showing significant cell death only following treatment with both MEK and a PI3K inhibitors but with neither agent alone (Fig. 2B).

AKT/PI3K and MAPK are the 2 most important cancer pathways downstream of most tyrosine kinase receptors. Cross-talk between these 2 pathways has been reported (29), and it is conceivable that for cell lines with mutations in both pathways, activated AKT can confer survival signal upon MEK pathway inhibition, whereas simultaneous inhibition of both pathways causes cancer cell death. This observation not only suggests combination studies using a PI3K inhibitor and a MEK inhibitor but also carries significance in further stratifying colon cancer patients for response. Approximately 40% of tumors of colon cancer patients have *BRAF* or *RAS* mutations, and half of these tumors would have concomitant *PI3K*/*PTEN* mutations, whereas the other half would be wild-type for *PI3K*/*PTEN* and would have greater probability to respond to single-agent MEK inhibitor (30, 31).

Mutant *KRAS* can activate multiple downstream effector pathways (29) besides the MEK pathway. As a result, the consequence of inhibiting MEK pathway in *RAS* mutant tumors can be influenced by the activity of other pathways downstream of RAS. In contrast, MEK pathway seems to be the only physiologically relevant pathway activated in *BRAF* mutants (32). Therefore, cells with mutant *BRAF* would presumably be truly addicted to MEK pathway activation. In an earlier study using CI-1040, it was reported that only *BRAF* mutation predicted sensitivity to MEK inhibition but not *RAS* mutation (11). Using GSK1120212, we found that overall cell lines with *RAS* mutations tend to be more sensitive (Fig. 1A). However, among 27 *BRAF V600E* mutant cell lines (the non-*V600E* mutations are known to have less activity), most were highly sensitive with GI_{50} of 5 nmol/L or below (20 of 27, 74%) regardless of *PI3K*/*PTEN* status. On the contrary, only 13 of 40 (32.5%) *KRAS* mutant cell lines gave GI_{50} values of 5 nmol/L or below, and a wider range of response was observed (Supplementary Table S3).

Because there is potential for some, but not all, patients with *RAS* mutant tumors to benefit from MEK inhibitor therapy, it is important to identify predictive biomarkers in the *RAS* mutant background. Even with GI_{50} alone, there seemed to be an association between *PI3K*/*PTEN* mutation and higher GI_{50}, although this is not statistically significant (Fig. 3A). A previous study using RNAi to knockdown *KRAS* in *KRAS* mutant cell lines showed that not all *KRAS* mutant cell lines require KRAS to maintain cell viability (21). The gene signature derived from this study to predict true KRAS dependency was associated with epithelial phenotype. Through analyzing mRNA expression patterns, we found that *KRAS* mutant tumors with an expression pattern suggesting more mesenchymal features tended to be more resistant (Fig. 3B and C). This relationship may be mirrored in the observation that the only *BRAF V600E* mutant cells lines that showed even moderate resistance were derived from sarcomas, a mostly mesenchymal tumor type. In a recent publication on predictive biomarkers for another MEK inhibitor AZD6244, the authors observed an association between Wnt pathway and resistance in *KRAS* mutant colon cell lines (24). Both MAPK and Wnt pathway activation are known to play an important and interconnected role in EMT transition in cancer, maybe through RKIP (33) or BMP4. However, despite this observed relationship between epithelial or mesenchymal expression pattern and MEK sensitivity, the underlying mechanism is not clear. EMT transition has been shown to be an acquired resistance marker to chemotherapy (20) and EGFR inhibitors (34) in multiple epithelial cancers (20). It could be that more advanced tumors tend to have undergone EMT and also to have gathered more upregulated growth pathways, which may compensate for MEK inhibition. Another possible explanation is that the MAPK pathway is an epithelial lineage pathway and, perhaps, as cells progress through EMT and become more mesenchymal, they also become less dependent on the MAPK pathway. This would suggest that *RAF*/*RAS* mutation would not be a route to oncogenesis in hematologic malignancies. *RAF*/*RAS* mutations are less common but certainly not absent from this class of cancers.

We decided to profile cell lines derived from many different tissue types and found tumor types that are known to have a low frequency of *RAF*/*RAS* mutations but unexpectedly showed sensitivity toward GSK1120212. Predictive biomarkers identifying tumors without mutations in *RAF*/*RAS* gene that still respond to MEK inhibition offer the opportunity to allow more patients to benefit from GSK1120212. All the available mutation data for the *RAF*/*RAS* wild-type cell lines was collected from Sanger database in hope of finding some other mutations that can explain the observed sensitivity. However no statistically significant association was observed, probably because most of the mutations occur at low frequency. Among breast cancer subtypes, triple negative breast cancer is considered to be EGFR driven and requires MEK pathway activation (35). Indeed, triple negative breast cell lines showed higher sensitivity toward GSK1120212 compared

