Human Cancer Biology

C-Raf Is Associated with Disease Progression and Cell Proliferation in a Subset of Melanomas


Abstract

Purpose: Raf-kinases include three major isoforms. Although the role of B-Raf in melanoma is well established, little is known about C-Raf. We studied effects of C-Raf knockdown in vitro and assessed expression of C-Raf in a large cohort of melanomas and nevi.

Experimental Design: Using specific siRNAs, we knocked down C-Raf expression, and determined the effect on viability, MAP extracellular signal-regulated kinase (ERK)/ERK kinase signaling, and apoptosis in seven melanoma cell lines. We determined the IC50 of the C-Raf inhibitors sorafenib and GW5074, and studied the effects of GW5074 on cell signaling. Using an automated method to measure in situ protein expression, we quantified C-Raf expression in 263 nevi and 523 melanomas.

Results: C-Raf was knocked down in three cell lines with detectable phospho-C-Raf, resulting in decreased viability in two of the three (YULAC and YUROB). This resulted in decreased Bcl-2 expression and phospho-Bad cleavage, without affecting phospho-MEK and phospho-ERK. Sensitivity to sorafenib and GW5074 varied. GW5074 inhibited mitogen-activated protein kinase signaling without Bcl-2 and phospho-Bad down-regulation. C-Raf was highly expressed in melanomas compared with nevi (P < 0.0001), and no nevi had high C-Raf expression. C-Raf expression was higher in metastatic than primary specimens (P = 0.0225).

Conclusions: C-Raf siRNA knock-down results in decreased viability of YULAC (B-RafV600K) and YUROB (B-RafWT) melanoma cells, likely mediated by Bcl-2 inhibition rather than mitogen-activated protein kinase inhibition. Cotargeting C-Raf and parallel pathways might be an effective therapeutic approach for melanoma. C-Raf expression is up-regulated in a subset of melanomas but not in nevi, suggesting that it might be a valuable diagnostic marker and therapeutic target. (Clin Cancer Res 2009;15(18):5704–13)

Melanoma is the sixth most common malignancy, with an estimated 62,480 new cases and 8,420 deaths in the United States in 2008 (1). Once it metastasizes, melanoma is associated with a very poor prognosis and is refractory to current treatment regimens, which induce responses in only 10% to 20% of patients (2).

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One of the major current primary focuses in clinical research is the development of targeted therapy, in particular, drugs that specifically inhibit proteins which are overexpressed and/or constitutively activated in melanoma. It is anticipated that targeted therapy specifically administered to patients based on tumor characteristics will improve response rates, whereas the selectivity of targets in malignant cells will decrease toxicity. One of the necessary first steps in developing new targeted therapies is careful characterization of the target in the disease population.

In the vast majority of melanomas, the mitogen-activated protein kinase (MAPK; Ras-Raf-ERK) and the phosphatidylinositol 3-kinase-AKT pathways are up-regulated, and alteration of signaling through both pathways plays a major role in melanoma progression (3, 4). Signaling through the MAPK pathway is initiated by binding of growth factors to their receptors or by constitutive activation of pathway members. Through a series of specific phosphorylations of Ras, Raf, MAP/extracellular signal-regulated kinase (ERK) kinase (MEK), and ERK proteins, growth factor signals are transmitted to transcription factors in the nucleus and are translated into antiapoptotic, protumorigenic, and proangiogenic effects.

Raf serine/threonine protein kinases are pivotal members of the MAPK pathway and comprise three isoforms: A-Raf, B-Raf,
and C-Raf (Raf-1). All RAF kinase isoforms have a common structure (consisting of an NH2-terminal regulatory domain, an activation loop, and a COOH-terminal kinase domain), share three homology regions, and have overlapping as well as distinct functions (5). RAF proteins differ in their tissue distribution; whereas C-Raf is ubiquitously expressed, A-Raf is highly expressed in urogenital tissues and B-Raf is predominantly found in neural, splenic, testicular, and hematopoietic tissues (5). The fact that RAF isoforms have distinct roles is suggested by knockout studies in which 

araf, braf, and craf null mice have different phenotypes (6). C-Raf knockout studies have shown that C-Raf is not necessary for activation of the MAPK pathway, which can still occur through B-Raf, but despite this, the 

craf null phenotype is associated with higher susceptibility to apoptosis (7).

