KRAS/BRAF mutation status and ERK1/2 activation as biomarkers for MEK1/2 inhibitor therapy in colorectal cancer

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Abstract

Phase II clinical trials of MEK inhibitors are ongoing and ERK1/2 activation is frequently used as a biomarker. In light of the mutational activation of *BRAF* and *KRAS* in colorectal cancer (CRC), inhibitors of the Raf-MEK-ERK mitogen-activated protein kinase are anticipated to be promising. Previous studies in pancreatic cancer have found little correlation between *BRAF/KRAS* mutation status and ERK1/2 activation, suggesting that identifying biomarkers of MEK inhibitor response may be more challenging than previously thought. The purpose of this study was to evaluate the effectiveness of MEK inhibitor therapy for CRC and *BRAF/KRAS* mutation status and ERK1/2 activation as biomarkers for MEK inhibitor therapy. First, we found that MEK inhibitor treatment impaired the anchorage-independent growth of nearly all *KRAS/BRAF* mutant, but not wild-type, CRC cells. There was a correlation between *BRAF*, but not *KRAS*, mutation status and ERK1/2 activation. Second, neither elevated ERK1/2 activation nor reduction of ERK1/2 activity correlated with MEK inhibition of anchorage-independent growth. Finally, we validated our cell line observations and found that ERK1/2 activation correlated with *BRAF*, but not *KRAS*, mutation status in 190 patient CRC tissues. Surprisingly, we also found that ERK activation was elevated in normal colonic epithelium, suggesting that normal cell toxicity may be a complication for CRC treatment. Our results suggest that although MEK inhibitors show promise in CRC, *KRAS/BRAF* mutation status, but not ERK activation as previously thought, may be useful biomarkers for MEK inhibitor sensitivity.

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Introduction

The genetic events that occur in the stepwise progression from benign epithelium to colorectal cancer (CRC) are well established with mutational inactivation of the *APC*, *TP53*, and *SMAD4/ DPC4* tumor suppressor genes and mutational activation of the *KRAS*, *PIK3CA* and *BRAF* oncogenes (1–3). *KRAS* mutations occur early and are detected in up to 50% of CRCs (3,4). Functional studies in cell culture (5) and mouse models (6) support a critical role for *KRAS* mutation in CRC progression and maintenance. Therefore, it is widely believed that therapeutic approaches to block Ras will be effective for CRC treatment. However, to date, efforts to develop effective anti-Ras therapies continue to be elusive.

Recent efforts to develop anti-Ras therapies have focused on Ras downstream effector pathways. The frequent mutational activation of two key effectors with validated roles in Ras-mediated oncogenesis, encoded by *BRAF* and *PIK3CA*, support the importance of aberrant effector signaling in mutant K-Ras function in CRC (7,8). In particular, the nonoverlapping occurrence of *BRAF* and *KRAS* mutations suggested that aberrant B-Raf signaling is the critical mechanism for KRAS-mediated oncogenesis in CRC. B-Raf phosphorylates and activates the MEK1 and MEK2 protein kinases, and activated MEK1/2 phosphorylate and activate the ERK1 and ERK2 mitogen-activated protein kinases (MAPKs). Therefore, much emphasis has been placed on treatment strategies that target this protein kinase cascade (9–11). In particular, potent and selective inhibitors of MEK1 and MEK2 have been developed and are currently in Phase I/II clinical trials (AZD6244, XL51, and ARRY-162; www.clinicaltrials.gov).

Studies in experimental cell culture models showed that ectopic expression of activated Ras causes ERK activation and ERK-dependent growth transformation (12). Since the only known substrates of Raf are MEK1 and MEK2, and the only known MEK1/2 substrates are ERK1 and ERK2, a logical hypothesis is that MEK1/2 (MEK) inhibitors will be potent inhibitors of Ras- and Raf-mediated activation of ERK. These observations prompt several working hypotheses for the application and effectiveness of MEK inhibitors in CRC. First, K*RAS* and *BRAF* mutation positive CRC tumor cells are expected to exhibit elevated ERK activation. Second, CRC cells with elevated ERK activation should possess ERK-dependent growth transformation, and hence, elevated ERK activity should correlate with sensitivity to growth inhibition by MEK inhibitor treatment. Consequently, previous Phase I/II trials of the MEK1/2 inhibitor CI-1040 have used ERK1/2 (ERK) inhibition as a biomarker of response to MEK inhibitor treatment (13,14).

Whether KRAS mutation status and ERK activity are accurate biomarkers for MEK inhibitor treatment of CRC has not been rigorously evaluated and validated. Recent observations in other cancer types suggest that the application of MEK inhibitors for CRC treatment may not be so straightforward. First, in addition to Raf, Ras interacts with multiple downstream effectors with demonstrated roles in Ras-mediated oncogenesis (15). Second, studies in pancreatic cancer cell lines have demonstrated that there is no correlation between *KRAS* mutational status and ERK activation in some tumors, suggesting that a Raf-independent function of Ras is important or that ERK activation occurs through a Ras-independent mechanism (16,17). Several recent studies have addressed *RAS* mutation status and MEK inhibitor sensitivity. One study evaluated primary and established human ovarian tumor cell lines and found that both *KRAS* and *BRAF* mutant cells showed preferential sensitivity to CI-1040 inhibition of anchorage-dependent growth (18). In a second study focused on melanoma cell lines, *BRAF* but not *NRAS* mutation status correlated with sensitivity to CI-1040 growth inhibition of anchorage-dependent growth (19). In contrast, another study of a panel of human tumor cell lines, including 7 CRC cell lines, found that AZD6244 inhibition of anchorage-dependent proliferation showed a strong but incomplete correlation with *BRAF* or *KRAS* mutation status (20). However, ERK activation and inhibition were not evaluated in this study. Finally, a study

using CI-1040 suggested that *KRAS* activation may be a mechanism of resistance to MEK inhibitor therapy in murine CRC (21). Thus, it remains unclear whether *KRAS* or *BRAF* mutation status or ERK activation will correlate with MEK inhibitor activity for CRC treatment. Determining the appropriate biomarkers for MEK inhibitor activity will be critical for the evaluation of MEK inhibitors in clinical trials.

Since MEK inhibitors remain a potentially effective therapeutic approach for CRC treatment, with three inhibitors in clinical trials and more in the pipeline, we evaluated CRC cell lines for sensitivity to two MEK inhibitors, U0126 and CI-1040. U0126 is a highly specific non-ATP competitive inhibitor of MEK1 and MEK2 and has been widely used in cell culture studies, but its pharmacologic limitations have restricted its use to *in vitro* analyses. The structurally distinct CI-1040 (PD184352) is also a highly specific non-competitive MEK1 and MEK2 inhibitor that showed anti-tumor activity against HT-29 CRC cell line induced tumor xenografts (22). CI-1040 evaluation in Phase I trials suggested that inhibition of ERK in tumor tissue correlated with anti-tumor activity (14), leading to its evaluation in Phase II trials in patients with colon and other solid tumors (13). However, the limited potency and lack of anti-tumor activity seen with CI-1040 prompted the generation of a second analog of CI-1040 (PD0325901) which demonstrated some anti-tumor efficacy, but was terminated due to toxicity (10). Currently, alternate MEK inhibitors, such as AZD6244 (23), an alternate and highly specific MEK1 and MEK2 inhibitor, similar to CI-1040 in structure and function, show promise and are currently in Phase I/II clinical trials (www.clinicaltrials.gov).

