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Expression of sorafenib targets in melanoma patients treated with carboplatin, paclitaxel and sorafenib

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Abstract

Background—Sorafenib, a multi-target kinase inhibitor, inhibits members of the MAPK pathway and receptor tyrosine kinases, including VEGF-R2. Sorafenib, carboplatin and paclitaxel (SCP) has anti-tumor activity in melanoma patients, but no association was found between response and activating B-Raf^{V600E} mutations. We assessed expression of sorafenib targets in SCP-treated patient specimens and evaluated the association with response and progression free survival.

Experimental Design—Using Automated QUantitative Analysis (AQUA®), we quantified expression of VEGF-R1, VEGF-R2, VEGF-R3, FGF-R1, PDGF-Rβ, c-Kit, B-Raf, C-Raf, MEK1 and ERK1/2 in pre-treatment specimens from 46 patients. Furthermore, we assessed ERK1/2 expression in 429 archival melanomas.

Results—VEGF-R2 expression was significantly higher in patients with a complete or partial response ($P=0.0435$), whereas ERK1/2 was higher in patients who did not respond ($P=0.0417$). High ERK1/2 was an independent predictor of poor survival. High ERK1/2 was associated with decreased survival in the archival melanoma cohort, suggesting that high ERK1/2 expressing tumors are biologically more aggressive. All of the six patients with both high VEGF-R2 and low ERK1/2 responded to SCP.

Conclusions—High VEGF-R2 expression is associated with response to SCP in melanoma, whereas high ERK1/2 is associated with resistance. Collection of specimens from SCP-treated melanoma patients in a cooperative group phase III trial comparing this regimen to the chemotherapy alone is ongoing, and confirmation of these findings is necessary. These markers might be useful for predicting response to sorafenib when given with other chemotherapies and in other diseases, resulting in possible elimination of unnecessary treatment of patients unlikely to respond.

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Keywords

Sorafenib; ERK; VEGF-R2; melanoma

INTRODUCTION

Melanoma is the sixth most common cancer, and the malignancy with the highest rise in incidence over the past decades, with an estimated 62,480 new cases and 8,420 deaths in the United States in 2008 (1, 2). The increase in mortality from melanoma is believed to be partially due to an increase in incidence, but also due to the limited efficacy of current treatment regimens for unresectable melanoma, which induce responses in a mere 10–20% of patients. No single agent or combination of agents have been shown to improve survival when compared to dacarbazine alone (3). Therefore, new approaches are required to improve outcome in unresectable melanoma.

There is increasing evidence that melanoma is a heterogeneous disease, which can arise from various distinct genetic aberrations, some related to environmental factors such as sun exposure, while others are related to anatomic location of the lesion. Examples include B-Raf mutations found in melanomas arising in sun damaged skin and c-Kit mutations found in mucosal and acral lentiginous melanomas (4, 5). Similarly, immune system attributes differ among patients (6). Future classification of melanoma patients based on their tumor and immune characteristics will likely result in selective targeted therapies for subsets of patients, which will improve response rates and outcome.

Two signaling pathways are known to be constitutively activated in the majority of melanomas; the MAPK pathway (Ras-Raf-MEK-ERK) and the AKT pathway (PI3K-AKT) (7, 8). Alteration of signaling through both the MAPK and AKT pathways plays a major role in melanoma development and progression. B-Raf is a key member of the MAPK pathway, and activating B-Raf mutations are found in approximately 70% of the cutaneous melanomas. The mutations typically involve a substitution of glutamate to a valine at position 600 (formerly described as V599E) (9, 10). Although the V600E mutation constitutively activates B-Raf and results in downstream MAPK signaling that translates into pro-angiogenic and pro-tumorigenic effects, it is not the only mechanism of oncogenic transformation, since the MAPK pathway is also activated in melanomas that lack this activating mutation (11). In addition, B-Raf^{V600E} mutations are found as frequently in atypical nevi, indicating that additional genetic alterations are needed for malignant transformation (12). Taken together, these data suggest that B-Raf activating mutations play a significant role in melanoma progression, but are neither essential nor sufficient for its development, and diverse pathways and mechanisms coexist that result in signaling through the MAPK pathway.

As with other types of cancer, targeted therapy (where proteins over-expressed and/or constitutively activated are the specific targets of drug-inhibition) is one of the current focuses of drug development for melanoma, with the hope that targeting underlying tumor characteristics will result in higher response rates than classical DNA and microtubule damaging cytotoxic agents. Sorafenib (BAY 43-9006, Nexavar) is an orally administered

multikinase small molecule inhibitor, developed jointly by Bayer Pharmaceuticals (West Haven, CT) and Onyx Pharmaceuticals (Emeryville, CA). Sorafenib inhibits a number of proteins involved in tumor neovascularization and tumor cell proliferation and survival, including C-Raf, B-Raf, B-Raf^{V600E}, VEGF-R2, VEGF-R3, Flt3, FGF-R1, PDGF-R β , c-Kit and p38 α (13). It inhibits activation of the MAPK pathway, slowing cell proliferation in melanoma cell lines and xenograft models (13–17).

