Development of an Integrated Genomic Classifier for a Novel Agent in Colorectal Cancer: Approach to Individualized Therapy in Early Development

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Abstract

Background: A plethora of agents is in early stages of development for colorectal cancer (CRC), including those that target the insulin-like growth factor I receptor (IGFIR) pathway. In the current environment of numerous cancer targets, it is imperative that patient selection strategies be developed with the intent of preliminary testing in the latter stages of phase I trials. The goal of this study was to develop and characterize predictive biomarkers for an IGFIR tyrosine kinase inhibitor, OSI-906, that could be applied in CRC-specific studies of this agent.

Methods: Twenty-seven CRC cell lines were exposed to OSI-906 and classified according to IC₅₀ value as sensitive (≤1.5 μmol/L) or resistant (>5 μmol/L). Cell lines were subjected to immunoblotting and immunohistochemistry for effector proteins, IGFIR copy number by fluorescence in situ hybridization, KRAS/BRAF/phosphoinositide 3-kinase mutation status, and baseline gene array analysis. The most sensitive and resistant cell lines were used for gene array and pathway analyses, along with shRNA knockdown of highly ranked genes. The resulting integrated genomic classifier was then tested against eight human CRC explants in vivo.

Results: Baseline gene array data from cell lines and xenografts were used to develop a k-top scoring pair (k-TSP) classifier, which, in combination with IGFIR fluorescence in situ hybridization and KRAS mutational status, was able to predict with 100% accuracy a test set of patient-derived CRC xenografts.

Conclusions: These results indicate that an integrated approach to the development of individualized therapy is feasible and should be applied early in the development of novel agents, ideally in conjunction with late-stage phase I trials.

Colorectal cancer (CRC) is the third most prevalent cancer type in both men and women in the United States, accounting for 10% of estimated new cases, and is the third leading cause of cancer deaths (1). Insulin-like growth factor I receptor (IGFIR) and other components of the IGF system are overexpressed in colon cancer and are associated with advanced stage of disease, metastasis, and reduced survival (2, 3). Interestingly, obesity, physical inactivity, and diabetes are associated with an increased risk of colon cancer and this has been correlated with overexpression of the IGF system, whereas elevated plasma insulin or C-peptide concentrations and insulin resistance can increase the risk of cancer recurrence and death, suggesting involvement of the insulin receptor (IR) signaling pathway as well (4, 5). Taken together, these data suggest that targeting the IGFIR pathway in CRC may be attractive clinically. In preclinical systems, this has been confirmed in multiple in vitro and in vivo CRC models, where blockade of the IGFIR inhibited proliferation and induced cell cycle arrest and apoptosis (6, 7).

There are numerous strategies for targeting the IGF pathway, including targeting the ligands by using somatostatin, ligand-specific antibodies, or by targeting the receptor with receptor-specific antibodies or receptor tyrosine kinases. OSI-906 (cis-3-[8-amino-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-3-yl]-1-methyl-cyclobutanol) is a small-molecule inhibitor of the IGFIR with IC₅₀ 0.018 and 0.054 in cell-free fractions against the IGFIR and IR, respectively (8). In vitro, OSI-906 potently (EC₅₀ <400 nmol/L) inhibited proliferation among a panel of 15 cell lines representative of colorectal, non–small cell lung carcinoma, pancreatic, breast, and pediatric...
Insulin-like growth factor I receptor (IGFIR) and other components of the IGF system are overexpressed in colon cancer and are associated with advanced stage of disease, metastasis, and reduced survival. The objective of this study was to use preclinical models along with a systems biology approach to develop and characterize predictive biomarkers related to the small-molecule IGFIR tyrosine kinase inhibitor, OSI-906, in colorectal cancer. The intent was to develop a predictive classifier that could subsequently be tested and refined in early clinical studies of OSI-906 in colorectal cancer. These data show that an integrated approach to the development of predictive biomarkers in the early clinical development of novel agents is feasible. By using the same approach to the development of other anticancer drugs, individualized therapy will become a reality, hopefully leading to more efficient and successful drug development.

Translational Relevance

Insulin-like growth factor I receptor (IGFIR) and other components of the IGF system are overexpressed in colon cancer and are associated with advanced stage of disease, metastasis, and reduced survival. The objective of this study was to use preclinical models along with a systems biology approach to develop and characterize predictive biomarkers related to the small-molecule IGFIR tyrosine kinase inhibitor (TKI), OSI-906, in CRC. The intent was to develop a predictive classifier that could subsequently be tested and refined in early clinical studies of OSI-906 in CRC.