Because anchorage-independent growth potential may correlate better with tumorigenic growth *in vivo*, we determined if ERK inhibition correlated with inhibition of tumor cell growth in soft agar rather than on plastic. First, we found that MEK inhibitor treatment impaired soft agar colony formation for a majority of CRC cell lines. Second, we found that ERK activation correlated with *BRAF*, but not *KRAS* mutation status. However, we found that elevated ERK activity and inhibition of ERK did not reliably predict sensitivity to MEK inhibitor. Finally, unexpectedly, we found that ERK activation was highest in normal and not neoplastic colonic epithelium, suggesting possible concerns with normal cell toxicity for MEK inhibitor therapy.

Materials and Methods

Cell lines and culture

All cell lines were obtained from American Type Culture Collection and maintained in either RPMI-1640 or Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. Cell lines were treated with either U0126 (Promega) or CI-1040 (Pfizer) for 24 h and harvested.

Western blot analyses

Exponentially growing cultures of cells were harvested in a buffer containing a phosphatase (Sigma-Aldrich) and protease inhibitor cocktail (Roche) and were resolved by SDS-10% polyacrylamide gel electrophoresis, and transferred to Immobilon-P (Millipore) membranes. Immunoblots were then incubated with antibodies to activated phosphorylated ERK1 and ERK2 (pERK) (9106S, Cell Signaling) and total ERK1 and ERK2 (Total ERK) (9102, Santa Cruz Biotechnology). Vinculin (V9131, Sigma-Aldrich) or GAPDH (ab9483, Abcam Inc.) was used as a control for equivalent loading of total protein. Antibodies were detected with the appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (31432 and 31462, Pierce) by enhanced chemiluminescence (Pierce). Densitometry was performed using ImageJ (http://rsbweb.nih.gov/ij/). The relative intensity of pERK was normalized using total ERK1/2 as the standard. The fold change was then calculated between either MEK inhibitor or vehicle treated cells or tumors and normal tissues.

MEK inhibitor transformation analyses

Soft agar colony formation assays were used to evaluate the ability of MEK inhibition to impair the transformed growth properties of CRC cells. Briefly, 5×10^3 cells per well in 6-well plates were suspended in soft agar (24). The appearance of colonies >30 cells were scored after four weeks. Assays were performed independently at least two times in triplicate.

Tissue microarrays (TMAs)

TMAs were prepared from formalin-fixed paraffin embedded colorectal tissue sections using a 0.6 mm punch as described (25). The arrays contained triplicate cores of normal and tumor tissue from each patient. We prepared 5 µm sections from each TMA block. Hematoxylin and eosin stained slides from each TMA block were reviewed by a pathologist (TR) to ensure that normal and tumor tissues were cored accurately.

Mutational analyses

We evaluated tissue cores for mutations in *KRAS* (n = 126) and *BRAF* (n = 104). DNA was extracted from tumor cores. The regions of interest for each gene were amplified by polymerase chain reaction (PCR) with specific primers and sequenced (MWG-Biotech). For *KRAS*, we evaluated DNA from tumor cores for mutations in codons 12 and 13. For *BRAF*, we evaluated DNA from tumor cores for mutations in exon 15. Samples were scored for the presence or absence of mutation.

Immunohistochemistry (IHC)

We examined protein expression of pERK from four patients with whole sections and tissue cores from 190 patients in triplicate. First, slides were scored by a technician (JP or DW) and verified by an investigator (TOK). Second, the slides were scored independently by a blinded pathologist (TR). The results of each protein marker were expressed as intensity (I) and proportion (P) of positive epithelial cells and the product of I and P (26). Each triplicate TMA core was counted separately.

Statistical analysis

The Fisher's exact test was used to analyze associations between two variables and the Pearson Chi-square test was used to analyze association between more than two variables. The independent samples t-test was used to analyze associations between ordinal (IHC scores) and continuous (soft agar colony formation scores) variables.

Results

The success of signal transduction target-based anti-cancer treatment is dependent on the establishment of molecular markers to identify the patient population that will respond to treatment. Thus, we set out to test four hypotheses. First, what CRC cell lines are sensitive to MEK inhibitor treatment? Second, do CRC cell lines with *KRAS* and *BRAF* mutation or elevated ERK activity show the greatest sensitivity to MEK inhibitor treatment? Third, does the ability of MEK inhibitor treatment to block ERK activity correlate with the ability to block CRC cell growth? Finally, is ERK activity differentially elevated in human CRC tumors?

MEK inhibitors impair anchorage-independent growth of the majority of *BRAF/KRAS* mutant CRC cell lines

For our analyses, we obtained a panel of CRC cell lines from the ATCC. Previous studies assessing MEK inhibitor activity monitored anti-tumor activity by evaluating loss of cell viability when tumor cells were treated on plastic (18–20). Since anchorage-independent growth *in vitro* remains the best *in vitro* parameter to predict tumorigenic growth *in vivo*, for

our studies, we utilized soft agar colony formation assays to monitor the anti-tumor activity of MEK inhibitors. To determine whether the anchorage-independent growth of CRC cell lines was also dependent on activation of the ERK MAPK pathway, we treated CRC cell lines, when suspended in soft agar, with either U0126 or CI-1040.

To determine if sensitivity to MEK inhibitor treatment correlates with specific molecular alterations, we compiled the *KRAS* and *BRAF* mutation status, as well as the mutation status of the *TP53* and *APC* tumor suppressor genes, and the epidermal growth factor receptor (*EGFR*) from the Cancer Genome Project tumor cell line database (http://www.sanger.ac.uk/genetics/CGP/) (Table 1). We first evaluated cell lines treated with U0126 and then selected a subset of representative cell lines for our studies with CI-1040. All cell lines except COLO-320-HSR showed similar responses to treatment with each inhibitor (Figs. 1*A*, 1*C*). COLO-320-HSR showed limited growth inhibition with U0126, but not CI-1040, treatment. *KRAS/BRAF* WT cell lines exhibited limited to no inhibition of colony formation, using either U0126 (SW48 and SNU-C1) or CI-1040 (COLO-320-HSR and SNU-C1) (Figs. 1*A*, 1*C*). We did not use SW48 in our studies of CI-1040 due to its poor growth in soft agar. All *BRAF* mutation positive cell lines except NCI-H508 demonstrated growth inhibition with either MEK inhibitor (Figs. 1*A*, 1*C*). Similarly, *KRAS* mutation positive cell lines except HCT-116 demonstrated growth inhibition in soft agar (Figs. 1*A*, 1*C*). Therefore *KRAS* or *BRAF* mutation status was associated with sensitivity to one or both MEK inhibitors ($P = 0.027$).