Sorafenib has been evaluated in phase I, II and III clinical trials in several types of cancers including RCC, HCC, squamous cell carcinoma of the head and neck, colorectal cancer, non-small cell lung cancer and melanoma, where the agent was utilized alone or in combination with a range of standard chemotherapies (18–20). A survival benefit for sorafenib treated patients in RCC and HCC has been demonstrated in phase III trials (21, 22), and the drug has been approved by the Food and Drug Administration for treatment of these diseases.

In melanoma single-agent sorafenib had little activity with response rates of less than 10% (23, 24). However, responses were seen in a separate phase I study, where sorafenib was administered in combination with paclitaxel and carboplatin (25, 26). Therefore, a phase II expansion trial was conducted in patients with melanoma, enrolling a total of 105 patients, 76 of whom were enrolled at the University of Pennsylvania. The response rate was 27%, and one patient had a complete response. At six months follow-up, 73% of the patients remained either with stable disease, partial response or complete response (25, 27). Due to these promising results, a phase III clinical trial was opened by the Eastern Cooperative Oncology Group (ECOG), which was endorsed by the other United States cooperative groups. Eight hundred and twenty-four patients have been enrolled and randomized to receive first line therapy with either sorafenib, carboplatin and paclitaxel or placebo, carboplatin and paclitaxel. Accrual has just been completed, and the results are pending. A second smaller phase III trial was conducted by Bayer and Onyx Pharmaceuticals randomizing 270 patients whose disease had progressed on prior dacarbazine or temozolomide-containing chemotherapy regimens, to receive SCP or carboplatin, paclitaxel and placebo (28). This trial failed to meet its primary end-point of an 83% improvement in progression free survival, suggesting that the addition of sorafenib to chemotherapy might not be effective as second line treatment. However, results from the larger ECOG trial are awaited and will clarify the benefit of adding sorafenib to chemotherapy in the first line treatment setting.

In the phase I/II studies using SCP, no clear association was seen between the presence of B-Raf mutations and response to therapy (25). Thus subsequent randomized trials have been conducted in unselected patients.

In this study we assessed expression of several known direct and indirect sorafenib targets and their downstream mediators in 44 specimens from melanoma patients who received SCP in early phase clinical trials. We evaluated differences in marker expression between responders and non-responders using a novel, objective, method of automated, quantitative analysis, (AQUA) on tissue microarrays. This method has been validated for epithelial cancers and melanoma and shown to be more precise than pathologist-based scoring of 3,3'-

diaminobenzidine stain. AQUA is highly reproducible and quantitative (29, 30). Although the cohort used in this study is small, our results indicate that high VEGF-R2 and low ERK1/2 are associated with a greater likelihood of response to this drug regimen.

MATERIALS AND METHODS

Cell Lines and Western Blots

YUSAC, YUSOC, YUMAC, YUFIC and YUROB are cell lines derived from tumors of patients treated at Yale University. The MEL501 cell line was obtained from Dr. Steven Rosenberg at the Surgery Branch, National Cancer Institute, MD. Proteins from lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed by standard methods utilizing antibodies listed in Supplemental Table 1 (working dilution, vendor and catalog number are provided). β -Actin (A2066 or A5441, SIGMA-ALDRICH) was utilized as a control to standardize sample loading. Detection of proteins was done using anti-mouse or anti-rabbit IgG secondary antibodies (715-035-151 and 711-035-152, Jackson ImmunoResearch Laboratories, 1:5000) and ECL (PerkinElmer).

Patients and Study Design

This Phase I/II clinical study was conducted at the University of Pennsylvania. Enrolment began in August 2002 and accrual was completed in May 2004. The study enrolled 105 patients, and specimen collection was conducted among the 76 patients enrolled at the University of Pennsylvania with institutional review board approval. All patients had pathologically confirmed advanced unresectable or metastatic melanoma. The study design, including the patient details, has been described previously (26). Patients were required to have a good performance status, progressive disease that was measurable, adequate organ function and no evidence of brain metastases. More than 70% of these patients had failed prior therapies.