RAF proteins are recruited for activation at the plasma membrane by membrane-bound Ras. Although B-Raf has a higher degree of phosphorylated sites and a higher basal kinase activity requiring fewer steps and only Ras for its activation, A-Raf and C-Raf require multiple Ras-and SRC-dependent phosphorylation and dephosphorylation events for their activation (8). The most important sites of phosphorylation in C-Raf are S338 and Y341, which are critical for complete activation (9). Although A-Raf can activate only MEK1, B-Raf and C-Raf can activate both MEK1 and MEK2; however, B-Raf is by far the strongest MEK activator (10). Gene mutations associated with malignant transformation are nonexistent for A-Raf and extremely rare for C-Raf (11). In a large scale study of 545 cancer cell lines and over 100 primary tumor specimens, 4 C-Raf mutations (only 0.73% of the cell lines) were identified, which did not induce malignant transformation of NIH3T3 cells. However, in a different study, two C-Raf germ line mutations S427G and I448V were identified in patients with therapy-related acute myeloid leukemia. These mutations displayed weak transforming potential and were able to inhibit apoptotic cell death (12).

B-Raf somatic mutations (typically involving a substitution of glutamate to a valine at position 600) are found in 7% to 8% of all malignant tumors and in 60% of cutaneous melanomas. The mutation constitutively activates B-Raf inducing downstream MAPK signaling and is associated with a poor prognosis in metastatic melanomas (13). Therefore, targeting B-Raf in melanoma has become a focus of intense research. Because the MAPK pathway is also activated in melanomas that lack the B-RafV600E mutation, and because this mutation is found as frequently in atypical nevi as in melanomas, MAPK signaling is probably mediated by additional mechanisms (14, 15). Although the role of C-Raf in human cancer has been less evident, there are numerous studies that point to the oncogenic properties of wild-type or active C-Raf; however, thus far, this Raf isoform has been understudied in terms of prevalence in disease populations and drug development.

Elevated C-Raf mRNA or protein levels have been identified in acute myelogenous leukemia, head and neck cancer, prostate cancer, and ovarian cancer (16–19). In ovarian cancer cell lines, antisense oligodeoxynucleotides inhibited cell proliferation in vitro (19). Similar studies in cell lines derived from lung, cervical, prostate, and colon carcinomas showed the same phenomenon (20).

Although activated C-Raf was shown in vitro to be capable of activating MEK and ERK, studies indicate that besides its MAPK-dependent effects (which require its kinase activity), C-Raf has functions independent of the MAPK pathway (which sometimes do not require kinase activity) that can lead to cell proliferation and inhibition of apoptosis (21). Unlike B-Raf, C-Raf can undergo localization to the mitochondria and can directly inhibit cellular apoptosis through its interactions with Bad and Bcl-2 proteins, which it phosphorylates, contributing to increased survival and oncogenic transformation (22). Although MEK1 and MEK2 are the only two downstream substrates for B-Raf, several cellular effectors involved in cell cycle progression, cellular survival, and differentiation, interact with and are activated by C-Raf in a MAPK-independent fashion (23–27). In melanoma, in vitro studies have shown that although C-Raf does not contribute to ERK signaling in cells harboring the B-RafV600E mutation, it is required for proliferation and, although to a lesser degree than B-Raf, it does play a role in cell survival (28). Moreover, Dumaz et al. (29) recently showed that in melanoma cell lines harboring Ras mutations, which are found in approximately 15% to 20% of melanomas, MAPK activation is solely induced by C-Raf and not B-Raf, through a mechanism of molecular switch mediated by cyclic AMP. This provides additional evidence that Raf isoforms, which might have identical upstream targets, can respond differently to the same stimuli and therefore result in different downstream cellular functions.

Due to the recently reported importance of C-Raf in melanoma tumorigenesis, we studied the expression of C-Raf and its active form (phospho-C-Raf Ser338) in several melanoma cell lines and targeted reduction of C-Raf expression and/or activity by small interfering RNA. We found that C-Raf knockdown inhibited proliferation in two phopho-C-Raf–expressing melanoma cell lines. This effect was MAPK independent and involved down-regulation of phospho-Bad and Bcl-2, two C-Raf substrates. We studied the pharmacologic inhibition of C-Raf using sorafenib and GW5074, and found variable degrees of sensitivity among melanoma cell lines. GW5074 completely abrogated MAPK signaling in five cell lines, yet had little or no effect in the other two. To assess the prevalence of C-Raf overexpression in melanoma, we used a large cohort of human melanoma
specimens and benign nevi, and studied the association with several important clinical variables. To the best of our knowledge, this is the first large study looking at expression of C-Raf in melanoma specimens. To obtain quantitative, objective measures of expression, we used an automated, quantitative method (AQUA) to study tissue microarrays (TMA). 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 (30). We found that expression of C-Raf is increased in both primary and metastatic melanomas compared with nevi, and that no nevus overexpress C-Raf. Our results indicate that C-Raf is a relevant diagnostic and therapeutic target in melanoma.