Materials and Methods

Cell culture and proliferation

Twenty-seven human colon cancer cell lines were obtained from the American Type Culture Collection. The GEO cells were a generous gift from Dr. Fortunato Ciardiello (Cattedra di Oncologia Medica, Dipartimento Medico-Chirurgico di Internistica Clinica e Sperimentale “F Magrassi e A Lanzara,” Seconda Università degli Studi di Napoli, Naples, Italy). All cells, except GEO, were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin and maintained at 37°C in an incubator under an atmosphere containing 5% CO₂. GEO cells were grown in DMEM/F12 supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin. The cells were routinely screened for the presence of Mycoplasma (Mycoplet, Cambrex Bio Science) and exposed to OSI-906 when they reached ~70% confluence. All cell lines were tested and authenticated in the University of Colorado Cancer Center DNA Sequencing and Analysis Core. CRC cell line DNA was tested using the Profiler Plus kit (Applied Biosystems). The data obtained were compared with the American Type Culture Collection data to ensure that the cell lines have not changed. The cell lines were last tested in September 2009. OSI-906 was provided by OSI Pharmaceuticals and prepared as a 10 mmol/L stock solution in DMSO. Cytotoxic/proliferation effects were determined using the sulforhodamine B (SRB) method (18). Briefly, cells in logarithmic growth phase were transferred to 96-well flat-bottomed plates with lids. Cell suspensions (100 μL) containing 5,000 viable cells were plated into each well and incubated overnight before exposure with different concentrations of OSI-906 for 72 hours. After drug treatment, medium was removed and cells were fixed with cold 10% trichloroacetic acid for 30 minutes at 4°C. Cells were then washed with water and stained with 0.4% SRB (Fisher Scientific) for 30 minutes at room temperature and washed again with 1% acetic acid, followed by stain solubilization with 10 mmol/L Tris at room temperature. The plate was then read on a plate reader (Biotek Synergy 2) set at an absorbance wavelength of 565 nm. Cell proliferation curves were derived from the raw absorbance data.

Immunoblotting/ELISA

Cells were seeded into six-well plates and allowed to grow (without drug) for 48 to 72 hours until 70% confluent. Cells were then scraped into radioimmunoprecipitation assay buffer containing protease inhibitors, EDTA, NaF, and sodium orthovanadate. The total protein in samples was determined using the D₆₅ Protein Assay (Bio-Rad).
Thirty micrograms of total protein were loaded onto a 10% gradient gel, electrophoresed, and then transferred to polyvinylidene difluoride using the I-Blot (Invitrogen). The membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in TBS containing Tween 20 (0.1%) before overnight incubation at 4°C with the following primary antibodies: phospho-IGFIR, IGFIR, phospho-SHC, SHC, phospho-IRS-1, IRS-1, phospho-AKT, AKT, phospho–extracellular signal-regulated kinase (ERK), ERK, phospho-S6RP, S6RP, phospho–phosphoinositide 3-kinase (PI3K), and PI3K (Cell Signaling). After the primary antibody, blots were washed thrice for 20 minutes in TBS–Tween 20 (0.1%), incubated with the appropriate secondary anti-rabbit or anti-mouse IgG horseradish peroxidase–linked antibody at 1:20,000 (Jackson Immunoresearch) for 1 hour at room temperature, washed thrice, and developed using the Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore). Immunoblot experiments were done in triplicate for each antibody. IGF1 and IGFII in cell line supernatants were measured by ELISA and processed according to the manufacturer’s recommendations (R&D Systems).

**Immunohistochemistry**

IGFIR and downstream effector protein expression was assessed by immunohistochemistry using the following antibodies: IGFIR (Ventana Medical Systems), IGFII receptor (IGFIIr; Santa Cruz Biotechnology), phospho-ERK (Cell Signaling), survivin (Zymed), and Ki67 (Dako). The staining procedures were done according to our previously published methods (19). For scoring of proteins, a staining index calculated as percent of stained tumor cells × average staining intensity graded from 0 to 4 was used, resulting in an index value between 0 and 400 (19). Consistent with previous reports, samples with a staining index calculated as percent of stained tumor cells × average staining intensity graded from 0 to 4 was used, resulting in an index value between 0 and 400 (19). Consistent with previous reports, samples with a staining index of ≥200 were predefined as protein positive (19). The scoring was done by pathologists who were blinded to the exposure data.