Elevated ERK activity and inhibition of ERK does not predict sensitivity to MEK inhibitor growth inhibition

One logical hypothesis is that CRC cell lines with elevated ERK activity are more dependent on the activity of this pathway for growth, and hence, more sensitive to growth inhibition by MEK inhibitors. We therefore determined whether elevated ERK activation (pERK), and sensitivity to ERK inhibition, would be reliable molecular determinants to predict a growth-inhibitory response to MEK inhibitor treatment in our panel of CRC cell lines.

First, we treated CRC cell lines with either the U0126 or CI-1040 MEK1 and MEK2-specific inhibitors (Figs. 1*B* and 1*D*). We found that both MEK inhibitors demonstrated similar effects, with essentially complete pERK inhibition seen in the majority of lines that expressed activated ERK. Although both inhibitors are highly specific for MEK1 and MEK2, at higher concentrations both may have off-target activities, for example, inhibition of MEK5 (27). However, since the goal of our studies was to evaluate pERK as a biomarker for MEK1 and MEK2 inhibition, we did not evaluate MEK inhibitor reduction of MEK5 activity in our analyses. Our observations are similar to those made recently with CI-1040, where all melanoma and other tumor cell lines, independent of their *RAS/BRAF* genetic profile, were responsive to MEK inhibition as measured by pERK inhibition (19).

Next, we determined if elevated pERK levels correlated with sensitivity to growth inhibition by MEK inhibitor treatment. Surprisingly, we found inhibition of soft agar colony formation regardless of the level of pERK activity (p=0.170) (Fig 1). In cell lines with little to no detectable levels of pERK (T84 and LS-174T), there was significant growth inhibition when treated with U0126 or CI-1040 (Fig. 1). Yet in cell lines with clear pERK inhibition (SW48, SNU-C1, and HCT-116) there was no inhibition of growth in soft agar (Fig 1). NCI-H508 demonstrated pERK inhibition with treatment of CI-1040 and to a lesser degree U0126, yet showed no growth inhibition in soft agar (Fig. 1*C* and data not shown). *COLO*-320-HSR was growth inhibited by U0126 but not CI-1040 (Fig. 1*A, C*), suggesting that minor differences do exist between the two inhibitors. Thus, neither the level of elevated steady state pERK levels nor the sensitivity to pERK inhibition correlated with growth inhibition sensitivity in response to MEK inhibitor treatment.

Elevated ERK activation correlates with *BRAF*, but not *KRAS* mutation status of CRC cell lines

Since studies with ectopic stable expression of activated K-Ras and B-Raf causes sustained elevated activation of the ERK MAPK cascade (12), we evaluated the panel of CRC cell lines for steady-state levels of activated pERK (Fig. 2). Whereas activated ERK was not detected in one CRC cell line with wild-type (WT) *KRAS* and *BRAF* (COLO-320-HSR), the remaining two CRC lines with WT sequences showed elevated ERK activation (SNU-C1 and SW48). SW48 cells possess a mutant allele of the *EGFR* (Table 1) that may contribute to ERK activation, but no mutant genes that are known to promote ERK activation have been described for SNU-C1. Surprisingly, only 3 (HCT-116, SW480, and LoVo) of 6 *KRAS* mutant CRC lines showed greatly elevated levels of activated ERK, compared to *BRAF* mutant and *BRAF/KRAS* WT CRC lines. This result is similar with that seen in *KRAS* mutation positive pancreatic carcinoma cell lines and patient tumors, where elevated ERK activity was not associated with a majority of *KRAS* mutant cells (16,17). In contrast, elevated levels of activated ERK were seen in all five *BRAF* mutant cell lines. Thus, *BRAF* but not *KRAS* mutation status may provide a better genetic marker for elevated activation of the ERK MAPK cascade. Nevertheless, since some CRC cell lines with WT *BRAF* possess elevated ERK activation, and overall there was no correlation between ERK activation and *BRAF* or *KRAS* mutation status ($P = 0.117$), we conclude that ERK activation alone may not be a reliable surrogate marker for mutant *BRAF* or *KRAS* mutation positive CRC tumor cells.

Elevated ERK activation in 190 CRC matched normal and tumor tissues does not correlate with *KRAS* mutation status

To date, analyses of ERK activation have been done with patient tumors but with mixed conclusions. In a previous clinical trial with CI-1040, patient archival pathology samples were evaluated for pERK expression and a general trend of elevated activity was seen in colon, pancreas and other tumor tissues, although a broad range of activities was seen (13). However, no comparison of pERK activity in non-tumor tissue was done nor was pERK activity correlated with *KRAS* or *BRAF* mutation status. More recently, Haigis *et al.* (2008) found that no pERK was detected in 18 of 18 primary human CRCs, although *KRAS* and *BRAF* mutation status was not determined (6). Another study evaluated pERK activity and *KRAS*, but not *BRAF* mutation, in 135 CRC tumors, but no comparison with non-tumor tissue was done (28). As these were limited to archival specimens, the degree of pERK activity may have been underestimated. Therefore, we first used western blot analyses and examined a panel of 8 frozen CRC tumors with matched normal tissues across different American Joint Committee on Cancer (AJCC) stages (Fig. 3*A*). Although levels of pERK activation varied and our sample size was limited, there did appear to be a trend towards decreased ERK activation with advanced stage. Most surprisingly, the matched normal mucosa also exhibited high levels of pERK, that in some cases were much higher than that seen in tumor tissue. We also found high pERK expression in the normal colon mucosa of a patient without CRC (data not shown).

Because macrodissected frozen tumors may not be reflective of a pure population of colonic epithelial cells, and our findings of pERK activation may be in stroma, we examined these matched samples for pERK using immunohistochemistry. We found that for the most part, the pERK staining in these matched samples (Fig. 3*B*) correlated with pERK protein expression in snap-frozen tumors determined by blot analyses (Fig. 3*A*). In normal colonic tissue, strong pERK immunostaining was noted in the terminally differentiated cells in the superficial epithelial layer, similar to a recent report by Haigis et al. (6). In the majority of tumors, pERK immunoreactivity was less intense and more variably distributed throughout the tissue compared to the normal colonic epithelium. Thus, we found high pERK expression in both normal and tumor colonic tissue but the intensity and distribution of immunoreactivity was variable.

Since detection of pERK activity in normal tissue to this degree was not expected, in order to further quantify and determine whether pERK activity was indeed elevated in normal colonic epithelium, we examined pERK activity in a larger sample set of 190 patients using CRC tissue microarrays (TMAs) (Fig. 4*A*). Complete AJCC staging was not available for the CRC TMAs. pERK staining and intensity was evaluated and scored by a blinded colorectal pathologist (TR). We found that both nuclear and cytoplasmic pERK activity was significantly elevated in normal colonic epithelium in patients with CRC compared to the patient tumors ($P < 0.001$).