Treatment and Assessment of Response

Carboplatin and paclitaxel were administered at an AUC of 6 and 225 mg/m², respectively, every three weeks. Sorafenib was given at 400mg PO BID from day 2 to day 19 of each 21-day cycle. Patients were evaluated for response by RECIST (Response Evaluation Criteria In Solid Tumors) after every two cycles. CR was defined as no residual visible tumor; PR as > 20% tumor shrinkage; SD as a decrease of < 20% or increase of < 30%; PD as an increase of at least 30% or emergence of new lesions.

Tissue Microarray (TMA) construction

Pre-treatment formalin-fixed paraffin embedded tumor biopsies were obtained from 46 patients with the approval of an Institutional Review Board. All biopsies were obtained prior to enrollment on the clinical trial. The specimens included metastases from several sites; lymph nodes, skin, soft tissue and visceral metastases (lung, intestine, and liver). There were only two primary melanomas, from patients whose metastatic melanoma was confirmed by fine needle aspirate. A pathologist (DLR) examined each case and selected a representative region of invasive tumor to be included in the array. Two cores from each block were taken

to construct tissue microarrays as previously described (31, 32). A total of 67 tissue cores (from 46 metastatic melanoma patients enrolled in the trial with more than one tumor block for 11 patients), each measuring 0.6 mm in diameter, were spaced 0.8 mm apart on a tissue microarray using a Tissue Microarrayer. The tissue microarrays were then cut into 5- μ m sections and placed on glass slides using an adhesive tape-transfer system with UV cross-linking. To account for experimental variation and for normalization across array blocks, specimens from a series of 39 metastatic patients were obtained from the Yale University Department of Pathology archives and were placed on the slides. Similarly, pellets of 14 melanoma cell lines were embedded as described in all array blocks (33).

For ERK1/2 expression we also used our large cohort melanoma and nevi arrays that were constructed from a historical cohort as described previously (31, 32). Cores from 230 primary melanomas and 293 metastatic melanomas were included. Specimens and clinical information were collected with approval of a Yale University Institutional Review Board. The specimens were resected between 1959 and 2000, with a follow-up range between 2 months and 40 years, and a mean follow-up time of 6.7 years. Age at diagnosis ranged from 18 to 91 (mean age 52.4 years), 55% were males and 45% were females. The array contained cell lines, cored from pellets, as previously described (33).

Immunohistochemistry

One set of two slides (each containing a core from different areas of tumor for the same patient) was stained for all markers. Staining was done for AQUA as described previously (31, 32) and is depicted in Figure 1. Briefly, slides were deparaffinized in xylene followed by two rinses in 100% ethanol. Antigen retrieval was performed by boiling the slides in a pressure cooker filled with 6.5 mM sodium citrate (pH 6.0). Slides were incubated in a mixture of methanol and 2.5% hydrogen peroxide for thirty minutes at room temperature to block the endogenous peroxidase activity. To block non-specific staining, slides were then incubated at room temperature for 30 minutes in 0.3% bovine serum albumin/1X Tris-buffered saline. Slides were incubated with the primary antibody diluted in Tris-buffered saline containing 0.3% bovine serum albumin at 4°C overnight; antibody species and working dilution are provided in Supplemental Table 1. For VEGF-R2 we used a mouse monoclonal antibody from Santa Cruz (A-3), at 1:200; for ERK1/2 we used a mouse monoclonal antibody from Cell Signaling (L34F12) at 1:400. Slides were then washed three times in 1X Tris-buffered saline/0.05% Tween-20. Either Goat anti-mouse (for mouse primary antibodies) or goat anti-rabbit (for rabbit primary antibodies) horseradish peroxidase-decorated polymer backbone (Envision, Dako) was utilized to visualize the target protein. To create a tumor mask, slides were simultaneously incubated with either mouse or rabbit anti-S100 at 1:100. For visualization of S100 staining a goat anti-mouse or anti-rabbit IgG conjugated to Alexa 546 (Molecular Probes, Inc.) at 1:200 was utilized. Biomarker staining was visualized with Cy5-tyramide (NED Life Science Products). Coverslips were mounted with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen).

Automated Image Acquisition and Analysis

Images were analyzed using algorithms that have been extensively described (29, 30). Briefly, monochromatic, high-resolution (1280×1024 pixel) images were obtained of each histospot. Tumor was distinguished from stromal elements by S-100 signal. Coalescence of S-100 at the cell surface was used to localize cell membrane/cytoplasmic compartment within the tumor mask, and DAPI was used to identify the nuclear compartment within the tumor mask. Targets were visualized with Cy5. Multiple monochromatic, high-resolution ($1,024 \times 1,024$ pixel, $0.5\text{-}\mu\text{m}$) grayscale images were obtained for each histospot using the 10x objective of an Olympus AX-51 epifluorescence microscope (Olympus) with automated microscope stage and digital image acquisition driven by a custom program and macrobased interfaces with IPLabs software (Scanalytics, Inc.). The signal intensity of the target biomarker was scored on a scale of 0–255 (the AQUA score).