**Materials and Methods**

**Cell lines and Western blots.** YUSAC, YUSOC, YIMAC, YIFIC, YUROB, YUGEN, and YULAC are cell lines derived from tumors of patients treated at Yale University. MEL501, MEL624, and MEL928 cell lines were obtained from Dr. Steven Rosenberg at the Surgery Branch, National Cancer Institute, MD.

DNA was isolated from cell lines using standard methods. Sequencing of cell lines to identify mutations in B-Raf and N-Ras were conducted as described previously (31). Protein concentrations of lysates were calculated by the bicinchoninic acid assay (Pierce Biotechnology). Proteins (50 μg) were diluted in a sample buffer [2.5% SDS, 10% glycerol, 5% β-mercaptoethanol, 50 mMol/L Tris (pH 6.8), and 0.1% bromophenol blue] and subjected to SDS-PAGE. Western blotting was done by standard methods using antibodies to C-Raf (1:1,000; BD Transduction Laboratories; 1:500; Upstate) or to phospho-C-Raf (Ser338; 1:1,000; Cell Signaling). In this study, we also used the following antibodies: B-Raf (1:200; Santa Cruz), phospho-Bad (1:1,000; Cell Signaling), Bcl-2 (1:500; Dako), phospho-ERK1/2 (1:2,000; Cell Signaling), and phospho-MEK1/2 (1:1,000; Cell Signaling). β-Actin (A2066 or A5441; Sigma; 1:200 or 1:5,000, respectively) was used to standardize sample loading. Detection of proteins was done with peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (711035-015 and 711-035-152; Jackson ImmunoResearch Laboratories; 1:5,000) and enhanced chemiluminescence (PerkinElmer).

**siRNA transfection.** Human melanoma cells (YUSAC, YUSOC, YIMAC, YIFIC, YUROB, YUGEN, YUEIC, YUIROB, and MEL501) were cultured under standard conditions (37°C in 5% CO2 atmosphere) and grown in OPTI-MEM and enhanced chemiluminescence (PerkinElmer). Values of treated cells were compared with cells treated with Oligofectamine only and reported as percent viability.

Cell viability assays. For cell viability assays, 1 x 104 cells were plated in triplicate in a 96-well microtiter plate (BD Bioscience) and allowed to grow for 24 h to an approximate confluence of 30%. For C-Raf silencing, 0.7 μL of oligofectamine was incubated with 5 μL of OPTI-MEM for 10 min at room temperature and added to siRNA duplexes prediluted in 14 μL OPTI-MEM, followed by 15-min incubation at room temperature. The siRNA solution was then added to each well containing 80 μL OPTI-MEM and cells were starved for 4 h at which point 50 μL (10%) fetal bovine serum was added.

Cell viability was evaluated at 96 h using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay, according to the manufacturer’s instructions (Promega). In brief, following treatment, 20 μL of CellTiter 96 Aqueous One Solution was added to each well, and the plate was incubated at 37°C for 2 h. Optical densities were measured at 490 nm using the Universal Microplate Reader EL 800 (BIO-TEK Instruments, Inc.). Values of treated cells were compared with cells treated with Oligofectamine only and reported as percent viability.

For drug inhibition studies, sorafenib (LC Laboratories, Inc.) or GW5074 (Sigma-Aldrich, Inc.) dissolved in DMSO were used to treat the cells at concentrations ranging from 1.28 nmol/L to 100 μmol/L. Cell viability was evaluated at 72 h using the CellTiter-Glo Luminescence Cell Viability Assay, according to the manufacturer’s instructions (Promega); an equal volume of the Cell Titer-Glo reagent was added to the wells, and after a short 10-min incubation, luminescence was recorded using a Victor X multibar plate reader (Perkin-Elmer). The IC50 values were determined by the XLife software (MathIQ version 2.2.2, IDBS).

**Caspase-3, -casepase-8, and -casepase-9 activity.** Following siRNA transfection of cells, 10 μg of protein in 50 μL of HEPES buffer were combined with Caspase-Glo 3/7, 8, or 9 reagents (Promega). After incubation for 1 h at room temperature, luminescence was measured using a TD 20/20 Luminometer (Turner Designs). Blank values were subtracted and fold-increase in activity was calculated based on activity measured from untreated cells. Each sample was measured in duplicate.