Finally, we determined whether mutations in *BRAF* or *KRAS* were associated with ERK MAPK pathway activation in patient-derived tissues. We evaluated 126 patient tumors for *KRAS* and 104 tumors for *BRAF* mutations and pERK activation using CRC TMAs. We found that 21% (26/126) of CRCs had *KRAS* and 4% (4/104) of CRCs had *BRAF* mutations. We found that pERK was significantly elevated in tumors with *BRAF* and not *KRAS* mutations (Fig. 4*B*, $P = 0.014$). Tumors without *BRAF* mutations had a mean cytoplasmic and nuclear score of 1.9 compared to a mean cytoplasmic score of 2.7 and nuclear score of 0.2 in tumors with *BRAF* mutations.

Discussion

Despite intensive effort, to date, no anti-Ras therapies have demonstrated clinical efficacy. Currently, one promising approach involves small molecule kinase inhibitors of MEK and the Ras-Raf-MEK-ERK effector pathway (9,11). A key requirement for successful development of MEK inhibitors will be the identification of molecular determinants that identify patient tumors that will be responsive to MEK inhibition, and to establish reliable biomarkers to monitor drug efficacy. Therefore, we evaluated a panel of CRC cell lines for their response to MEK inhibitor treatment, and whether ERK activation and sensitivity to ERK inhibition or *KRAS* or *BRAF* mutation status, are accurate biomarkers for MEK inhibitor treatment and response. We determined that the majority of CRC cell lines demonstrate growth inhibition using MEK inhibitors, specifically those that are *BRAF* or *KRAS* mutation positive. However, ERK activation did not correlate reliably with *BRAF* and *KRAS* mutation status. In addition, MEK inhibitor suppression of ERK activity did not correlate with suppression of anchorage-independent growth. Finally, we found that ERK is not differentially activated in tumor tissue. Our results reveal complexities that will need to be considered and overcome for successful development of MEK inhibitors as anti-Ras therapy.

Similar to our observations with pancreatic cancer cell lines and tumors (16), we found that ERK activation did not correlate strongly with the presence of a *KRAS* mutation in CRC cell lines or tumors. Our conclusion may seem to contrast that of Schmitz *et al.* (2007), who concluded that ERK activation was associated with *KRAS* mutation (28). However, they found that 10 of 20 (50%) high pERK expression tumors lacked *KRAS* mutations, and 26 of 114 (23%) *KRAS* mutation positive tumors had low pERK activity. The authors also found that there was a significant correlation with high pERK expression and poor survival, but this association was not seen with *KRAS* mutation status, suggesting that *KRAS*-independent activation of the Raf-MEK-ERK pathway may occur. In a second study, Sakakura *et al.* (1999) found that ERK activation was found in only 4 of 21 advanced CRCs, with no correlation with *KRAS* mutation status (29). Consequently, these observations are consistent with our conclusion that ERK activation is not a reliable biomarker for *KRAS* mutational activation. We evaluated codon 12 and 13 mutations in *KRAS* and therefore may be underestimating the frequency of *KRAS* mutations in our study. However, genome wide studies in CRC have found that less than 3% of *KRAS* mutations are outside of codon 12 and 13 (4). Therefore, the frequency of non-codon 12 and 13 mutations would not affect our findings.

The biochemical basis for our findings that ERK activation does not correlate with *KRAS* mutational activation is not clear and may reflect the fact that the Ras-Raf-MEK-ERK cascade is not a simple linear pathway, with a multitude of positive and negative regulatory components operating at all levels of this protein kinase cascade (30). For example, upregulated ERK phosphatase activity was determined to account, in part, for the lack of a direct relationship between *KRAS* mutation and ERK activation in pancreatic and murine colonic tumor cells (6, 31). Alternatively, it may be possible that activated K-Ras does not engage this effector pathway in some CRC tumor cells, and instead, preferentially activates other effector pathways to promote oncogenesis.

We did find that *BRAF* mutation correlated strongly with ERK activation in CRC cell lines and patient tumors. The significance of the change in pERK localization from the nucleus to cytoplasm in *BRAF* mutation positive tumors is interesting and will require further investigation. We have previously shown that in the *BRAF* mutant CaCo-2 line, ERK activation is *BRAF*-independent (32). Furthermore, ERK activation was also associated with WT *KRAS/ BRAF* CRC cell lines. Thus, although ERK activation is associated with the presence of *BRAF* mutation in CRC, both *BRAF*- and *KRAS*-independent mechanisms of ERK activation exist. Therefore it remains to be determined whether ERK activation will be a reliable biomarker for mutant B-Raf function in CRC.

Similar to the findings of Rosen and colleagues (19) where *BRAF* mutant melanoma cell lines exhibited exquisite sensitivity to CI-1040 when compared to some *NRAS* mutant and all WT lines, we also found that 4 of 5 *BRAF* mutant CRC cell lines were sensitive to MEK inhibitor suppression of anchorage-independent growth. Similarly, all except one *KRAS* mutant CRC cell line showed sensitivity to MEK inhibitor treatment. Therefore, we did find that *KRAS* and *BRAF* mutation status provided a strong, but incomplete, correlation with sensitivity to MEK inhibitor treatment growth inhibition.

A logical hypothesis is that CRC cell lines with elevated ERK activity are more dependent on the activity of this pathway for growth, and hence, more sensitive to growth inhibition by MEK inhibitors. An earlier study with a small number of human tumor cell lines, including two CRC cell lines, supported this possibility (22). However, we found that cell lines with little or no evidence of ERK activation (COLO-320-HSR, T84, and LS-174T) were sensitive to MEK inhibition, yet cell lines with elevated pERK (HCT-116, NCI-H508, SNU-C1, and SW48) were resistant to MEK inhibition of anchorage-independent growth. Similar observations in cell culture were made by Rosen and colleagues, who found that WT *RAS/BRAF* tumor cell lines showed elevated ERK activity, and were sensitive to CI-1040 inhibition of ERK activity, yet showed resistance to growth inhibition (19). Therefore ERK activation may not be a reliable biomarker to predict a therapeutic response to MEK inhibitor treatment. Limited analyses of patients treated with AZD6244 suggest that this will be the case, where the level of activated pERK, as well as the degree of inhibitor reduction of pERK, did not correlate with clinical benefit (23). Since it has been reported that MEK inhibitors may inhibit ERK5 (12), perhaps off-target activities of these inhibitors may contribute to their growth inhibitory activities.