**Fluorescence in situ hybridization**

Dual-color fluorescence in situ hybridization (FISH) assays were done on the prepared slides of the CRC cell lines using 120 ng of Spectrum Red–labeled IGFIR (University of Colorado Cancer Center Cytogenetics Lab) and 0.3 μL of Spectrum Green–labeled CEP15 (Abbott Molecular) per 113 mm² hybridization area according to our previously published procedures (20). The slides were first washed in 70% acetic acid for 20 to 30 seconds and then incubated in 0.008% pepsin/0.01 mol/L HCl at 37°C for 3 to 5 minutes and in 1% formaldehyde for 10 minutes and dehydrated in a graded ethanol series. The probe mix was applied to the selected hybridization areas, which were covered with glass coverslips and sealed with rubber cement. DNA codenaturation was done for 9 minutes at 85°C, and hybridization was allowed to occur at 37°C for 40 to 48 hours. Posthybridization washes were done with 2× SSC/0.3% NP40 at 72°C and with 2× SSC for 2 minutes at room temperature and dehydrated in a graded ethanol series. Chromatin was counterstained with 4’,6-diamidino-2-phenylindole (0.3 μg/mL in Vectashield mounting medium; Vector Laboratories). Analysis was done on epifluorescence microscope using single interference filter sets for green (FITC), red (Texas red), and blue (4’,6-diamidino-2-phenylindole) as well as dual (red/green) and triple (blue, red, green) band pass filters. Approximately 20 metaphase spreads and 100 interphase nuclei were analyzed in each cell line, and ploidy was assessed along with identification of the chromosomes harboring homologous sequences to the IGFIR/CEP15 probe set. To determine occurrence of genomic imbalances, IGFIR copy number per cell was compared with expected by the ploidy of the cell line (e.g., two copies in diploid lines and three copies in triploid lines). For documentation, images were captured using a charge-coupled device camera and merged using dedicated software (CytoVision, Applied Imaging).

**KRAS/BRAF/PI3K mutation analyses**

For both CRC cell lines and human tumor explants, DNA was isolated using the DNA extraction kit (Qiagen). KRAS mutations were analyzed by one of two methods. The human primary tumor explants were assayed by the University of Colorado Cancer Center Pathology Core with the Scorpion method (DxS Ltd.) using the manufacturer’s instructions. Briefly, template DNA was analyzed for a set of seven known KRAS point mutations using the Therascreen KRAS Mutation Detection kit (DxS). Reactions and analysis were done on a LightCycler 480 real-time PCR instrument (LC480) that was calibrated using a dye calibration kit provided by the kit manufacturer. Reactions were done on a 96-well plate in 20 μL reactions using ~60 ng of each DNA template. Sample DNA was amplified with eight separate primer sets [one for the wild-type (WT) sequence and one for each of seven different point mutations] with an internal Scorpion reporter probe. Cycle cross-point (Cp) values were calculated using the LC480 Fit-point software suite, and the control Cp was subtracted from the Cp of each mutation-specific primer set. Because there may be spurious low-level amplification in the absence of mutant template, amplification products are often visible at later cycle numbers for most of the primer sets. To avoid false-positive results due to background amplification, the assay was considered valid only if the control Cp value was ≤35 cycles. ΔCp thresholds were calculated to compensate for this background amplification. Mutations were scored positive when the ΔCp was less than the statistically set 5% confidence value threshold (21).

The CRC cell lines were analyzed for KRAS mutations by the University of Colorado Cancer Center Pathology Core with a high-resolution melting (HRM) temperature method using custom primers and the LC480 real-time PCR machine (Roche). Briefly, template DNA was tested by HRM analysis using a LightCycler 480 real-time PCR instrument (Roche Applied Science). Approximately
60 ng of tumor template DNA, WT control DNA, and mutant control DNA were amplified on the LightCycler 480 instrument using a HRM master mix (Roche Diagnostics), with the RASO1 and RASA2 primers and 1.75 mmol/L MgCl2 in 10 μL on a 96-well plate, using a two-step cycling program (95°C melting, 72°C annealing and extension) for 45 cycles. PCR products were analyzed by HRM with 25 data acquisitions per degree of temperature increase, from 40°C to 90°C. LightCycler 480 Gene Scanning software using the known WT control samples for baseline calculation was used for these analyses (21). BRAF and PI3K mutations were analyzed by PCR amplification and direct sequencing of the products as described previously (22). Primers used were AACACATTTCACGCCCTAAA-TATTGT (reverse) for amplification of exon 15 of BRAF, GCCCTTCTGTAAATCATCTGTG (forward) and CTGAGATCAGCCAATTCAGT (reverse) for exon 9 of PIK3CA, and CATTTATCTCACAAGTCCCA (forward) and TACCTCGAGAAGCTTGT (reverse) for codon 1023 mutation of exon 20 of PIK3CA, and ACAATTCAGAGAGAAAATCCTGCC (forward) and CAATTTCTATGCAATCGGTCT (reverse) for codon 1047 mutation of exon 20 of PIK3CA.