**TMA construction.** The melanoma and nevi arrays were constructed from a historical cohort as described previously (32, 33). The cohort was constructed from paraffin-embedded, formalin-fixed tissue blocks obtained from the Yale University Department of Pathology Archives. An experienced pathologist examined each case and selected a representative region of invasive tumor to be included into the array. Cores from 230 primary melanomas and 293 metastatic melanomas, each measuring 0.6 mm in diameter, were spaced 0.8 mm apart on slides using a Tissue Microarrayer (Beecher Instruments). The TMAs were then cut to 5-μm sections and placed on glass slides using an adhesive tape-transfer system with UV cross-linking. Specimens and clinical information were collected with approval of a Yale University Institutional Review Board. The cohort has been used in prior publications (32, 33). The specimens were resected between 1959 and 2000, with a follow-up range between 2 mo and 40 y, and a mean follow-up time of 6.7 y. Age at diagnosis ranged from 18 to 91 y (mean age, 52.4 y). The cohort included 55% males and 45% females. Similarly, a TMA was made containing cores from 263 benign nevi. The nevus array contained 40 metastatic and primary specimens from patients that were also represented on the melanoma array. Both arrays contained identical cell lines, cored from pellets, as previously described (34). The overlapping metastatic and primary specimens and cell lines were used for normalization of the scores obtained from the benign and malignant arrays.

**Immunohistochemistry.** One set of two slides (each containing a core from different areas of tumor for the same patient) was stained for C-Raf. Staining was carried out for AQUA as described previously (32, 33). Briefly, slides were deparaffinized in xylene followed by two rinses in 100% ethanol. Antigen retrieval was done by boiling the slides in a pressure cooker filled with 6.5 mmol/L sodium citrate (pH 6.0). Slides were incubated in a mixture of methanol and 2.5% hydrogen peroxide for 30 min at room temperature to block the endogenous peroxidase activity. To block unspecific staining, slides were then incubated at room temperature for 30 min in 0.3% bovine serum albumin/1% TBS. Slides were incubated with the primary antibody (rabbit monoclonal anti-Raf-1 IgG; Upstate) diluted 1:100 in TBS containing 0.3% bovine serum albumin at 4°C overnight. Slides were then washed thrice in 1× TBS/0.05% Tween 20. Goat anti-rabbit horseradish peroxidase–decorated polymer backbone (Envision; Dako) was used as a secondary reagent. To create a tumor mask, slides were simultaneously incubated with a cocktail of mouse anti-S100 at 1:100 (BioGenex) and mouse anti-HMB45 at 1:100 (BioGenex). For visualization
of S100 and HMB45 staining, goat anti-mouse IgG conjugated to Alexa 546 (Molecular Probes, Inc.) at 1:200 was used. C-Raf 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).

Automated image acquisition and analysis. Images were analyzed using algorithms that have been extensively described (30). Tumor was distinguished from stromal elements by S-100 and HMB45 signal. Coalescence of S-100 and HMB45 at the cell surface was used to localize cell membrane/cytoplasm compartment within the tumor mask, and 4',6-diamidino-2-phenylindole was used to identify the nuclear compartment within the tumor mask. Targets were visualized with Cy5. Multiple monochromatic, high-resolution (1,024 × 1,024 pixel; 0.5-μm) grayscale images were obtained for each histostop using the ×10 objective of an Olympus AX-51 epifluorescence microscope (Olympus) with an automated microscope stage and digital image acquisition driven by custom program and macro-based interfaces with IPLabs software (Scanalytics, Inc.). The signal intensity of the target was scored on a scale of 0 to 255 (the AQUA score).

Staining was cytoplasmic and membranous. We excluded histospots with few tumor cells, selecting ≥3% as a threshold for inclusion.

Data analysis. JMP version 5.0 software was used (SAS Institute). The prognostic significance of parameters was assessed using the Cox proportional hazards model with survival as an end point. The association between continuous AQUA scores and other clinical/pathologic parameters was assessed by ANOVA.

Results

C-Raf siRNA decreases cell viability in a B-Raf–mutated and a B-Raf wild-type melanoma cell line. Recent studies suggest that N-Ras (Q61K, Q61R, and Q61L) and low-activity B-Raf–B-Raf wild-type melanoma cell line. Recent studies suggest that specific siRNAs inhibited the expression of C-Raf by about 68% to 90% in all melanoma cell lines at a concentration of 75 nmol/L. Of note, expression of B-Raf was not affected by C-Raf siRNA treatment; treatment with a control siRNA (TS1058) at a concentration of 75 nmol/L had absolutely no effect on C-Raf or the other proteins, thereby demonstrating the specificity of the siRNA knockdown.

To determine the effect of C-Raf siRNA on viability of our melanoma cell lines, we used the CellTitre 96 Aqueous One Solution Cell Proliferation Assay. As shown in Fig. 2A, at 96 hours of incubation, 75 nmol/L of both C-Raf siRNA clones inhibited cell proliferation of the YULAC and YUROB cell lines by 43% to 58% and 50% to 65%, respectively. The effect on the other cell lines was less pronounced, indicating that the effect is independent of the B-Raf mutational status. At a concentration of 12.5 nmol/L, C-Raf siRNA did not significantly affect protein expression or cell viability (data not shown). At 75 nmol/L, the control siRNA did not affect cell viability in any of the cell lines tested. Shorter transfections with the siRNAs had little to no effect: at 72 hours of incubation, C-Raf siRNA inhibited cell proliferation of the YULAC cell line by 23% to 39%, whereas it had negligible effects on the other cell lines (Fig. 2B). At 48 hours of incubation, C-Raf silencing did not significantly alter cell viability in any of the seven melanoma cell lines (Fig. 2C).