In light of the substantial experimental evidence that ERK activation is associated with mitogenic stimulation, we were surprised to find ERK activation more consistently associated with normal rather than tumor colonic epithelium. The issue of whether ERK activity is elevated in CRC cancer has been addressed in previous studies, but with contrasting conclusions. Brenner and colleagues (1997) found greatly elevated ERK activity in carcinogen-induced tumor but not normal rat intestinal tissue (33). However, their later analyses using an *in vitro* kinase assay to monitor Elk phosphorylation did not find greater ERK activity in the majority of human CRC tumor tissue when compared to matched nontumor tissue (34). Using *in vitro* kinase assays, Hoshino *et al* (1999) found that ERK activation was consistently high

in only a subset of CRC cell lines and tissues, and additionally that ERK activation was frequently higher in adjacent nontumor tissue (35). Eggstein *et al.* (1999) utilized both pERK and *in vitro* kinase analyses and evaluated paired samples of colorectal mucosa and adenocarcinoma derived from 22 patients and concluded that ERK was inactivated in tumor tissue (36). Wang *et al.* (2000) evaluated CRC and adjacent normal mucosa from 21 patients by *in vitro* ERK activity and concluded that kinase activity was reduced in tumor tissue (37). More recently, Nemoto *et al.* (2005) determined that pERK levels in paired colorectal tumor and adjacent non-tumor tissue and found elevated ERK in only 9 of 33 (27%) tumors (38). Similarly, Schmitz *et al.* (2007) recently reported that ERK activation was found in only 20 of 115 (15%) CRC tumors and noted that ERK activation was seen in normal colonic epithelium (28). Finally, Haigis *et al.* (2008) found that no pERK was detected in 18 of 18 primary human CRCs, but 4 of 18 (22%) normal adjacent colonic crypts demonstrated pERK staining (6). The different conclusions reached in these studies may reflect differences in how ERK activation was determined, sample preparation to avoid contamination by non-colonic epithelial tissue, as well as differences in the size and nature of the patient population evaluated.

Taken together, these studies show that there is increasing evidence to suggest that ERK activation is elevated in normal colonic epithelium compared to CRC tumors. Our study is the first to quantify ERK activation in a large number of matched normal and tumor tissues in CRC patients and provides definitive evidence that ERK activation is significantly higher in normal colonic epithelium compared to matched tumors. Our findings of elevated ERK activation in the normal colonic mucosa of a non-CRC patient and CRC patients are consistent in both frozen and archival specimens, suggesting that this is not a fixation artifact. This suggests that normal colonic tissue toxicity may be a concern in patients receiving MEK inhibitor therapy and may therefore compromise the therapeutic efficacy of these inhibitors. However, it remains to be determined whether ERK activity is critical for normal colonic epithelial cell proliferation and survival, and consequently, whether normal cell toxicity will be seen with MEK inhibitor therapy.

In summary, our results suggest that *KRAS* or *BRAF* mutation status may correlate with CRC sensitivity to MEK inhibitor treatment. Our *in vitro* analyses together with similar observations with AZD6244 (20), and the colon tumor xenograft analyses described previously (22), support the possibility that this subset of CRCs will be responsive to MEK inhibitor treatment. Our results together with those of other studies (19) indicate that although the degree of ERK activation and inhibition will not provide useful biomarkers to reliably predict patient response to MEK inhibitor therapy, *KRAS* and *BRAF* mutation status may. Our studies suggest that tumors of CRC patients currently enrolling in clinical trials of MEK inhibitor therapy should be tested for *KRAS* and *BRAF* mutation status. Finally, although the presence of differentially high levels of activated ERK in normal colonic epithelium in patients with CRC tumors is concerning, the theoretical toxicity to normal tissues remains to be seen in the clinical setting.

Abbreviations List

CRC, colorectal cancer; pERK, phosphorylated ERK; TMA, tissue microarray; IHC, immunohistochemistry; PCR, polymerase chain reaction.

Acknowledgments

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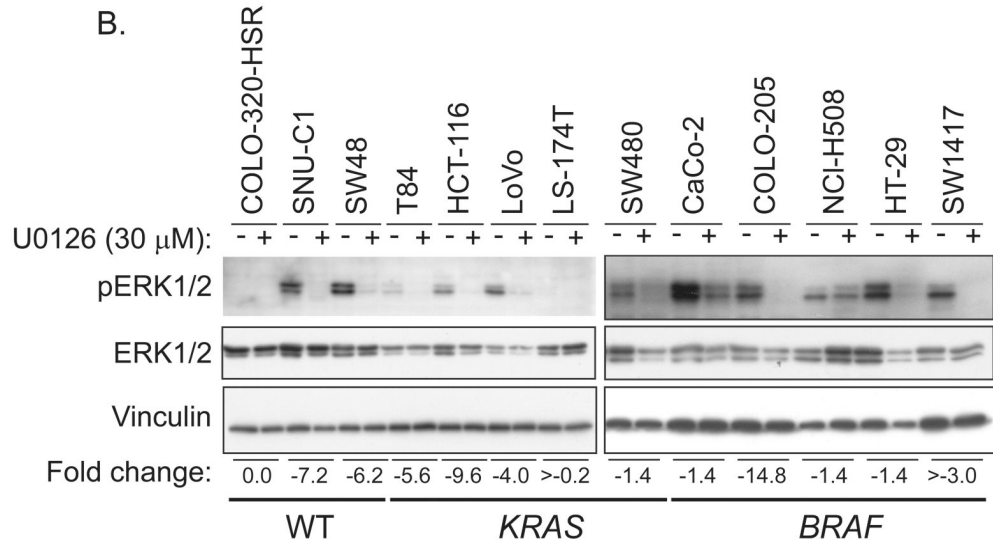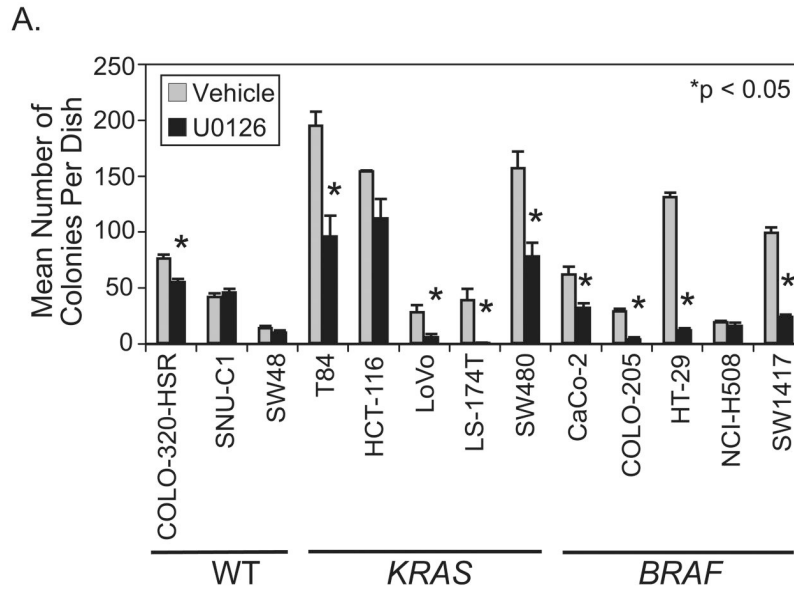
Figure 1AB

