Original contribution

Localization of active, dually phosphorylated extracellular signal-regulated kinase 1 and 2 in colorectal cancer with or without activating *BRAF* and *KRAS* mutations ☆,☆☆

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Summary Colorectal cancers (CRC) often show activating mutations of the *KRAS* or *BRAF* genes, which stimulate the extracellular signal-regulated kinase (ERK) pathway, thus increasing cell proliferation and inhibiting apoptosis. However, immunohistochemical results on ERK activation in such tumors differ greatly. Recently, using a highly optimized immunohistochemical method, we obtained evidence that high levels of ERK activation in rectal adenocarcinomas were associated with resistance to radiochemotherapy. In order to determine whether *KRAS* and/or *BRAF* mutations correlate to immunohistochemically detectable increases in phosphorylation of ERK (pERK), we stained biopsies from 36 CRC patients with activating mutations in the *BRAF* gene (*BRAF*V600E: BRAF^m), the *KRAS* gene (KRAS^m) or in neither (BRAF/KRAS^n) with this optimized method. Staining was scored in blind-coded specimens by two observers. Staining of stromal cells was used as a positive control. BRAF^m or KRAS^m tumors did not show higher staining scores than BRAF/KRAS^n tumors. Although BRAFV600E staining occurred in over 90% of cancer cells in all 9 BRAF^m tumors, 3 only showed staining for pERK in less than 10% of cancer cell nuclei. The same applied to 4 of the 14 KRAS^m tumors. A phophorylation-insensitive antibody demonstrated that lack of pERK staining did not reflect defect expression of ERK1/2 protein. Thus, increased staining for pERK does not correlate to *BRAF* or *KRAS* mutations even with a highly optimized procedure. Further studies are required to determine whether this reflects differences in expression of counterregulatory molecules, including ERK phosphatases.
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1. Introduction

Stimulation of growth factor receptors activates signaling pathways, including the ERK/MAPK cascade. Dimerization

and autophosphorylation of such receptors recruit adaptor and docking proteins like GRB2, which, in turn, attracts the guanine nucleotide exchange factor SOS. This leads to activation of RAS, which initiates the ERK cascade by activating the MAPK/ERK kinase kinase RAF. In turn, RAF activates the MAPK/ERK kinases MEK 1 and 2, which activate ERK 1 and 2 by dual threonine/tyrosine phosphorylation [1,2]. Most of the phosphorylated ERK1 and 2 translocate rapidly to the nucleus [3] and stimulate transcription of genes involved in cell cycle progression and cell survival [1,2]. Additionally, ERK activation is important to stromal cell proliferation and angiogenesis [4,5] and affords protection against radiation therapy in vitro [6,7]. In concert with this, radiochemotherapy has been found to be less efficient in gliomas [8] and rectal adenocarcinomas [9], which express high levels of activated ERK (pERK).

Overexpression and/or aberrant activation of the epidermal growth factor (EGF) receptor, (EGFR; ErbB-1; HER1), is frequent in colorectal carcinoma (CRC) and constitutes a therapeutic target for monoclonal antibodies and kinase inhibitors [10]. Activating mutations downstream of EGFR are most common in RAS or RAF and result in constitutive activation of ERK. Tumors harboring such mutations may not respond to EGFR-directed therapies but might respond to RAF or MEK inhibitors [11,12]. The RAS family of small GTPases includes three members (KRAS, NRAS and HRAS), and the RAF family of signal transduction protein kinases also includes three members (ARAF, BRAF and CRAF). Activating *KRAS* mutations, which contraindicate EGFR-directed therapy, occur in around 30%–40% and *BRAF* mutations occur in around 10% of CRC [12]. With rare exceptions, *KRAS* and *BRAF* mutations are mutually exclusive [12]. The predominant activating mutation in the *BRAF* gene substitutes valine with glutamic acid at position 600 (BRAFV600E), and the resulting mutant protein can be immunohistochemically detected [13–15]. In contrast, immunohistochemical procedures for detecting *KRAS* mutations are not available. Currently, *BRAF* mutations are not considered contra-indicative of EGFR-directed therapies, but detection of such mutations opens up alternative treatment modalities like RAF inhibitors [12].