C-Raf siRNA alters Bcl-2 and phospho-Bad expression in the YULAC and YUROB cell lines without affecting MAPK signaling. To study the intracellular effect of C-Raf knockdown in melanoma cells, we transfected melanoma cells with 75 nmol/L of C-Raf–targeted siRNA and assessed its effect on activation of apoptotic mediators, including caspase-3/7, caspase-8, and caspase-9 using the Caspase-Glo reagents (Promega). We did not detect any significant increase in caspase activity at 24, 48, 72, or 96 hours after transfection (data not shown). As C-Raf has been shown by other investigators (22) to inhibit apoptosis at the level of the mitochondria through its direct interactions with Bcl-2 and Bad, we next looked at the expression of these proteins by Western blots, following C-Raf siRNA transfection. C-Raf knockdown led to down-regulation of both Bcl-2 and phospho-Bad expression in the YULAC and YUROB cell lines, but not in YUMAC, YUSAC, and YUGEN cell lines; weak inhibition of Bcl-2 protein was also observed in YUFIC and MEL501 cells (Fig. 3). The protein levels of phospho-MEK and phospho-ERK1/2 were unchanged in all cell lines following C-Raf knockdown, demonstrating that the effects seen are MAPK independent and therefore mediated through parallel pathways.
sensitive cell line YUGEN (IC$_{50}$, 1.1 μmol/L) to the most resistant YULAC (IC$_{50}$, 6.1 μmol/L; Supplementary Table S2).

**GW5074 selectively blocks MAPK signaling in melanoma cell lines.** To determine the time-dependent effects of GW5074 on cell signaling, we treated our panel of melanoma cells with 10 μmol/L of GW5074 for 1, 6, or 24 hour. We collected lysates at these specific time points and did Western blot analysis. As seen in Supplementary Fig. S2, at 1-hour exposure, GW5074 blocked MAPK signaling in YLUMAC, YUSAC, YUGEN, YULIC, and MEL501 cell lines as shown by the decline of both phospho-MEK and phospho-ERK1/2 expression. Interestingly, little or no activity was seen in YULAC and YUROB cell lines. The protein levels of phospho-Bad and Bcl-2 were not altered in all cell lines.

**C-Raf expression is high in a subset of melanoma specimens, but not in any of the nevi.** Given the role of C-Raf in melanoma cell proliferation and survival, we used a historical cohort of melanomas and nevi and assessed the association between C-Raf expression and melanoma tumor progression. To assess the intratumor heterogeneity, two separate melanoma TMA slides, each containing a core from a different area of the tumor for each patient, were used to evaluate the expression of C-Raf. C-Raf did not show nuclear staining in our specimens, as seen in Fig. 4A and B, and therefore, we analyzed only the membranous/cytoplasmic compartment. Staining within the tumor mask within a histospot was homogenous. Expression in the corresponding specimens from the two arrays was highly correlated ($R = 0.75$), as shown in Fig. 4C. AQUA scores from both sets of slides were combined to give a single data set. Tumor spots were deemed interpretable if they had sufficient 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 data set had scores for a total of 440 melanoma patients (261 metastatic and 179 primary melanomas) and 235 scores for benign lesions (nevi). AQUA scores ranged from 2.755 to 84.606 (with a median value of 11.779) in the malignant specimens and from 5.212 to 14.725 (with a median value of 6.061) in the benign specimens. C-Raf staining showed a relatively bimodal distribution, where an appreciable number of spots had low AQUA scores and only a subset of specimens had high AQUA scores (Fig. 5A). We assessed the difference in C-Raf expression between benign and malignant tissues using unpaired $t$ tests. Expression was significantly higher in malignant versus benign tissue cores ($P < 0.0001$), as shown in Fig. 5B. Given our score distribution, we sought to classify tumors as high or low expressers, based on expression in our large cohort of benign nevi, and the assumption that levels of expression in benign nevi are normal from a biological perspective. Here, we define "low" or "normal" AQUA scores as those that fall at or below the 95th percentile score for nevi. This score was 7.537. C-Raf expression was found to be high, or above the 95th percentile for nevi in ~78.95% of malignant tissues of which 80.46% of metastatic and 76.67% of primary spots were classified as high expressers (Fig. 5C).