**Gene expression profiles**

Cells were plated at 2 × 10^6 in six-well plates 24 hours before harvest. After 24 to 72 hours, cells were rinsed twice with PBS, and RNA was prepared using a RNeasy Plus Mini kit (Qiagen). RNA stabilization, isolation, and microarray sample labeling were carried out using standard methods for reverse transcription and one round of in vitro transcription. Total RNA isolated from CRC cell lines and tumor xenografts was hybridized on Affymetrix U133 Plus 2.0 gene arrays in triplicates. To integrate the profiling data via the National Cancer Institute Cancer Genes and Genomes (KEGG) database as the gene set in this study (23).

**shRNA knockdown**

The pRS-shE2F6 gene-specific shRNA expression cassettes, along with control shRNA plasmids including the original pRS vector (TR20003), were purchased from OriGene. The sequences are as follows: metallothionein 2A (MT2A)--specific 29-mer shRNA, GTAAGAAGCAGCTAACCCAAACCTGGA; caldesmon, GCCACCCCAAATTAGAAATCTGCTGCT; MT1E, ACCTCCGTCCCTAATAGACCCAGT; aldehyde dehydrogenase 1A1 (ALDH1A1), GTATGCGAGTGGACAATGTGTTGAA; and mitogen-activated protein kinase (MAPK) kinase 6, TGCTGACCTGCAAGAGAAACCTGACT. Stable clones were generated by transfecting the OSI-906–resistant (HCT116 and SW480) and OSI-906–sensitive (HT29 and LS513) cells in six-well dishes with 1 μg of each of the shRNA plasmids using Fugene 6 (Roche) according to the manufacturer’s recommendations. Seventy-two hours after transfection, the cells were placed under selection with 2.0 μg/mL puromycin, splitting 1:5 when the cells reached confluency. Multiple clones from the same transfection were pooled and grown under puromycin selection. Successful knockdown of specific genes and gene products was confirmed by semiquantitative reverse transcription-PCR and immunoblotting with specific antibodies. Each experiment was conducted in triplicate.

**Gene set enrichment analysis**

Gene set analysis was done using the GSEA software version 2.0.1 obtained from the Broad Institute (http://www.broad.mit.edu/gsea; PMID: 16199517). Gene set permutations were done 1,000 times for each analysis. We used the nominal P value and normalized enrichment score to sort the pathways enriched in each phenotype. We used the pathways defined by BioCarta (http://www.biocarta.com) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database as the gene set in this study (23).

**k-Top scoring pair classifier**

We used the k-top scoring pair (k-TSP) algorithm (24) to construct a discriminative classifier in predicting tumors sensitive to OSI-906. In brief, the algorithm exploits the information contained in the rank-based matrix by focusing on “marker gene pairs” (i, j) for which there is a significant difference in the probability of the event (R_i < R_j) across the n samples from class Y = 1 (OSI-906 sensitive) to Y = −1 (OSI-906 resistant), where the event (R_i < R_j) equivalent to the rank of gene i is less than the rank of gene j if and only if gene i is expressed less than gene j (relative expression). Here, the quantities of interest are p^k_m = Prob(R_i < R_j | Y = m), m = (1, −1) (i.e., the probabilities of observing R_i < R_j in each class). These probabilities are estimated by the relative frequencies of occurrences of R_i < R_j within profiles and over samples. Let Δ_i denote the “score” of gene pair (i, j), where Δ_i = | p^k(1) − p^k(−1)|. A score Δ_i is computed for every pair of genes i, j ∈ {1, …, P}, i ≠ j. Gene pairs with high scores are viewed as most informative for classification. Using an internal leave-one-out cross-validation, the final k-TSP classifier uses the k disjoint pairs of genes, which achieve the k best scores from the training set. In this study, maximum number of pairs (k_max) was fixed as 10.
**In vivo xenograft studies**

Five- to six-week-old female athymic nude mice (Harlan Sprague Dawley) were used. Mice were caged in groups of five and kept on a 12-hour light/dark cycle and provided with sterilized food and water ad libitum. Animals were allowed to acclimate for at least 7 days before any handling. All CRC cells were harvested in an exponential phase growth and resuspended in a 1:1 mixture of serum-free RPMI 1640 and Matrigel (BD Biosciences). Five to 10 million cells per mouse were injected s.c. into the flank using a 23-gauge needle. Mice were monitored daily for signs of toxicity and weighed twice weekly. Tumor size was evaluated twice per week by caliper measurements using the following formula: tumor volume = [length × width²]/0.52. When tumors reached 150 to 300 mm³, mice were randomized into two groups with at least 10 tumors per group. Mice were then treated for 14 days with either vehicle control (25 mmol/L tartaric acid) or OSI-906 (40 mg/kg) once daily by oral gavage. The shorter duration of treatment of the cell line xenografts was used only to establish that the tumors exhibited similar in vitro and in vivo responsiveness to OSI-906 (sensitive or resistant).