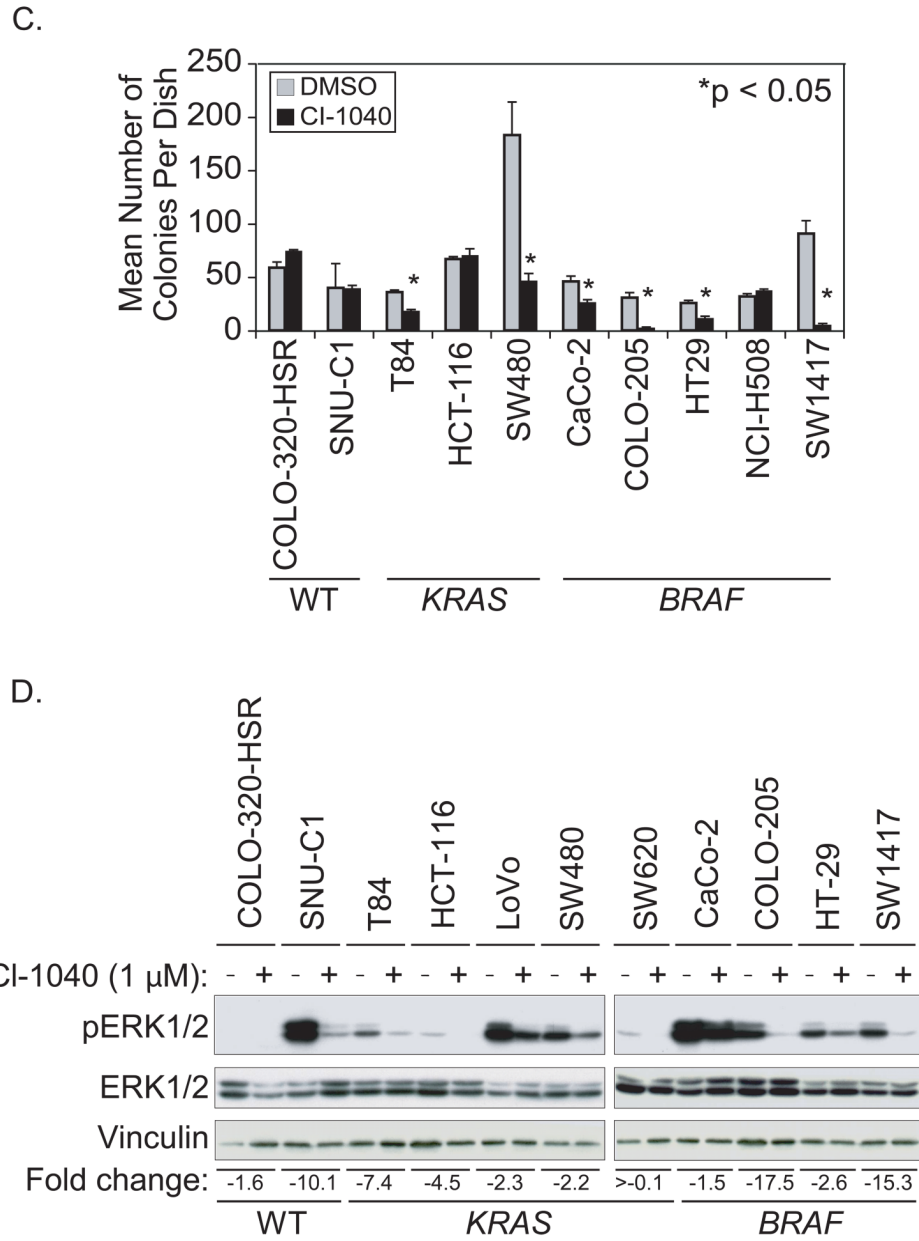A.

B.

Figure 1CD

C.

D.

Figure 1.
CRC cell lines exhibit differential sensitivity to ERK and anchorage-independent growth inhibition by MEK inhibitor treatment. *A*, Inhibition of anchorage-independent growth of CRC cell lines by treatment with the U0126 MEK1/2 inhibitor. The indicated cells were suspended in soft agar with vehicle (DMSO) or 30 µM U0126. The number of colonies of proliferating cells was quantitated after 30 days. *B*, Inhibition of ERK1/2 activation in CRC cell lines by U0126 MEK inhibitor treatment. Cells were treated for 24 h with vehicle (DMSO) or 30 µM U0126, then lysed and immunoblotted with anti-pERK1/2, anti-total-ERK1/2 or anti-vinculin sera. *C*, Inhibition of anchorage-independent growth of CRC cells by treatment with the CI-1040 MEK inhibitor. The indicated cells were suspended in soft agar with vehicle (DMSO)