For all markers, staining was mostly cytoplasmic and membranous, and therefore we only analyzed the cytoplasmic expression. For ERK1/2 a few spots showed both nuclear and cytoplasmic staining. We excluded histospots with few tumor cells, selecting >3% as an arbitrary threshold for inclusion.

Data Analysis

JMP version 5.0 software was used (SAS Institute, Cary, NC). The prognostic significance of parameters was assessed using the Cox proportional hazards model with progression free survival and overall survival as an end point. Univariate survival analyses were depicted using the Kaplan-Meier method. The association between continuous AQUA scores and other clinical/pathological parameters was assessed by analysis of variance. Pearson's correlation coefficient was used to assess associations between marker expression. All reported p-values are based on two-sided significance testing. ANOVA and t-test were used to compare continuous measurements. Chi-square tests were used to assess associations of contingency table.

RESULTS

Antibodies were validated by Western blots of lysates obtained from a panel of melanoma cell lines to verify presence of a single or dominant band corresponding to the correct molecular weight (data not shown). Normalization to β -Actin loading showed that all targets were variably expressed in the melanoma cell lines.

To account for technical variation in AQUA analysis, 53 control cell lines and metastatic melanoma tissue blocks were included in the arrays. Using the Pearson correlation test we compared scores from the control cases from the two arrays. For all markers we found a high degree of correlation between the matching specimens. The correlations for VEGF-R2 and ERK1/2 were $R = 0.7$ and $R = 0.9$, respectively; $P < 0.0001$ for both markers.

To assess for intratumor heterogeneity, two separate sets of slides, each containing a core from a different area of the tumor for each patient, were utilized to evaluate the expression of each marker. With the exception of ERK1/2, all markers did not show significant amounts of nuclear staining in our specimens, and we therefore analyzed only the

membranous/cytoplasmic compartments. ERK1/2 staining was mostly membranous/cytoplasmic with several specimens showing both nuclear and cytoplasmic staining (Figure 1). Staining patterns within the tumor mask within a histospot were fairly homogenous for all markers analyzed. AQUA scores ranged from 10.624 to 64.345 for VEGF-R2, from 15.13 to 57.706 for VEGF-R1, from 10.208 to 67.175 for VEGF-R3, from 18.042 to 78.891 for FGF-R1, from 23.924 to 76.271 for PDGF-R β , from 26.445 to 88.988 for c-Kit, from 34.045 to 101.83 for B-Raf, from 9.449 to 74.389 for C-Raf, from 2.283 to 42.7 for MEK1 and from 3.851 to 69.36 for ERK1/2. The Pearson correlation test was utilized to compare scores from matching spots from the two arrays. For all markers, expression on two matching specimens from the different arrays was found to be highly correlated ($P < 0.0001$ for all). For patients with multiple spots cored from separate tumor blocks, AQUA scores were averaged to give one AQUA score per patient. For each of the markers, the AQUA scores from both sets of slides were combined to give a single dataset. Tumor spots were deemed un-interpretable if they had insufficient tumor cells, loss of tissue in the spot, or an abundance of necrotic tissue. For patients who had two interpretable histospots, a composite score was formed by calculating the mean of the two scores. For patients with only one interpretable core, the single score was used for analysis. The combined dataset had scores for 44 melanoma patients for VEGF-R2, FGF-R1, PDGF-R β and C-Raf, 42 for c-Kit, 41 for VEGF-R1 and B-Raf, 39 for VEGF-R3 and ERK1/2 and 38 for MEK1.

For survival analysis and analysis of response to therapy, three patients with an overall survival (OS) of less than 42 days (two therapy cycles) were eliminated from the study, as they were deemed not assessable. To assess the association between marker expression and response to therapy we performed analysis of variance. We compared expression of each biomarker in two patient groups; responders (complete response (CR) and partial response (PR)) and non-responders (stable disease (SD) and progressive disease (PD)). In this trial, achieving a CR or PR was associated with a significantly longer PFS compared to patients who achieved stable disease as a best response (median 15.2 months vs. 6.7 months). Unpaired t-tests showed that expression of VEGF-R2 was significantly higher in patients who responded to therapy (CR + PR) than in non-responding patients ($P = 0.0435$) (Table 1 and Figure 2A.). Conversely, non-responders had significantly higher levels of ERK1/2 than patients with PR and CR ($P = 0.0417$) (Table 1 and Figure 2B). None of the other markers analyzed showed a statistically significant association between tumor expression and response to therapy (Table 1).