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with breast cell lines from other subtypes (Fig. 4). For other tumor types, we investigated expression of MEK pathway genes downstream of RAF/RAS to identify tumors that have an activated MEK pathway but not identifiable with a *RAF/RAS* mutation. Multiple transcriptomics signatures have been reported either for *RAF/RAS* mutations or sensitivity toward MEK inhibitors (10, 23, 24). Most of these signatures include a *DUSP* gene, a family of dual-specificity phosphatases that regulate the MEK pathway. We found that a single gene *DUSP6*, a DUSP that specifically dephosphorylates and inactivates ERK2, predicts response to GSK1120212 with sensitivity of 79% and specificity of 82% ($P = 0.0027$; Fig. 5). DUSP6 is known to be involved in a feedback loop with ERK2 at the transcriptional level via ETS2 (25). Presumably its expression would reflect ERK2 activity and thus MAPK pathway activation status. In fact, it is among the genes identified by Pratilas and colleagues to be the transcriptional output of MEK pathway (36). Indeed, when the MAPK pathway was inhibited by treatment with GSK1120212, a sharp reduction in DUSP6 level was also observed (Fig. 5D). Measuring a single gene instead of a group of genes in the clinical samples requires less tumor material. The fact that in cell lines DUSP6 transcription expression is usually below detection (in resistant cells) or significantly expressed (in sensitive cells) makes the assay almost binary. This has a significant advantage translating to clinical specimens when cellular heterogeneity of tumors complicates the interpretation of multifactorial gene signatures. In cell lines, the protein level of DUSP6 correlated well with mRNA level (Fig. 5C), making it a possibility to use immunohistochemistry to measure DUSP6 levels. With the sharp reduction of DUSP6 expression upon GSK1120212 treatment, it also has the potential to be used as a pharmacodynamic marker, as well as a predictive marker.

Among hematologic malignancies, the growth of the majority of myelogenous lines (AML and CML) was inhibited at low concentrations of GSK1120212 with GI_{50} values of less than 50 nmol/L. These sensitive lines included *RAF/RAS* mutant and wild-type AML, whereas all of the CML lines were *RAF/RAS* wild type. Among these tumors, other frequent oncogenic events including *BCR-ABL* translocation and *FLT3* internal tandem duplications can lead to alternative routes to constitutive RAS activation and MAPK pathway dependency (26, 27).

Overall, we have conducted a comprehensive, in-depth predictive biomarker study for the MEK inhibitor GSK1120212 in solid tumor cell lines as well as cell lines from hematologic malignancies. This work has identified additional predictive biomarkers in cancer cell lines with a *RAF/RAS* mutant background, as well as biomarkers that would identify *RAF/RAS WT* tumors that might respond to GSK1120212. The ultimate validation of these data will be the analysis of these biomarkers in cancer patients who have received GSK1120212.

Disclosure of Potential Conflicts of Interest

Y. Degenhart received other commercial support from and has ownership interest in GlaxoSmithKline.

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References

1. Montagut C, Settleman J. Targeting the RAF-MEK-ERK pathway in cancer therapy. Cancer Lett 2009;283:125–34.
2. Moasser MM. Targeting the function of the HER2 oncogene in human cancer therapeutics. Oncogene 2007;26:6577–92.
3. Takeuchi K, Ito F. EGF receptor in relation to tumor development: molecular basis of responsiveness of cancer cells to EGFR-targeting tyrosine kinase inhibitors. FEBS J 2010;277:316–26.
4. Yap TA, de Bono JS. Targeting the HGF/c-Met axis: state of play. Mol Cancer Ther 2010;9:1077–9.
5. Weinstein IB. Cancer. Addiction to oncogenes–the Achilles heal of cancer. Science 2002;297:63–4.
6. Weinstein IB, Joe A. Oncogene addiction. Cancer Res 2008;68:3077–80; discussion 80.
7. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 2001;344:1031–7.
8. Rusnak DW, Alligood KJ, Mullin RJ, Spehar GM, Arenas-Elliott C, Martin AM, et al. Assessment of epidermal growth factor receptor (EGFR, ErbB1) and HER2 (ErbB2) protein expression levels and response to lapatinib (Tykerb, GW572016) in an expanded panel of human normal and tumour cell lines. Cell Prolif 2007;40:580–94.
9. Spector NL, Xia W, Burris H 3rd, Hurwitz H, Dees EC, Dowlati A, et al. Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. J Clin Oncol 2005;23:2502–12.
10. Garon EB, Finn RS, Hosmer W, Dering J, Ginther C, Adhami S, et al. Identification of common predictive markers of *in vitro* response to the Mek inhibitor selumetinib (AZD6244; ARRY-142886) in human breast cancer and non-small cell lung cancer cell lines. Mol Cancer Ther 2010;9:1985–94.
11. Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A, et al. BRAF mutation predicts sensitivity to MEK inhibition. Nature 2006;439:358–62.
12. Rinehart J, Adjei AA, Lorusso PM, Waterhouse D, Hecht JR, Natale RB, et al. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. J Clin Oncol 2004;22:4456–62.
13. Sznol M. Molecular markers of response to treatment for melanoma. Cancer J 2011;17:127–33.
14. Gilmartin AG, Bleam MR, Groy A, Moss KG, Minthorn EA, Kulkarni SG, et al. GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic properties for sustained *in vivo* pathway inhibition. Clin Cancer Res 2011;17:989–1000.
15. Greshock J, Bachman KE, Degenhardt YY, Jing J, Wen YH, Eastman S, et al. Molecular target class is predictive of *in vitro* response profile. Cancer Res 70:3677–86.
16. Charafe-Jauffret E, Ginestier C, Monville F, Finetti P, Adelaide J, Cervera N, et al. Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene 2006;25:2273–84.