By Cox univariate survival analysis of raw AQUA scores, we found no association between survival and expression of C-Raf in either the primary or metastatic subsets of specimens ($P > 0.05$ for both). To assess the association between C-Raf expression and commonly used clinical and pathologic parameters, we used
ANOVA. C-Raf expression was associated with disease stage; C-Raf expression was lower in primary specimens than in metastatic specimens by unpaired t tests ($P = 0.0225$). We found no association between C-Raf and age, gender, Breslow depth, Clark's level, and presence of ulceration.

### Discussion

In this work, we investigated the effect of siRNA on viability in seven patient derived, early passage melanoma cell lines harboring wild-type or activating V600K(E) B-Raf mutations. Whereas C-Raf expression was variable among cell lines, phosphorylated C-Raf was detectable in three of the cell lines. C-Raf silencing by siRNAs was specific and effective, and resulted in a decline in cell viability in the YULAC and YUROB melanoma cell lines; however, siRNA inhibition of C-Raf in the other cell lines only had a minor effect on viability. In addition, we found that in YULAC and YUROB cells, C-Raf–specific silencing induced down-regulation of Bcl-2 and phospho-Bad, but not phospho-MEK1/2 or phospho-ERK1/2. We studied the pharmacologic inhibition of C-Raf by sorafenib and GW5074 and found small variations in the degree of sensitivity among cell lines. GW5074 completely inhibited MAPK signaling in five of seven melanoma cell lines, but not in YULAC and YUROB cell lines, the two cell lines that are most sensitive to C-Raf silencing by siRNA.

Given the effect of C-Raf targeting on apoptosis, we next assessed expression of C-Raf in clinical melanoma specimens and nevi. We used an objective, automated method that provides quantitative output scores. We found that expression of C-Raf was elevated in a subset of melanomas, whereas it was low or undetectable in all benign specimens. Elevated C-Raf expression was found in both primary and metastatic specimens, suggesting that C-Raf up-regulation can be an early event in melanoma progression. Although expression was associated with disease stage, we found no correlation between the level of expression and survival within the primary or metastatic patient subsets. We found no significant association between C-Raf and age, gender, Breslow depth, Clark's level, presence of tumor infiltrating lymphocytes, and presence of ulceration.

Our TMA studies show that although nevi expressed undetectable levels of C-Raf, in a subset of melanomas, C-Raf is highly expressed. This finding could have important diagnostic implications in cases in which the diagnosis of malignancy is questionable, and morphologic features are not sufficient to differentiate between atypical nevi and melanoma. High C-Raf expression could be used, perhaps in combination with other markers such as HSP90 (32) to determine the surgical approach to these ambiguous lesions.

Although elevated C-Raf levels are found in all stages of melanoma, its precise role in malignant tissues has yet to be determined. Our in vitro studies further support the notion that inhibiting C-Raf might be a useful strategy in some, but not all melanomas. Our results are consistent with findings by other investigators, which show that in a subset of melanoma cell lines, C-Raf is required for proliferation and plays a role in cell survival (29, 35).

Our in vitro findings also suggest that C-Raf might have anti-apoptotic effects that are not necessarily dependent on its kinase activity, as shown by other investigators (21). This was shown in our study by the fact that although YUSAC cells had the highest levels of phosphorylated C-Raf, silencing by targeted C-Raf siRNA, inhibited proliferation of YULAC and YUROB, but not YUSAC cells.

It has been established that in RAS-mutated melanoma cells, activation of MAPK is reliant on C-Raf signaling; however, when B-Raf is mutated, signaling through the MAPK pathway

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**Table 1.** IC$_{50}$ of GW5074 in a panel of melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC$_{50}$ of GW5074 (μmol/L)</th>
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<tbody>
<tr>
<td>YUSAC</td>
<td>19.79</td>
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<tr>
<td>YUMAC</td>
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<tr>
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<td>19.67</td>
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<tr>
<td>MEL501</td>
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**Fig. 3.** Effect of C-Raf silencing on protein expression. YUSAC, YUMAC, YULAC, YUGEN, YUFIC, YUROB, and MEL501 cells were transfected with one of the C-Raf siRNA clones at 75 nmol/L. Following 96 h of treatment, cells were lysed and proteins were extracted, electrophoresed, and probed for expression of B-Raf, C-Raf, phospho-MEK1/2 (Ser 221), phospho-ERK1/2 (Thr202/Tyr204), Bcl-2, and phospho-Bad (Ser112) following stripping of the blots. Equal protein gel loading was confirmed by probing for expression of β-actin. C-Raf knockdown resulted in decreased levels of Bcl-2 and phospho-Bad in YULAC and YUROB cells, whereas it had less or no effect at all in other cell lines.
Fig. 4. Membranous and cytoplasmic C-Raf expression in melanoma histospots. A, AQUA uses a cocktail of S100 and HMB45 to define the tumor mask (bottom left), 4,6-diamidine-2-phenylindole to define the nuclear compartment (bottom right), and Cy5 (top right) for the target (C-Raf). The cytoplasmic compartment is generated by subtracting the nuclear compartment from the S100/HMB45 mask (top left). C-Raf 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. C-Raf expression in this example corresponds to an AQUA score of 35.922 (measured in the cytoplasmic compartment) and is shown at x10 magnification (top right; A) and x40 (B) magnification. C, regression plots for scores from the two sets of melanoma arrays for C-Raf. Each array contains histospots from the same patients, taken from different areas of the tumor.