The human CRC explant xenografts were generated according to previously published methods (25). Briefly, surgical specimens of patients undergoing either removal of a primary CRC or metastatic tumor at the University of Colorado Hospital were reimplanted s.c. into five mice for each patient. CUCRC006, CUCRC007, CUCRC010, CUCRC021, and CUCRC027 were obtained from primary tumor sites, whereas CUCRC001, CUCRC012, and CUCRC026 originated from peritoneal, pelvic, or omental metastatic sites, respectively. Tumors were allowed to grow to a size of 1,000 to 1,500 mm³ (F1), at which point they were harvested, divided, and transplanted to another five mice (F2) to maintain the tumor bank. After a subsequent growth passage, tumors were excised and expanded into cohorts of ≥25 mice for treatment. All experiments were conducted on F3 to F5 generations. Tumors from this cohort were allowed to grow until reaching 150 to 300 mm³, at which time they were equally distributed by size into the two treatment groups (control and OSI-906 treated). Mice with tumors from this treatment stage were treated for 28 days with either vehicle control (25 mmol/L tartaric acid) or OSI-906 (40 mg/kg) once daily by oral gavage. Monitoring of mice and measurements of tumors were conducted as described above. The relative tumor growth index was calculated by taking the relative tumor growth of treated mice divided by the relative tumor growth of control mice since the initiation of therapy (T/C) as described previously (25). Tumors with a T/C of <50% were considered sensitive.

All of the xenograft studies were conducted in accordance with the NIH guidelines for the care and use of laboratory animals, were conducted in a facility accredited by the American Association for Accreditation of Laboratory Animal Care, and received approval from University of Colorado Animal Care and Use Committee before initiation. Obtaining tissue from CRC patients at the time of removal of a primary tumor or metastasectomy was conducted under a Colorado Multi-Institutional Review Board–approved protocol.

**Fig. 1.** Cell proliferation assay on the panel of 27 CRC cell lines. Cell proliferation was evaluated by SRB following exposure to OSI-906 for 72 h. Cells were plated at an optimized density in 96-well plates, incubated overnight at 37°C, and then exposed to a serial dilution of OSI-906. After 72 h of incubation, cells were fixed with trichloroacetic acid and a SRB assay was done as described in Materials and Methods. Green lines, sensitive cell lines; yellow lines, intermediate cell lines; red lines, resistant cell lines.
Statistical methods
To determine the statistical significance of mutational status and FISH analysis, a contingency table was constructed and the Fisher’s exact test was done using GraphPad Prism software. The bioinformatics approach and relevant references are stated above. Differences were considered significant at $P < 0.05$.

Results

Assessment of responsiveness of a panel of CRC cell lines to OSI-906
To evaluate the sensitivity of CRC cell lines to OSI-906, a panel of 27 CRC cell lines was exposed to increasing concentrations and assessed for proliferation using a SRB assay (18). As depicted in Fig. 1, there was a broad range of sensitivity of the CRC cell lines to OSI-906. For categorization, a sensitive cell line was classified as one with an IC$_{50}$ of $\leq$1.5 μmol/L, whereas resistant cell lines had IC$_{50}$ values of $>5$ μmol/L; 6 cell lines met the criteria as being sensitive, and the remaining 21 were resistant.

IGFIR pathway analysis by immunoblotting and immunohistochemistry
The use of protein biomarkers as predictive tools has been fraught with controversy for both EGFR1-directed and EGFR2 (HER2/NEU)–directed therapies (10, 26); nonetheless, we thought it important to analyze upstream and downstream effectors of the IGFIR pathway by immunoblotting and immunohistochemistry at baseline. As depicted in Supplementary Fig. S1, none of the major components of the IGFIR pathway seemed to predict sensitivity to OSI-906. For example, activated (phosphorylated) IGFIR, ERK, AKT, IRS-1, P13K, SHC, and S6 kinase were variably present at baseline in both sensitive and resistant cell lines. Similarly, baseline expression of phospho-IGFIR, IGFIR, IGFIIR, IGFI, phospho-ERK, survivin, and Ki67 by immunohistochemistry did not correspond to sensitivity to OSI-906 (Supplementary Table S1). Only two cell lines, GEO and LS1034 (both sensitive to OSI-906), showed IGFII in the cellular supernatant, whereas none of the cell lines had detectable IGFI secretion (data not shown).