Thus, activating *RAS* and *RAF* mutations lead to increased ERK activation. ERK activation can be immunohistochemically detected by antibodies, which simultaneously detect dual phosphorylations on both ERK1 (on Thr202/Tyr204) and ERK2 (on Thr185/Tyr187). However, in melanomas, such staining does not correlate to the presence of the *BRAF*V600E mutation [16]. Interestingly, heterogeneity of BRAFV600E expression has been detected in some melanomas, suggesting that this mutation may be a secondary event in such tumors [17]. Moreover, studies of ERK activation in CRC have yielded divergent results. In one study, cancer cells of all CRC tumors studied stained [18], whilst others observed a more differentiated pattern [19,20]. One study reported only partial correlation to mutation status [19], whilst another study reported a positive correlation to *KRAS* mutations [20]. These discrepancies may reflect the use of different types of antibodies, use of inefficient demasking techniques (discussed by Holck et al [9]), of large tissue blocks and/or aged or inappropriately fixed material [21–23]. This is particularly important with respect to phosphoproteins, which rapidly are dephosphorylated. Thus, surgical resections that are not immediately fixed may fail to stain for pERK and other phosphoproteins [9,22,23]. Fortuitously, stromal cells also show staining for pERK and this can be used as an internal positive control for the quality of the fixation and staining in cases where cancer cells are unreactive [9,16].

In previous studies of pERK in BRAF- or KRAS-mutated CRC [18–20], material from surgical resections appear to have been used throughout and use of stromal cells as internal controls was not reported. Different pERK antibodies were used in the three studies, two monoclonals [18,19] and one polyclonal [20]. Although all three antibodies were derived from animals immunized with the relevant phospho-epitope, different results may ensue because different antibodies may require different primary or secondary structures surrounding the phosphorylated residues. Moreover, antibodies differ in avidity and low-avidity antibodies may dislocate during washings, resulting in weakened or indistinct staining [24]. Additional problems are inherent with polyclonal antibodies, including the potential presence of antibodies to contaminating proteins, like keratins from animal keepers [24]. This necessitates the use of negative controls to document antibody specificity. Moreover, positive internal controls, showing staining of stromal cells, are needed to document that delays in fixation have not affected the results [9,16,24]. As discussed above, this is particularly important when surgical resections are examined. In two of the studies cited [19,20] no controls at all were reported and in the third study [18], replacement of the primary antibody with phosphate-buffered saline served as the only (negative) control. Unfortunately, this control only examines whether the secondary antibodies and detection reagents react with the tissue and tells us nothing about the specificity of the primary antibody [24]. Finally, epitope demasking was carried out using citrate buffers at pH 6 in all three studies [18–20]. This may have compromised detection efficiency because careful comparisons recently carried out in our laboratory documented that alkaline demasking (pH 9) is far superior to acidic demasking in citrate buffer at pH 6 (compare Fig. 1 A and C with Fig. 1 B and D by Holck et al [9]).