Fig. 5. C-Raf expression in nevi and malignant specimens. A, comparison of C-Raf expression (Cytoplasmic AQUA scores) in nevi, primary, and metastatic specimens. AQUA scores are represented as percentages of total nevi, primaries, and metastases, respectively, by deciles. Nevi (99.57%) have AQUA scores between 0 and 10, whereas only 0.43% had scores between 10 and 20. AQUA scores higher than 20 correspond only to either primary or metastatic specimens. B, comparison of C-Raf expression in nevi and all malignant specimens (metastatic and primary melanomas). Unpaired t-tests showed that C-Raf expression was significantly higher in malignant versus benign tissue cores. C, comparison between high and low C-Raf expression in primary and metastatic melanoma specimens. We defined high C-Raf expression for malignant specimens as expression that was higher than the 95th percentile score for nevi. This score was 7.537.
is sustained primarily by activated B-Raf (28, 29). Although activating B-Raf mutations contribute to melanoma progression, they are neither necessary nor sufficient for its development and accessory pathways, and mechanisms coexist and are used by melanoma cells to ensure oncogenic signaling and tumorigenesis. Our in vitro data suggest that at least in a subset of B-Raf(V600E) melanoma cells, C-Raf contributes to cell survival and proliferation, independently of MAPK activation, via a parallel pathway that entails an interaction with Bad and Bcl-2.

Recent studies show that Raf isoforms have distinct, non-redundant oncogenic functions and that C-Raf, unlike B-Raf, is capable of activating molecules involved in cell survival signaling and cell cycle regulation independently of MAPK activation (23–27). Moreover, C-Raf was shown to translocate to the mitochondria, where it can directly inhibit cellular apoptosis by phosphorylating Bad and activating Bcl-2 proteins (22, 35). Smalley et al. (35) most recently reported that C-Raf inhibition by either shRNA or sorafenib treatment reduces expression of Bcl-2 and phospho-Bad and induces apoptosis in low-activity mutants of B-Raf [B-Raf(D594G) and B-Raf(G469E)] but not in high-activity B-Raf(V600E) melanoma cells. Low-activity B-Raf mutants were previously described by Wan et al. (36) and shown “in vitro” to have only weak kinase activity; nevertheless, they stimulate ERK1/2 activation when transfected into COS cells, an effect mediated by C-Raf. Although both mutants have similar basal kinase activity, only the B-Raf(D594G) mutant was found by Smalley et al. (35) to be highly sensitive to growth inhibition induced by sorafenib. Both low-activity B-Raf mutants were shown to have constitutive levels of phospho-ERK but very low levels of phospho-MEK1/2, and were highly resistant to MEK1/2 inhibition by U0126. Altogether, their data suggest that B-Raf(D594G) and B-Raf(G469E) melanoma cells rely on C-Raf signaling for their growth and survival, and that C-Raf antiapoptotic effects are independent of MEK1/2 activation, but depend instead on its direct interactions with Bcl-2 and Bad at the mitochondrial membrane (35). Here, we show the same phenomenon with siRNA knockdown in one B-Raf(V600E) phospho-C-Raf–expressing cell line (YULLAC), but not in the other (YLJAC). This implies that active B-Raf and C-Raf signaling might occur through parallel nonredundant pathways, which can coexist and be equally important in cell survival and proliferation. This idea is also supported by a study by Montagut et al. (37), who reported that in a human melanoma cell line harboring the B-Raf(V600E) mutation, elevated C-Raf levels were responsible for the primary insensitivity to the RAF kinase inhibitor AZD628 and accounted for the acquired resistance to this drug in another B-Raf(V600E) melanoma cell line. This suggests that in a subset of melanomas, growth inhibition and cellular apoptosis might be effectively induced by combining two potent drugs that specifically target both Raf isoforms. In addition, C-Raf expression could be used as a predictor of resistance to drugs that target mutated or non-mutated B-Raf.