Evaluation of IGFIR gene copy number by FISH
Based on prior studies suggesting that increased EGFR copy number may be predictive for EGFR-directed agents (27), we assessed IGFIR gene copy number in the panel of cell lines using a specific probe set. Although gene amplification was not observed in any of the CRC cell lines, several of them displayed an unbalanced IGFIR copy number gain based on ploidy, which showed a statistically significant relationship ($P = 0.0152$) between the presence of unbalanced gain and sensitivity to OSI-906 (Supplementary Table S2). Representative spreads/interphase nuclei are depicted in Fig. 2 for one sensitive (Colo205; Fig. 2A) and one resistant (SW1463; Fig. 2B) cell line.

Assessment of KRAS/BRAF/P13K gene mutation status by sequencing
Because tumors with mutant KRAS/BRAF or P13K show resistance to EGFR-based therapies, we characterized the KRAS/BRAF/P13K mutational status of the CRC cell lines (28–31). Although no significant correlation was observed between KRAS status and OSI-906 sensitivity, there was a trend toward KRAS WT tumors being more sensitive. There was no relationship between either BRAF or P13K mutation status and responsiveness to OSI-906 (Supplementary Table S3).

Identification of differentially expressed genes between CRC cell lines sensitive or resistant to OSI-906
To initially identify genes that correlated with sensitivity to OSI-906, we analyzed the basal gene expression profiles of the four sensitive and the four most resistant CRC cell lines. Using the two-sample t test, 139 genes were identified as differentially expressed in OSI-906–sensitive and OSI-906–resistant CRC cell lines. Of these,
there were 61 top-scoring ($P < 0.002$) genes (Supplementary Table S4). Strikingly, genes that encode MTs, a family of ubiquitous, low molecular weight intracellular proteins that bind and detoxify heavy metal ions, were increased in the resistant cells and represented the highest ranked group (9- to 36-fold increase) of differentially expressed genes. MT can be induced by a variety of stimuli; are involved in other cellular functions such development, differentiation, proliferation, and carcinogenesis; and have been associated with a poor prognosis and metastasis in cancer as well as drug resistance (32–34).

Genes that were upregulated in the sensitive lines included ALDH1A1 (83-fold), an enzyme involved in the metabolism of alcohol and the oxidation of all-trans retinal to all-trans retinoic acid, and the MAPK kinase 6 (MAP2K6; 11-fold), a protein that activates p38 MAPK and mediates stress-induced cell cycle arrest, transcriptional activation, invasion/migration, and apoptosis (35, 36).

shRNA knockdown of potential predictive biomarker genes

To determine whether any of the highly ranked genes had a functional role in mediating responsiveness to OSI-906, we did knockdown experiments with shRNA. OSI-906–resistant (HCT116 and SW480) CRC cell lines were transfected with shRNA for caldesmon, MT2A, or MT1E. OSI-906–sensitive (HT29 and LS513) CRC cell lines were transfected with shRNA for ALDH1A1 or MAP2K6. The phenotype was analyzed by exposing the CRC cell lines to increasing concentrations of OSI-906. As depicted in Fig. 3, shRNA knockdown of MT2A resulted in a robust decrease in MT2A RNA and protein in the resistant HCT116 and SW480 cells, which was associated with a statistically significant increase in the antiproliferative effects of OSI-906 in the HCT116 but not the SW480 cells. Similar shRNA knockdown of the other genes noted above did not show a functional role in mediating sensitivity (ALDH1A1 and MAP2K6) or resistance (Caldesmon and MT1E) to OSI-906 (data not shown).

Pathway analysis of OSI-906–sensitive and OSI-906–resistant CRC cell lines

To determine whether any particular pathway was associated with responsiveness to OSI-906, pathway enrichment analysis was done on the baseline CRC cell lines. In the sensitive cell lines, p53, insulin, and IGFIR signaling pathways were among the top 25 pathways upregulated according to BioCarta annotations. Among the core genes in the insulin and IGFIR signaling pathways were IRS-1, PIK3CB, RASA1, and FOS. Individually, these core genes were not in the differentially expressed gene list in Supplementary Table S4; however, coordinate
overexpression of these core genes in the insulin and IGFIR signaling pathways correlated with OSI-906 sensitivity (Fig. 4). In contrast, the EGFR, MAPK, vascular endothelial growth factor, Wnt, and multiple cell adhesion/motility signaling pathways were prominently represented in the top 25 upregulated pathways of resistant cell lines. Similar results were also observed when using KEGG gene set annotations in the pathway analysis.