In the present study, we examined biopsies from CRC tumors harboring activating mutations in the *BRAF* gene (*BRAF*V600E: BRAF^m), the *KRAS* (KRAS^m) gene or in neither (BRAF/KRAS^n) with our highly optimized immunohistochemical method for pERK detection [9]. As documented by Holck et al [9], the method was optimized by use of small endoscopic biopsies that were immediately fixed upon removal, by the use of alkaline epitope demasking and by the use of stromal cell staining as an internal positive control. Additionally, we included a number of specificity controls (dephosphorylation, type-matched monoclonal antibodies and Western blotting) and pERK staining results were compared
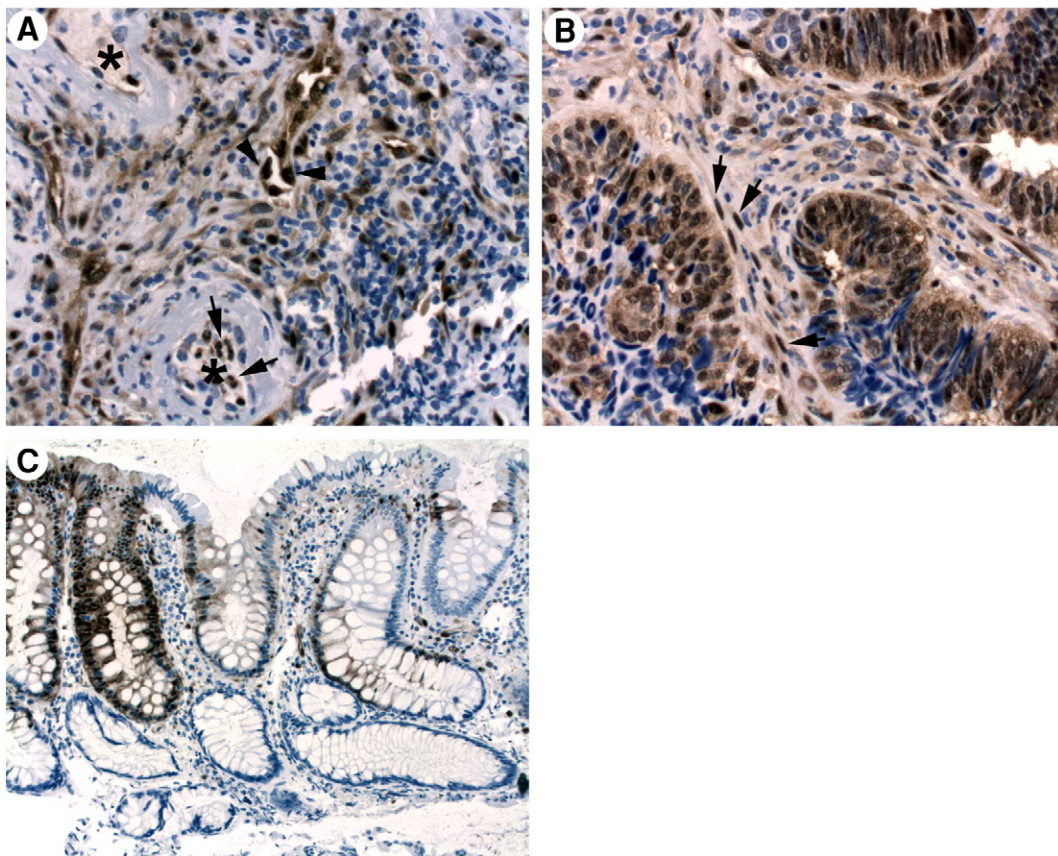
Fig. 1 CRC biopsies stained for pERK with the Milan8R antibody (A-D). A, Juxtatumoral area of a BRAF/KRAS^n tumor (#1536) showing strong staining of endothelial cell nuclei in capillaries and venules (exemplified by arrowheads) as well as of myofibroblast-like cells. In contrast, endothelial cells of arterioles and small arteries (asterisks) show more variable staining. Note staining of inflammatory cells (arrows) in the lumen of the arteriole at the bottom. B, Strong staining in nuclei of a majority of tumor cells as well as of myofibroblast-like cells (arrows) in a BRAF/KRAS^n tumor (#1536). C, Variable pERK staining in mucosa adjacent to a BRAF/KRAS^n tumor (#1536). Note staining of part of the surface epithelium and of some, but not all, crypts. In addition, staining occurs in cells of the lamina propria.

to stainings with an antibody that does not discriminate between phosphorylated and non-phosphorylated ERK1/2. In order to exclude a heterogeneous expression of the BRAFV600E mutation in CRC [as noted in melanomas in 17], the pattern of ERK activation was compared to the expression pattern of the BRAFV600E protein in BRAF^m tumors.

2. Materials and methods

2.1. Tumor material

Endoscopic biopsies from 36 cases of CRC were immediately fixed in 10% formalin and routinely embedded in paraffin. The material was assessed by molecular analysis for mutations in *BRAF* and *KRAS* and tumors with mutations in the *BRAF* (n = 9) or the *KRAS* (n = 14) gene or in neither of these (n = 13) were selected. Pathological tumor staging, nodal metastasis, vascular invasion and differentiation were

assessed on hematoxylin and eosin–stained sections of surgical resections. Patient data are summarized in the Table. The study was approved by the Danish Data Protection Agency (2008–41-2252) and Ethical Committee (H-KF-26,288/KF-01-164/03).

2.2. Mutation analysis

KRAS and *BRAF* mutation analysis was performed using Roche cobas® K-RAS Mutation Kit (product number 05,852,170,190) (Roche, Pleasanton, CA). The Roche K-RAS mutation assay is a CE-IVD real-time PCR melting curve mutation assay without genotyping, detecting 6 specific mutations in codon 12 (exon 2), 5 mutations in codon 13 (exon 2) and 6 mutations in codon 61 (exon 3) of the *KRAS* oncogene. *BRAF* mutation analysis was performed using the Roche cobas® BRAF V600 mutational analysis (product number 05,985,595,190), which evaluates the *BRAF* V600 site in exon 15 and detects wild-type or mutated V600E. The analyses were performed according to the manufacturer's instructions on the z480 Light cycler (Roche).