We assessed the association between site of metastatic biopsy and expression of VEGF-R2 and ERK1/2 by analysis of variance. Sites of metastatic disease were divided into three groups: skin and soft tissue metastases, lymph node metastases and visceral metastases. Both VEGF-R2 and ERK1/2 expression were not associated with the site of metastases ($P = 0.84$; $P = 0.855$, respectively).

We next assessed the association between either dichotomized scores or continuous AQUA scores for each marker and progression free survival (PFS) or overall survival (OS) by Cox univariate analysis. High ERK1/2 expression was associated with decreased PFS ($P = 0.0035$) and decreased overall survival (OS) ($P = 0.0189$). No association between

expression of other markers and melanoma-specific survival, PFS or OS was found (data not shown).

To visualize the association between continuous ERK1/2 scores and survival, we divided the AQUA scores into quartiles, reflecting the use of routine statistical divisions in the absence of an underlying justification for division of expression. Kaplan-Meier survival curves were generated for ERK1/2 expression and PFS and OS (Figure 3A and 3C). The *P*-values corresponding to these curves were obtained by the Mantel-Cox log-rank method. Low ERK1/2 expression is associated with better PFS and OS ($P = 0.0027$ and $P = 0.01$, respectively). The Kaplan-Meier curves demonstrate clear convergence of the top three quartiles, to define these patients as “low” ERK1/2 expressers and the fourth quartile as “high” expressers. This cut-point corresponded to an AQUA score of 35.485. The dichotomized Kaplan-Meier survival curves for PFS and OS are shown in Figure 3B and 3D ($P = 0.0007$, $P = 0.0084$).

Using the Cox proportional hazards model, we performed multivariable analysis, and found that high ERK1/2 expression remained an independent predictor of poor survival ($P = 0.0002$ for PFS and $P = 0.0015$ for OS). Data are only shown for PFS. The only other variable associated with PFS by multivariate analysis was administration of prior therapies ($P = 0.0081$). All other variables included in the model (age, gender, and AJCC M stage) were not associated with PFS (Table 2).

To assess the association between marker expression and other clinical parameters, we used analysis of variance. We found that low C-Raf expression was significantly correlated with advanced AJCC M stage ($P = 0.0182$). For all other markers, there was no significant association between expression and age, gender, administration of prior therapies or AJCC M stage. To assess the association between ERK1/2 expression and presence of the V600E B-Raf mutation, we performed analysis of variance. No significant differences in ERK1/2 expression were seen in B-Raf mutant and wild type tumors ($P = 0.468$). Similarly B-Raf mutation status was not associated with any of the other sorafenib targets studied.

To assess the combined effect of high VEGF-R2 and low ERK1/2 on response, we generated a composite VEGF-R2/ERK1/2 score by arbitrarily dichotomizing AQUA scores by the associated median score for each marker (24.426 for ERK1/2 and 32.207 for VEGF-R2). We then defined three groups of patients: one group had low VEGF-R2 and high ERK1/2 expression (six patients), the second group had either low VEGF-R2 and low ERK1/2 or high VEGF-R2 and high ERK1/2 (24 patients), and the third group had high VEGF-R2 and low ERK1/2 (six patients). We found a strong association between this composite VEGF-R2/ERK1/2 score and response to therapy, as shown in Figure 2C; all six patients with high VEGF-R2 and low ERK1/2 expression had a partial or complete response ($P = 0.0012$, $\chi^2 = 13.484$).

We assessed associations between markers by Pearson correlation test, as demonstrated in Figure 4A. Expression of most of the markers was found to be correlated. Yellow shades represent positive correlations, and turquoise shades represent anti-correlations. The strongest correlations were found among B-Raf, VEGF-R1, PDGF-R β and c-Kit.

Given that ERK1/2 was associated with survival in the treated cohort, we attempted to ascertain whether this is related to the natural history of the disease or treatment effect, by employing our historical cohort of patients who were not treated with sorafenib. This cohort included 230 primary and 293 metastatic melanomas. For ERK1/2, the AQUA scores from two corresponding sets of slides was found to be highly correlated ($R = 0.78$), and were combined to give a single dataset with a total of 254 AQUA scores for metastatic specimens and 175 scores for primary melanomas. AQUA scores ranged from 2.89 to 125.47. By two-sample t-tests we found higher ERK1/2 expression in metastatic specimens than primary specimens (Figure 4B). High ERK1/2 expression correlated with high Clark level (stage IV and V) ($P = 0.0055$) and advanced stage of disease ($P < 0.0001$), but not with other clinical variables including age, gender, Breslow depth, presence of ulceration or presence of tumor infiltrating lymphocytes. By Cox univariate survival analyses of raw AQUA scores, we found that high ERK1/2 expression correlated with decreased survival in the entire cohort ($P = 0.0484$) as well as in the primary subset of specimens ($P = 0.0209$). On multivariate analysis ERK1/2 expression did not retain its independent predictive value. The only variable associated with survival by multivariate analysis was Clark level ($P = 0.016$). We then dichotomized the continuous ERK1/2 AQUA scores arbitrarily by the median score. Kaplan-Meier survival curves are shown in Figure 4C. Log-rank analysis revealed a significant association between high ERK1/2 expression and poor survival in the primary subset of patients ($P = 0.0327$). These results indicate that high ERK1/2 is associated with biologically aggressive disease, and might not be related to resistance to SCP.