or 1 µM CI-1040. The number of colonies of proliferating cells was quantitated after 30 days. *D*, Inhibition of ERK activation in CRC cell lines by CI-1040 MEK inhibitor treatment. Cells were treated for 24 h with vehicle (DMSO) or 1 µM CI-1040, then lysed and immunoblotted with anti-pERK, anti-total-ERK or anti-vinculin sera. Analyses with total anti-vinculin were done to verify equivalent loading of cellular protein. Fold change in the relative intensity of pERK was calculated for MEK inhibitor treatment compared to vehicle control (+/−) using total ERK1/2 as the standard. Data shown are representative of at least three independent experiments. (WT, *KRAS/BRAF* wild-type; *KRAS*, *KRAS* mutation positive, *BRAF*, *BRAF* mutation positive).
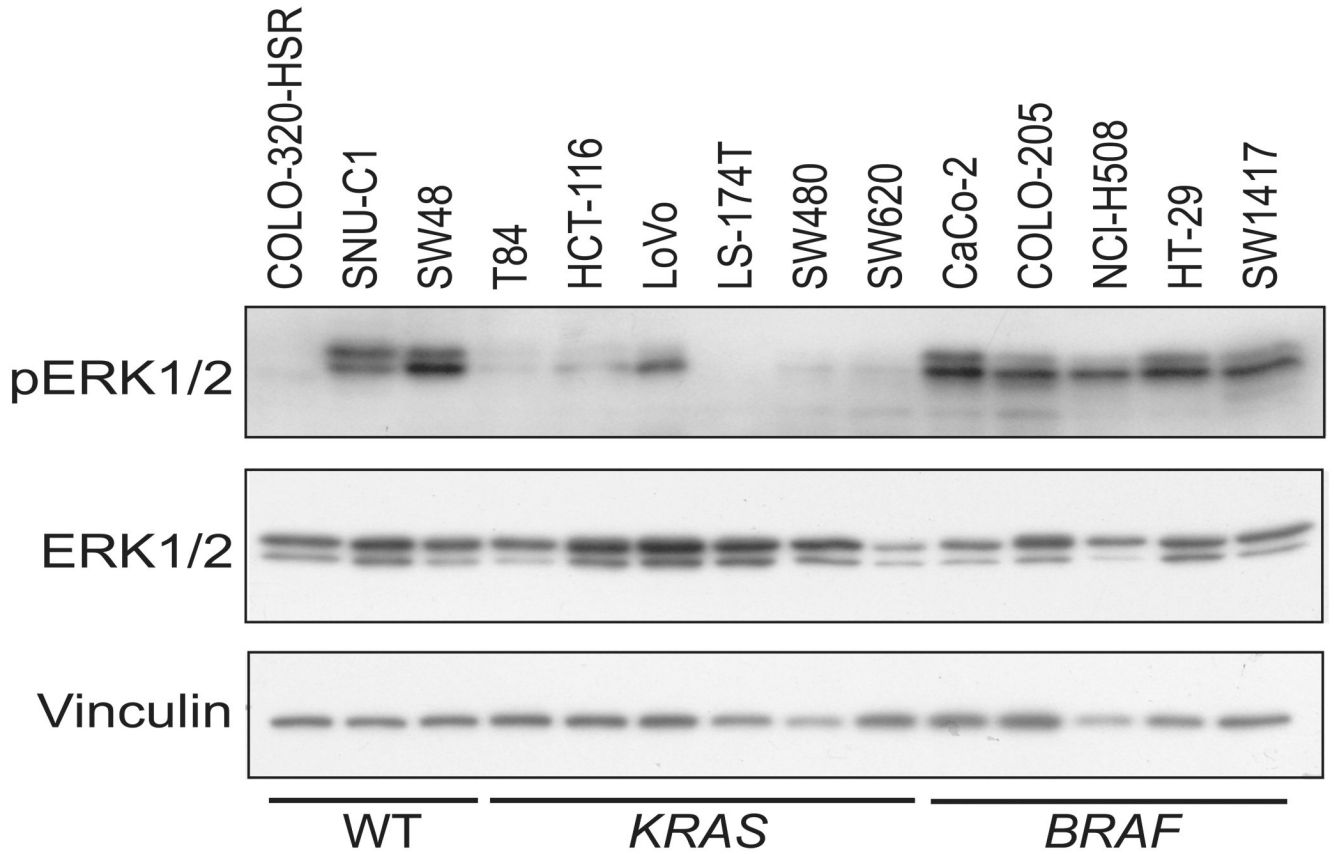
Figure 2.
ERK activity does not appear to be associated with *KRAS* and *BRAF* mutation status. Western blot analyses with phospho-specific anti-ERK1 and ERK2 antibody were done to evaluate ERK1/2 phosphorylation and activation. Parallel blotting analyses with total anti-vinculin were done to verify equivalent loading of cellular protein. Data shown are representative of at least three independent experiments. (WT, *KRAS/BRAF* wild-type; *KRAS*, *KRAS* mutation positive, *BRAF*, *BRAF* mutation positive).
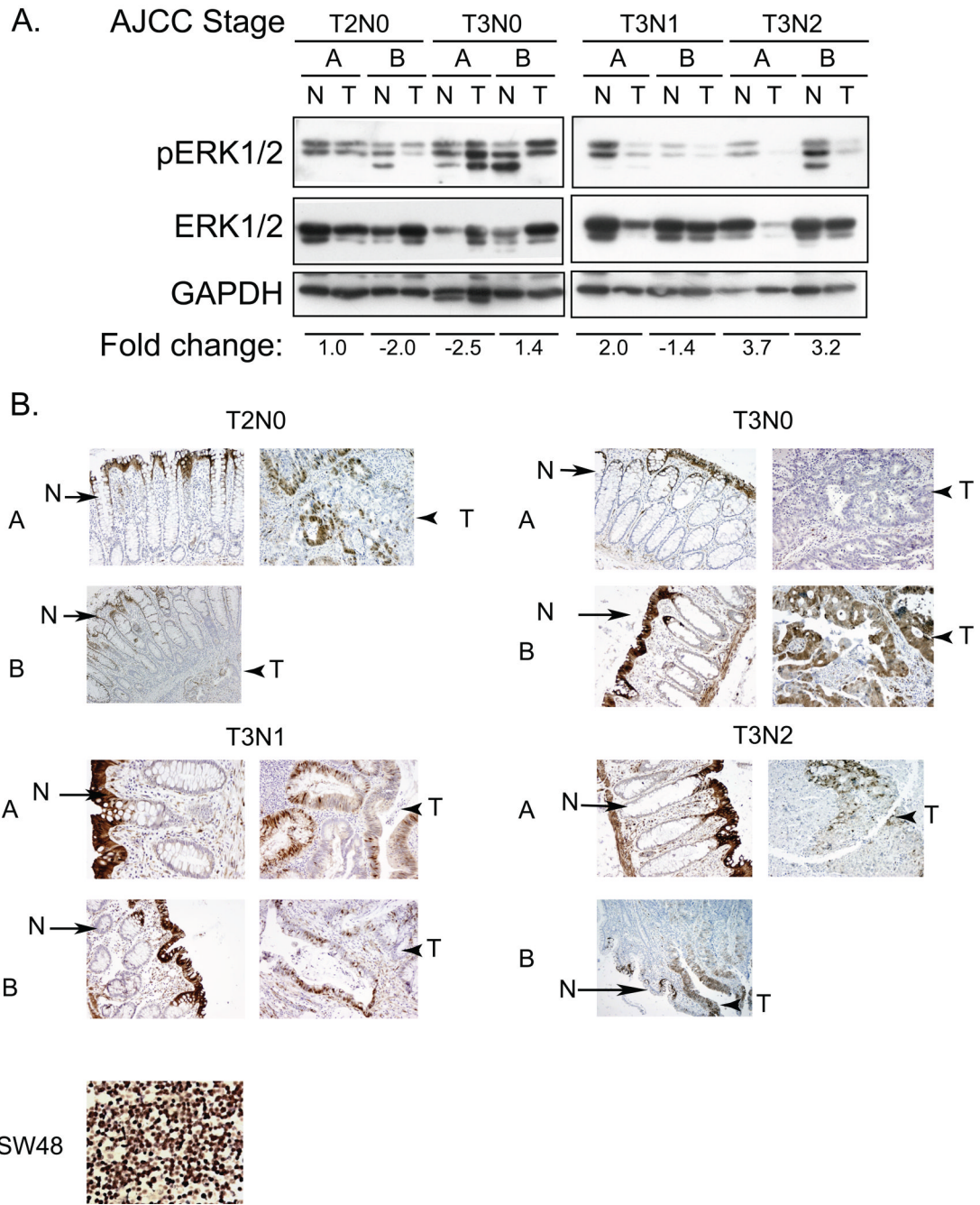
A.

AJCC Stage	T2N0		T3N0		T3N1		T3N2	
	A	B	A	B	A	B	A	B
	N T	N T	N T	N T	N T	N T	N T	N T

pERK1/2

ERK1/2

GAPDH

Fold change:	1.0	-2.0	-2.5	1.4	2.0	-1.4	3.7	3.2

B.