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17. Voskoglou-Nomikos T, Pater JL, Seymour L. Clinical predictive value of the *in vitro* cell line, human xenograft, and mouse allograft preclinical cancer models. Clin Cancer Res 2003;9:4227–39.
18. Wee S, Jagani Z, Xiang KX, Loo A, Dorsch M, Yao YM, et al. PI3K pathway activation mediates resistance to MEK inhibitors in KRAS mutant cancers. Cancer Res 2009;69:4286–93.
19. Brachmann S, Fritsch C, Maira SM, Garcia-Echeverria C. PI3K and mTOR inhibitors: a new generation of targeted anticancer agents. Curr Opin Cell Biol 2009;21:194–8.
20. Sabbah M, Emami S, Redeuilh G, Julien S, Prevost G, Zimber A, et al. Molecular signature and therapeutic perspective of the epithelial-to-mesenchymal transitions in epithelial cancers. Drug Resist Updat 2008;11:123–51.
21. Singh A, Greninger P, Rhodes D, Koopman L, Violette S, Bardeesy N, et al. A gene expression signature associated with "K-Ras addiction" reveals regulators of EMT and tumor cell survival. Cancer Cell 2009;15:489–500.
22. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. Mod Pathol 2011;24:157–67.
23. Dry JR, Pavey S, Pratilas CA, Harbron C, Runswick S, Hodgson D, et al. Transcriptional pathway signatures predict MEK addiction and response to selumetinib (AZD6244). Cancer Res 2010;70:2264–73.
24. Tentler JJ, Nallapareddy S, Tan AC, Spreafico A, Pitts TM, Morelli MP, et al. Identification of predictive markers of response to the MEK1/2 inhibitor selumetinib (AZD6244) in K-ras-mutated colorectal cancer. Mol Cancer Ther 2010;9:3351–62.
25. Furukawa T, Tanji E, Xu S, Horii A. Feedback regulation of DUSP6 transcription responding to MAPK1 via ETS2 in human cells. Biochem Biophys Res Commun 2008;377:317–20.
26. Sharma SV, Gajowniczek P, Way IP, Lee DY, Jiang J, Yuza Y, et al. A common signaling cascade may underlie "addiction" to the Src, BCR-ABL, and EGF receptor oncogenes. Cancer Cell 2006;10:425–35.
27. Takahashi S. Inhibition of the MEK/MAPK signal transduction pathway strongly impairs the growth of Flt3-ITD cells. Am J Hematol 2006;81:154–5.
28. Brufsky A. Trastuzumab-based therapy for patients with HER2-positive breast cancer: from early scientific development to foundation of care. Am J Clin Oncol 2010;33:186–95.
29. Halilovic E, She QB, Ye Q, Pagliarini R, Sellers WR, Solit DB, et al. PIK3CA mutation uncouples tumor growth and cyclin D1 regulation from MEK/ERK and mutant KRAS signaling. Cancer Res 2010;70:6804–14.
30. Parsons DW, Wang TL, Samuels Y, Bardelli A, Cummins JM, DeLong L, et al. Colorectal cancer: mutations in a signalling pathway. Nature 2005;436:792.
31. Velho S, Oliveira C, Ferreira A, Ferreira AC, Suriano G, Schwartz S Jr, et al. The prevalence of PIK3CA mutations in gastric and colon cancer. Eur J Cancer 2005;41:1649–54.
32. Halilovic E, Solit DB. Therapeutic strategies for inhibiting oncogenic BRAF signaling. Curr Opin Pharmacol 2008;8:419–26.
33. Shin SY, Rath O, Zebisch A, Choo SM, Kolch W, Cho KH. Functional roles of multiple feedback loops in extracellular signal-regulated kinase and Wnt signaling pathways that regulate epithelial-mesenchymal transition. Cancer Res 2010;70:6715–24.
34. Uramoto H, Shimokawa H, Hanagiri T, Kuwano M, Ono M. Expression of selected gene for acquired drug resistance to EGFR-TKI in lung adenocarcinoma. Lung Cancer 2011;73:361–5.
35. Siziopikou KP, Cobleigh M. The basal subtype of breast carcinomas may represent the group of breast tumors that could benefit from EGFR-targeted therapies. Breast 2007;16:104–7.
36. Pratilas CA, Taylor BS, Ye Q, Viale A, Sander C, Solit DB, et al. (V600E) BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. Proc Natl Acad Sci U S A 2009;106:4519–24.

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