DISCUSSION

In this study we quantitatively assessed the expression of targets of sorafenib in pre-treatment tumors of patients who were enrolled in a phase I/II trial of SCP, with the goal of identifying predictors of response to this multi-drug regimen. We used an objective, automated method which provides continuous output scores rather than arbitrary pathologist-based divisions of scores into “high/low” or “positive/negative”. We found that expression of VEGF-R2 was significantly higher in patients who obtained a complete or partial response to therapy than in patients with stable or progressive disease. Responders had significantly lower expression of ERK1/2 than non-responders, and we demonstrated a statistically significant association between high ERK1/2 expression and decreased PFS and OS. On multivariable analysis, ERK1/2 retained its independent prognostic value. High ERK1/2 and VEGF-R2 expression was not associated with the presence of B-Raf^{V600E} mutations. We generated a composite VEGF-R2/ERK1/2 score and found that all six patients in this cohort with both high VEGF-R2 and low ERK1/2 responded to this therapy.

Given the association between high ERK1/2 and worse survival in the treated cohort we assessed a historical cohort of melanoma patients to determine whether the association might be due to more aggressive tumor biology rather than resistance to therapy. We found a range of ERK1/2 expression in these tumors, with higher expression in metastatic than primary melanomas. Within the primary melanomas and within the entire cohort we found an association between low ERK1/2 and improved survival, although the association with survival did not retain its significance on multivariable analysis.

While it would have been interesting to study the predictive value of activated (phosphorylated) ERK1/2 in patients treated with sorafenib, we were unable to obtain reproducibility of results with antibodies to phosphorylated ERK1/2. This is likely due to activity of phosphatases in these tissues prior to their fixation and variability in tissue fixation times. These specimens were collected under non-uniform conditions from multiple institutions. In addition to phosphorylated ERK1/2, active forms of other proteins (such as phosphorylated-VEGF-R2) might be highly predictive of response to targeted therapy.

Sorafenib was empirically found to be effective in melanoma patients when given with carboplatin and paclitaxel in the phase I/II studies (26), yet the most important target/s and prognostic markers remain unknown. Sorafenib has also been administered with other chemotherapies, including dacarbazine and temozolamide, and these combinations also appear to be active (25–27, 34–36). Although intensively studied, the precise mechanism by which sorafenib induces cell death is still unclear. *In vitro* studies show that it dually targets cell proliferation, cell survival and angiogenesis by inhibiting B-Raf^{wt}, B-Raf^{V600E}, C-Raf, c-Kit, Flt-3, VEGF-R2 and VEGF-R3 and PDGF-R β (13, 37, 38). Inhibition of these targets occurs at drug concentrations that can be readily achieved in humans (IC₅₀ < 100 nM). Our results support the idea that response to therapy is associated with inhibition of the VEGF pathway, dependent on inhibition of VEGF signaling through its receptor VEGF-R2, as demonstrated by the fact that patients with high VEGF-R2 expression were more likely to respond. This is likely due to both inhibition of angiogenesis and inhibition of cell proliferation driven by the presence of a VEGF/VEGF-R2 autocrine loop in tumor cells, as indicated in other studies which showed that melanoma cells which overproduce both VEGF and its receptors display a higher ability to invade into the extracellular matrix, as well as an increased growth and survival via the MAPK and PI3K signaling pathways (39, 40). We and others have found that VEGF and VEGF-R2 are co-expressed in most melanoma tumor cells (41–43) and similar findings have been reported for other solid tumors as well (44, 45). In a previous study we analyzed expression of VEGF and its receptors in tumor cells using a large melanoma cohort of over 400 melanomas (Mehnert et. al., submitted). We showed that up-regulation of VEGF and VEGF-R2 was associated with disease progression, indicating that a VEGF/VEGF-R2 autocrine loop exists in tumor cells in advanced melanoma cases.