Figure 3.
ERK activation in matched normal and tumors from colorectal cancer patients. *A*, ERK is activated in normal tissue. Total cell lysates of matched normal (N) and tumors (T), identified by AJCC stage, were immunoblotted with anti-pERK and anti-GAPDH sera. Fold change in the relative intensity of pERK was calculated for matched tumors compared to normals (T/N) using total ERK as the standard. *B*, ERK is activated in normal colonic epithelial cells. CRC tissues, identified by AJCC stage, were immunohistochemically stained with anti-pERK serum. Arrows indicate pERK staining in normal adjacent colonic epithelial cells. Arrowheads indicate pERK staining in tumor. Paraffin embedded SW48 cell lines with high levels of pERK are shown as a positive control.
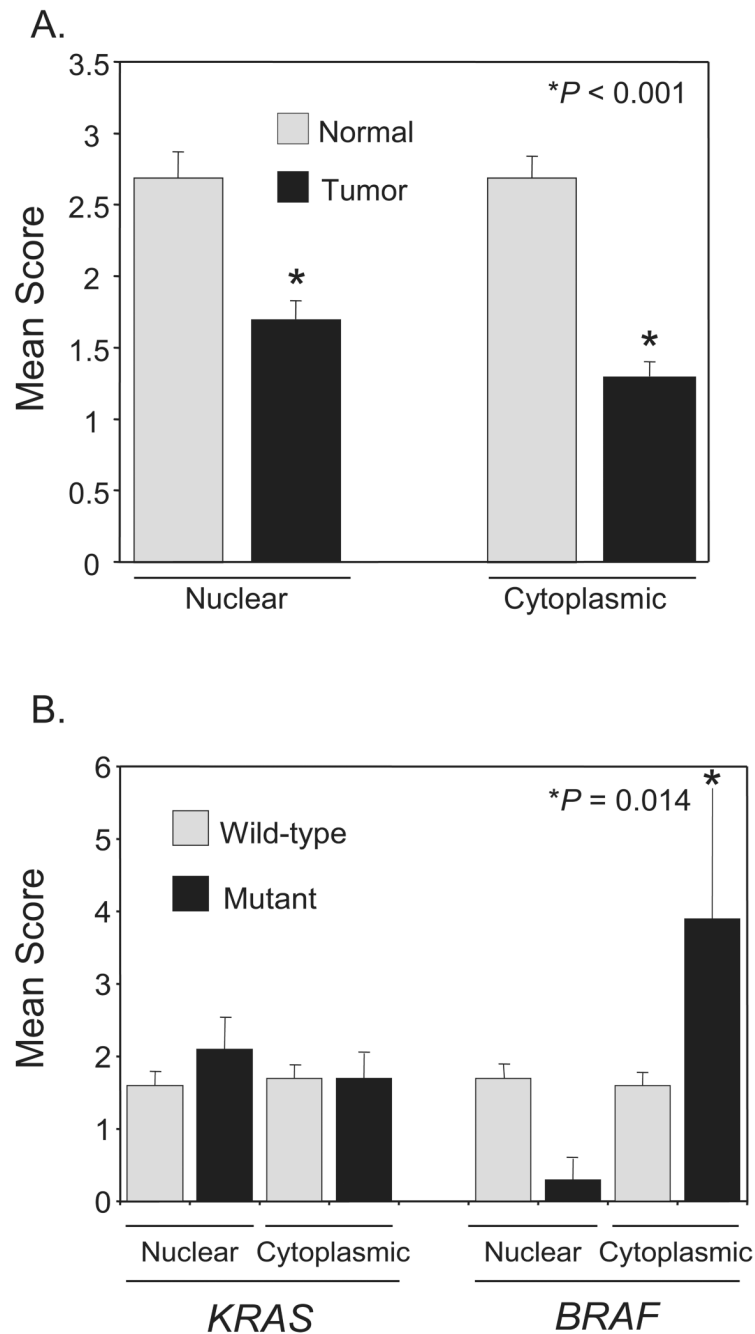
Figure 4.
ERK activation in colorectal cancer patient normal and tumor tissues. Tissue microarrays of 190 matched normal colon and tumors were prepared as described in Materials and Methods and immunohistochemically stained with anti-P-ERK serum. Mean scores were expressed as the product of intensity (I) and proportion (P) of positive epithelial staining. *A*, ERK activation in matched normal mucosa and colorectal tumors. *B*, ERK activation in patients with *KRAS* and *BRAF* mutations.

Table 1

Gene mutation and MEK inhibitor profile of human colorectal carcinoma cell lines[a]

Cell Line	MSI[b]	KRAS	BRAF	TP53	APC	Other[c]	MEK inhibitor[f]
COLO-320-HSR	Stable	WT	WT	R248W[†‡]	S811[*‡]		Insensitive[**]
SNU-C1	Stable	WT	WT	S166[*‡]	WT		Insensitive
SW48	Stable	WT	WT	WT	WT	EGFR G2155A[†] CTNNB1 S33Y[†]	Insensitive
T84	Stable	G13D[†]	WT	WT	L1488fs[*19§‡]	PIK3CA E542K[†]	Sensitive
HCT-116	Unstable	G13D[†]	WT	WT	WT	PIK3CA - H1047R[†] CTNNB1 - S45_L46>L[§] - if[†] BRCA2 - I2675fs[*]6 (Insertion)[†] CDKN2A - R24fs[*]20 (Insertion)[†]	Insensitive
LoVo	Unstable	G13D[†]	WT	WT	R1114[*†] M1431fs[*428§†]	None[d]	Sensitive
LS-174T	Unstable	G12D[†]	WT	WT	WT	PIK3CA - H1047R[†] CTNNB1 - S45F[†]	Sensitive
SW480[e]	Stable	G12V	WT	R273H and P309S[†‡]	Q1338[*‡]	WT for CDKN2A, CTNNB1, EGFR	Sensitive
SW620	Stable	G12V[‡]	WT	P309S[†] R273H[‡]	Q1338[*‡]	None[d]	ND
CaCo-2[e]	Stable	WT	V600E	V600E 204[*†‡]	Q1367[*‡]	MADH4 - D351H[‡] CTNNB1 - G245A/WT (Substitution - Missense) (?) WT for EGFR	Sensitive
COLO-205	Stable	WT	V600E[†]	Y103_L111>L if[§‡]	T1556fs[*3§‡]	None[d]	Sensitive
NCI-H508	Stable	WT	G596R[†]	R273H[†]	WT	PIK3CA - E545K[†]	Sensitive
HT-29	Stable	WT	V600E[†]	R273H[†]	T1556fs[*3§†] E853[*†]	PIK3CA - P449T[†] MADH4 - Q311[*]	Sensitive
SW1417	Stable	WT	V600E[†]	WT	R1450[*]	None[c]	Sensitive

[a] Mutation data compiled from Cancer Genome Project database http://www.sanger.ac.uk/genetics/CGP/CellLines/.

[*] nonsense mutation

†heterozygous

‡homozygous

§deletion

¶insertion; fs, frameshift; if, inframe

*b*Microsatellite instability

*c*Abbreviations: CTNNB1, β-catenin; PIK3CA, p110α; CDKN2A, cyclin-dependent kinase inhibitor 2A, isoform 4 (p14ARF) (p19ARF); MADH4, SMAD4.

*d*Reported for known cancer genes.

*e*Mutation data compiled from different studies cited in Cancer Genome Project database.

*f*Compiled from data presented in the current study where the same sensitivity was seen with either U0126 or CI-1040 treatment except. where indicated (**).