Our results may have important implications for future drug development and design of clinical trials using targeted therapy against the MAPK pathway in melanoma.

A limited number of drugs targeting C-Raf have been developed and tested in preclinical studies. RAF antisense oligonucleotides including ISIS 5132 or ISIS 13650 (ISIS Pharmaceuticals) and LEraFAON (NeoPharm) target C-Raf mRNA, and have shown promising results in preclinical studies, but were disappointing in later clinical studies in which no benefit was seen (38–43). The antisense technology as a treatment methodology has dose-limiting side effects and inadequate penetration into the tumor tissue, which have decreased its efficacy and use in the clinical setting. The disappointing results obtained with antisense agents could also be due to the fact that patient selection was not based on C-Raf tumor expression levels, and expression levels were not studied retrospectively.

Sorafenib (BAY 43-9006; Nexavar) is an orally administered multikinase small molecule inhibitor, developed jointly by Bayer Pharmaceuticals and Onyx Pharmaceuticals, and is a potent C-Raf inhibitor, with an IC50 for C-Raf of 6 nmol/L (44). However, sorafenib is not C-Raf specific, and in fact, it inhibits a number of other proteins involved in tumor neovascularization and tumor cell proliferation and survival, including B-Raf, B-Raf(V600E), VEGF-R2, VEGF-R3, Flt3, FGF-R1, PDGFR-R, c-kit, and p38α (44). In melanoma, single agent sorafenib had little activity with response rates of <10% (45, 46); however, promising results were obtained when used in combination with chemotherapy (47). In our in vitro assays, sorafenib displayed only moderate inhibition of cell viability (IC50 in μmol/L range), which was not dependent on the level of C-Raf activation, demonstrating that sorafenib inhibition is not C-Raf specific and involves multiple targets and mechanisms.

GW5074 is a benzylidine oxindole derivative that inhibits the Raf/MEK/ERK2 kinase cascade by presumably inhibiting the kinase activity of C-Raf (48). GW5074 has been used in vitro in several studies, but contradictory findings have been reported regarding its differential effects on the MAPK signaling cascade. Surprisingly, GW5074 was found to sometimes stimulate, rather than inhibit, the MAPK pathway and can even cause C-Raf and B-Raf activation in cultured neurons (49, 50). Although in our assays, GW5074 effectively inhibited MAPK signaling in five of seven melanoma cell lines, it displayed only weak effects on cell viability (IC50 values in μmol/L range). GW5074 inhibition was independent of the B-Raf mutational status or the degree of C-Raf activation, raising the possibility that GW5074 might target other kinases as well. Interestingly, GW5074 did not inhibit the MAPK cascade in YUROB and YULLAC cell lines, the two cell lines most sensitive to C-Raf mRNA silencing, suggesting once more that parallel pathways might be used by these cells to assure their survival and proliferation. Further studies are required to thoroughly analyze the effects of GW5074 on melanoma cells, as they do not parallel the effects of C-Raf siRNA inhibition. Pharmacologic inhibition and siRNA inhibition of C-Raf have different down-stream effects, and C-Raf clearly has antiapoptotic effects that are not necessarily dependent on its kinase activity, as shown by other investigators (21).

Current metastatic melanoma therapies have a number of inherent limitations: lack of specificity for melanoma cells and drug resistance. Melanoma oncogenesis is a very complex process that might involve simultaneous signaling through different pathways. In addition, subsets of melanomas can be driven by nonoverlapping pathways. Therefore, the use of rationally designed combinations of therapy, where two or more drugs are administered to specifically inhibit key effectors in those pathways in appropriately selected subsets of patients, would likely be more effective and improve response rates. Studies are under way in our laboratory to further identify mediators of sensitivity and resistance to C-Raf knockdown and assess combinations of other small molecule inhibitors and C-Raf inhibition, such as Bcl-2 inhibitors and MEK inhibitors.

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C-Raf in Melanoma


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In summary, our *in vitro* data further indicate that apoptosis is induced in some melanoma cells with C-Raf silencing. This seems to be independent of B-Raf mutational status. We have shown that a subset of melanomas has up-regulation of C-Raf, whereas levels were low in nevi, suggesting that it might be a relevant diagnostic marker and therapeutic target in melanoma. Assessment of baseline C-Raf expression in patients treated with C-Raf inhibitors might enable us to improve patient selection in future clinical trials using C-Raf inhibitors. These inhibitors are likely to be most effective when used in combination with other rationally selected inhibitors, such as Bcl-2 inhibitors and/or MEK inhibitors.

**Disclosure of Potential Conflicts of Interest**

R. Camp and D. Rimm are scientific cofounders, stockholders, and consultants for HistoRx. The other authors disclosed no potential conflicts of interest.

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