**Training and validation of a $k$-TSP classifier for predicting OSI-906 sensitivity**

The primary goal of this project was to develop a classifier to predict sensitivity to OSI-906. To accomplish this, we used the baseline gene arrays from the four sensitive and four most resistant cell lines grown in vitro and in vivo as xenografts. In this study, we used the previously described $k$-TSP algorithm as a discriminative classifier (24). Using an internal leave-one-out cross-validation, the final $k$-TSP classifier uses the $k$ disjoint pairs of genes, which achieve the $k$ best scores from the training set. In this study, the maximum number of pairs ($k_{\text{max}}$) was fixed at 10. The $k$-TSP classifier identified three gene pairs as the final classifier: (PROM1>MT1E), (LY75>OXCT1), and (HSD17B2>CALD1). Interestingly two of these genes, MT1E and CALD1, also appeared in the gene analysis above. From the training data, the $k$-TSP classifier achieved an estimated leave-one-out cross-validation of 85.8%. To potentially improve the predictive power of the $k$-TSP classifier, we integrated KRAS mutational (WT) status and IGFIR FISH (unbalanced gain). Figure 5 depicts diagrammatically how this integrated classifier is used. In order for a tumor to be predicted as sensitive, it must meet four of the five classifiers. In the validation set of 18 additional CRC cell lines (independent from the training set), the classifier correctly predicted responsiveness to OSI-906 in 89% (17 of 19) of cell lines.

**Validation of the integrated genomic classifier against human CRC explants in vivo**

To further validate the classifier, we assessed the baseline $k$-TSP from above, IGFIR by FISH, and KRAS status of eight...
human CRC explants (primary tumors or metastasectomy specimens) grown in vivo as xenografts before treatment with OSI-906. The classifier predicted six explants as resistant and two as sensitive. Following treatment with OSI-906, the two that were predicted to be sensitive were indeed sensitive [tumor growth index (TGI) = 21-28%] and the six that were predicted to be resistant were resistant (TGI = 69-178%), leading to an overall accuracy of prediction against the human CRC explants of 100% (Fig. 6).

Discussion

IGFIR inhibitors as therapeutic agents in cancer constitute a promising class of targeted cancer therapies (6, 37). Initial phase I studies of these agents (antibodies and small molecules) have shown mild but reversible toxicities, including hyperglycemia and fatigue. Early signs of activity have been observed in tumors where IGFIR is thought to play a role, including breast, liver, and CRC as well as soft tissue sarcoma (38). We chose to study OSI-906, a potent oral TKI that is currently in phase I clinical trials as a single agent and in combination. Whereas previously there was thought to be a selectivity advantage of antibodies against the IGFIR, recent data suggest that small-molecule TKIs may confer the biological benefit of inhibiting signaling through the IR in cancer cells, which is now thought to be a cooperative partner in mediating cellular processes associated with the malignant phenotype (39, 40). Hyperglycemia, initially thought to be associated only with TKIs, is a mechanism-based toxicity associated with this class of agents (antibodies and TKIs) and is manageable (37). Nonetheless, despite promising hints of efficacy, there has been limited evidence that receptor activation or overexpression can adequately select patients for treatment with IGFIR inhibitors (41, 42). Therefore, the goal of this study was to use an unbiased genomic approach in developing a predictive classifier that could be validated in human CRC explants before clinical testing.

As expected, we observed a broad range of sensitivity to OSI-906 among the 27 CRC cell lines in vitro that was recapitulated by the four sensitive and four most resistant cell lines in vivo. Similar to EGFR-based inhibitors, there did not seem to be a relationship between baseline quantity or activation status of relevant effector proteins and sensitivity to OSI-906, nor was there a pattern of posttreatment changes of selected proteins that was consistently
related to responsiveness to OSI-906. Interestingly, when
the IGFIR gene copy number (based on ploidy) was as-
sessed by FISH, there was a trend toward a gain in copy
number and sensitivity to OSI-906 that did not meet sta-
tistical significance. Previous studies have shown that CRC
cell lines and tumors are infrequently amplified for IGFIR,
but little has been reported with regard to unbalanced
gains of IGFIR (20). Of note, because OSI-906 targets IR
as well as IGFIR, and these can form hybrid receptors,
the role of IR amplification is also worth investigating
and is included in the ongoing clinical trial of OSI-906
in patients with CRC (5). Among the IGFIR therapeutic
antibodies and small-molecule TKIs, the most extensive
predictive biomarker investigations have been in sarco-
mas, where elevated expression of ligands and IGFIR has
been associated with a sensitive phenotype, whereas in-
creased expression of the binding proteins IGFBP-3 and
IGFBP-6 has been associated with resistance (41, 42). In-
terestingly, although a prior study of basal-like breast can-
cers in murine models suggested that IGFIR inhibition was
effective in KRAS mutant models, among our CRC cell lines,
there was a nonstatistically significant trend toward
cells with WT KRAS being more sensitive to OSI-906 (43).
Indeed, one could make the case that mutations down-
stream of the IGFIR (KRAS or PI3K) would confer resistance
to IGFIR-directed therapies in the same manner as has been
described with EGFR-directed antibodies in CRC (22).