Based on the observation that RNA interference targeted against mutated B-Raf induces growth arrest and apoptosis in human melanoma cells (46), it was initially thought that sorafenib exerts its anti-tumor activity by specifically inhibiting B-Raf^{V600E}. This notion was later refuted by studies showing that sensitivity to sorafenib in cancer cells is independent of K-Ras and B-Raf mutational status and by clinical studies showing that no clear association exists between the presence of B-Raf activating mutation and response to therapy, as response rates in metastatic melanoma were higher (75%) for patients with wild type B-Raf than for those harboring the activating mutation (55%) (14, 26). This reinforces the idea that B-Raf activating mutations although significant for melanoma progression, are not essential or sufficient for its development and that accessory pathways and mechanisms coexist and are utilized by melanoma cells to ensure oncogenic signaling through the MAPK pathway. Therefore, ascertaining the mutation status of B-Raf before treatment with sorafenib, carboplatin and paclitaxel is not a reliable means of predicting clinical efficacy.

High ERK1/2 expression, while not associated with B-Raf mutational status, was found to be associated with resistance to therapy. Although we realize that high ERK1/2 expression does not necessarily correlate with its level of activation, we speculate that elevated levels of ERK1/2 are at the least the consequence of cellular mechanisms that might reflect overproduction and activation of other mediators and pathways that induce tumor growth. Interestingly, several previous studies indicate that the B-Raf^{V600E} mutation is independent of nevo-melanoma progression, and does not correlate with ERK1/2 phosphorylation, suggesting that alternative signaling pathways for MAPK activation exist and can control melanoma progression (47, 48). The association between high ERK1/2 and decreased PFS and OS in our small treated cohort might either be due to resistance to treatment or due to a biologically more aggressive cell type. The latter possibility is supported by our findings of an association between high ERK1/2 expression and decreased survival in our large historical melanoma cohort.

While VEGF-R2 and ERK1/2 can be co-expressed (as VEGF-R2 signals through ERK1/2), when high expression of VEGF-R2 is associated with low expression of ERK1/2, the likelihood of response to SCP appears to be highest. This demonstrates once more that melanoma oncogenesis is a very complex process that might involve simultaneous signaling through different pathways and that these drugs might be most effective in melanomas where signaling through VEGF-R2 and its downstream effectors is predominant and less effective when other receptor tyrosine kinases not inhibited by sorafenib might be activating the MAPK pathway, bypassing VEGF-R2. Patients with high VEGF-R2 and low ERK1/2 might have biologically less aggressive disease, and the improved outcome seen in this subset of patients might be a reflection of disease biology.

In summary, two major limitations of current treatments for metastatic melanoma are lack of specificity for melanoma cells and drug resistance. Targeted therapies in melanoma are likely to be effective only for the subset of patients whose tumors express the relevant target. Therefore biomarker studies aimed at identifying key proteins that predict response or resistance to targeted therapies, are important and necessary for improving patient care and eliminating the unnecessary treatment of patients who are unlikely to respond to treatment. Other small melanoma studies have shown that serum and tissue biomarkers and DCE-MRI can be useful for predicting response to chemotherapy and VEGF-receptor targeted therapies (49, 50). While the results of the present study should be interpreted with caution due to the small size of the cohort analyzed, it is a necessary first step in identifying potential predictive biomarkers and establishing technical feasibility for biomarker studies that can be used in future analyses of patients treated with sorafenib and chemotherapy. Accrual to the ECOG trial comparing carboplatin, paclitaxel and sorafenib to carboplatin, paclitaxel and placebo in 824 patients was recently completed, and retrospective collection of tumors of patients treated on this trial is ongoing. The results of the present study will be applied in the future to this large randomized clinical trial, and this will enable us to validate the present findings. In addition, the randomized trial will enable us to determine whether high VEGF-R2 and low ERK1/2 are associated with sensitivity to SCP or sensitivity to chemotherapy alone. Furthermore, these pilot studies can also be applied to prediction of response to sorafenib in combination with other chemotherapies (dacarbazine and temozolamide) in

melanoma and to sorafenib-based treatment in other diseases such as renal cell carcinoma and hepatocellular carcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STATEMENT OF TRANSLATIONAL RELEVANCE

This manuscript details laboratory based findings utilizing specimens from patients treated in a phase I/II clinical trial assessing response to sorafenib, carboplatin and paclitaxel in metastatic melanoma. This regimen has clear clinical activity in a subset of melanoma patients, and characterization of pre-treatment specimens from these patients can identify predictors of response to this therapy. This will enable future selective treatment of patients more likely to respond. We found that high expression of VEGF-R2 and low expression of ERK1/2 were associated with a greater likelihood of response. While the cohort in this trial was small, these studies form the basis of future analyses of this regimen in an 824 patient randomized phase III trial, and can also be beneficial for predicting response to other sorafenib-based regimens in other diseases.