The single gene lists and pathway analysis between
CRC cells sensitive or resistant to OSI-906 yielded some
important observations. Highly overexpressed in the resis-
tant cells were caldesmon and MTs. Caldesmon is an actin-
binding protein that has recently been shown to play a
critical role in regulating the formation and dynamics of
podosomes and invadopodia, cell adhesion structures that
protrude from the plasma membrane and degrade the ex-
tracellular matrix (44). Although caldesmon is generally
thought to be a negative regulator of podosome formation by sequestering actin, phosphorylation of caldesmon by either cdc2 kinase or ERK1/2 MAPK reverses its inhibitory function, a situation that may dominate in cancer cells (45). Also highly represented in the resistant cells were the MTs, a family of cysteine-rich low molecular weight proteins with affinity for heavy metal ions that have been associated not only with drug resistance to a variety of anticancer compounds but also with a more invasive cancer phenotype (32–34). Reduction of MT2A expression by antisense or siRNA was associated with a reduction in proliferation and viability in breast cancer cell lines (34). Interestingly, MTs were also very prominent on the list of genes associated with resistance to the small-molecule IGFIR/IR inhibitor, BMS-536924, among a panel of 28 sarcoma and neuroblastoma cell lines, indicating that this may represent a disease-independent mechanism of resistance to these agents (42). Interesting trends noted in the pathway analysis indicate that cell cycle and cell adhesion/communication pathways were prominent among resistant cells, whereas IGFIR/EGFR/insulin pathways including downstream effectors were dominant in sensitive cells. Similar dependence on IGFIR signaling has been described for breast and CRC cell lines when exposed to the IGFIR antibody h10H5 (41). Taken together, these results indicate potential mechanisms to overcome inherent resistance to OSI-906, including modulation of cell cycle, MAPK, and cell adhesion/invasion pathways, perhaps by rational combinations with inhibitors of cyclin-dependent kinases, MAPK/ERK kinase, focal adhesion kinase, or Src, all of which are clinically available. Based on data indicating that inhibition of mammalian target of rapamycin leads to IGFIR-dependent activation of AKT, combinations of agents that inhibit these pathways also seem warranted (46).

To develop the k-TSP classifier, we used both in vitro and in vivo baseline gene array for a given cell line in the training set so that differences relating purely to the tumor microenvironment would be minimized. We acknowledge that there are limitations to the k-TSP classifier, as it is dependent on gene expression and may not reflect the complex biology that mediates inherent drug resistance; therefore, to increase accuracy, we developed an algorithm that combines gene expression, copy number, and mutation. Our integrative genomic classifier differs from other published drug-response gene expression signatures in two important aspects. First, as opposed to other studies where the gene signatures remain unvalidated, we validated our integrative genomic signature on a set of independent direct human CRC explants as an intermediate step before moving into clinic. Second, the classifier is composed of the relative expression of three gene pairs, KRAS mutation status, and IGFIR FISH analyses, all of which can be readily applied to clinical settings (41, 47). Interestingly, the rate of responsiveness (and presence of a “sensitive” biomarker pattern) in this small sample was 25%, which is similar to the rate of HER2 gene amplification in breast cancer, used for selecting patients for treatment with trastuzumab (48). Clearly, these results require validation in a clinical trial, which has recently been initiated in CRC patients.

In summary, these data show that an integrated approach to the development of predictive biomarkers in the early clinical development of novel agents is feasible. By using the same approach to the development of other anticancer drugs, individualized therapy will become a reality, hopefully leading to more efficient and successful drug development.

Disclosure of Potential Conflicts of Interest

T.M. Pitts, A.C. Tan, S.G. Eckhardt: commercial research grant, and ownership interest (including patents). S.G. Eckhardt: consultant/advisory board, OSI Pharmaceuticals. The other authors declare no conflicts.

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References


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