Figure 1. Membranous and cytoplasmic ERK1/2 expression in melanoma histospots
 AQUA uses S100 to define the tumor mask (*bottom-right panel A*), 4,6-diamidine-2-phenylindole to define the nuclear compartment (*bottom-left panel A*) and Cy5 (*top-right panel A*) for the target (ERK1/2). The cytoplasmic compartment is generated by subtracting the nuclear compartment from the S100 mask (*top-left panel A*). ERK1/2 expression is then measured within the nuclear and cytoplasmic compartments within the tumor mask, and each spot is assigned a score based on pixel intensity per unit area. ERK1/2 expression in this example corresponds to an AQUA score of 67.38 and it is shown at 10X magnification (*top-right panel A*) and 60X magnification (*panel B*).

Figure 2. Association of VEGF-R2 and ERK1/2 expression and response to therapy

A) VEGF-R2 expression is higher in patients with CR and PR. B) ERK1/2 expression is higher in patients with SD and PD. C) Chi-square analysis was utilized to compare high/low VEGF-R2/ERK1/2 expression and response to therapy. VEGF-R2 and ERK1/2 scores were arbitrarily dichotomized to high and low categories by the associated median score for each marker (24.426 for ERK1/2 and 32.207 for VEGF-R2). The three groups of patients were defined as follows: one group had low VEGF-R2 and high ERK1/2 expression, the second group had either low VEGF-R2 and low ERK1/2 or high VEGF-R2 and high ERK1/2, and the third group had high VEGF-R2 and low ERK1/2. All of the patients in the high VEGF-R2 and low ERK1/2 group responded to therapy.

Figure 3. Association between high ERK1/2 expression and decreased survival

Panels A and C show Kaplan–Meier curves for ERK1/2 expression and PFS or OS where AQUA scores were divided into quartiles. The survival for the highest quartile was shorter than the other three quartiles. Panels B and D show Kaplan–Meier curves for ERK1/2 expression and PFS or OS where scores were dichotomized into “low” (the top three quartiles) and “high” (the fourth quartile). This cut-point corresponds to an AQUA score of 35.485.

Figure 4. A) Pearson correlations for all pairs of markers

Yellow shades represent positive correlations, and turquoise shades represent anti-correlations. The intensity of color in each square represents the strength of the association. Almost all correlations correspond to a p -value of less than 0.0004 (corresponding to the Bonferroni correction for 112 multiple tests at an alpha of 0.05). Note that for ease of reading, scores are represented twice. **B and C) High ERK1/2 expression is associated with disease progression and poor prognosis in archival melanoma specimens. B)** Analysis of variance comparing the AQUA scores for primary and metastatic specimens. ERK1/2 expression is higher in metastatic specimens than primary specimens. **C)** Kaplan-Meier survival curves for ERK1/2 AQUA scores divided by the median score for the primary subset of patients. High ERK1/2 expression is associated with poor survival.

TABLE 1

Association between marker expression and response to therapy.

Markers	Mean Score (95% Confidence Interval)		<i>P</i> value
	PD + SD	PR + CR	
MEK1	13.276 (9.791–16.762)	16.032 (11.498–20.567)	0.3816
B-Raf	60.838 (53.063–68.613)	62.406 (52.227–72.586)	0.7958
C-Raf	26.534 (21.610–31.458)	26.263 (19.781–32.746)	0.9399
ERK1/2	29.891 (23.221–36.563)	20.140 (11.777–28.503)	0.0417
VEGF-R1	34.148 (29.442–38.856)	34.926 (29.098–40.755)	0.8228
VEGF-R2	29.067 (25.408–32.727)	35.459 (30.641–40.277)	0.0435
VEGF-R3	41.666 (35.942–47.392)	42.671 (35.494–49.848)	0.8152
FGF-R1	48.040 (41.483–54.597)	48.846 (40.214–57.480)	0.8771
PDGF-Rβ	48.474 (42.833–54.115)	47.744 (40.318–55.171)	0.8671
CD117, c-Kit	53.519 (45.791–61.249)	51.761 (41.985–61.538)	0.7548

TABLE 2

Multivariable Cox proportional hazards analysis of ERK1/2 and other clinical variables.

Variable	Hazard Ratio	95% Confidence Interval	P value
ERK1/2 (high vs. low)	2.965	(1.691–5.296)	0.0002
Gender (male vs. female)	1.219	(0.797–1.852)	0.3547
Age (>50 vs. younger)	1.398	(0.932–2.147)	0.1052
AJCC M Stage [B][C] vs. [A]	2.430	(0.966–5.487)	0.0744
Administration of Prior Therapies	1.961	(1.189–3.365)	0.0081