Table   Patient characteristics

	BRAF[m]	KRAS[m]	BRAF/KRAS[n]
Age: median (range)	76 (55–84)	67 (47–87)	65 (49–83)
Sex	8 female/1male	6 female/8male	4 female/9 male
Location [a]	7 right/2 left	11 right/3 left	5 right/8 left
T stage	8 T3/1 T4	7 T3/6 T4/1 ND	1 T2/8 T3/4 T4
N stage	1 N0/6 N1/2 N2	1 N0/4 N1/8 N2/1 ND	3 N0/3 N1/7 N2
Vascular invasion	2 cases	5 cases/1 ND	6 cases
MMRP expression	3 normal/6 abnormal	11 normal/1 abnormal/2 ND	13 normal
Differentiation	3 m/5 p/1 u	8 m/6 p	12 m/1 p

Abbreviations: BRAF[m], tumors with a *BRAF*V600E mutation; BRAF/KRAS[n], tumors with no mutations in the *BRAF* or *KRAS* genes according to the testing panel; KRAS[m], tumors with activating *KRAS* mutations; m, moderate glandular differentiation; MMRP, mismatch repair proteins; ND, not determined; p, poor or mucinous differentiation; u: undifferentiated carcinoma.

[a] Right denotes caecum, ascending and transverse colon, left denotes descending colon, sigmoid colon and rectum.

2.3. Immunohistochemistry

Three-micrometer sections (routine thickness in our laboratory) were demasked (PTLink, Dako, Glostrup, Denmark) at high pH [9] and stained with either of two different mouse monoclonal antibodies specific for dually phosphorylated ERK1/2 (clone Milan8R; mouse IgG1, eBioscience/Affymetrix, San Diego, CA as well as clone E10; mouse IgG1, #9106, Cell Signaling Technology, Danvers, MA, both monoclonals recognize phosphorylation of Thr202/Tyr204 in ERK1 and Thr185/Tyr187 in ERK2) [9] as well as with mouse monoclonals specific for total ERK1/2 protein, regardless of phosphorylation status (clone L34F12; mouse IgG1, #4696, Cell Signaling Technology), BRAFV600E (VE1, Ventana Medical Systems, Tucson, AZ) or mismatch repair proteins (MSH6: IR086; MSH2: IR085; MLH1: IR079; PMS2, Dako). The site of antigen–antibody reaction was detected by a three- (the MSH2 monoclonal) or two-layer (all remaining monoclonals) polymer peroxidase detection system (Envision, Dako) using a Dako autostainer, followed by counterstaining with hematoxylin. Negative controls included substitution of the pERK antibodies with a type-matched control mouse IgG1 to *Aspergillus niger* glucose oxidase, which is absent from mammals (DAK-GO1, Dako) or dephosphorylation with 4800 units of λ protein phosphatase (New England Biolabs, Ipswich, MA) in 0.3 ml NEB buffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35 pH 7.5, supplemented with 1 mM MnCl2) for 60 min at 30°C prior to pERK staining.

2.4. Scoring and statistics

Staining intensities of cancer cell nuclei were scored by two independent observers in blind-coded specimens as absent (0), moderate (1) or strong (2), and numbers of stained cancer cell nuclei were graded as 0% (0), below 10% (1), 10%–60% (2) and above 60% (3) [8]. The intensity scores were multiplied with the number scores in order to characterize the overall staining as absent (0), weak (≤ 1; exemplified in Fig. 2A, D and E), intermediate (>1–3) or strong (>3; exemplified in Figs. 1B, 2B and F). Averages of the original scores from both observers were used for statistical analysis. Statistics were calculated using SAS (version 9.3, SAS Institute, Cary, NC) and GraphPad Prism (version 4, GraphPad Software, Inc., La Jolla, CA) with the significance level set at 5%.

2.5. Cell culture and Western blotting

Colonic carcinoma (CaCo-2) cells (ATCC/LGCC, Wesel, Germany), grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Invitrogen, Naerum, Denmark) and 10% fetal calf serum, were lysed with the MCL1 kit (Sigma, St. Louis, MO). Protein determination employed the QuantiPro BCA kit (Sigma). Following electrophoresis on Novex 4%–12% Bis-Tris gels, transfer to PVDF membranes (Invitrogen) and staining with the pERK monoclonal antibodies, antigen–antibody reactions were detected with alkaline phosphatase-conjugated antimouse antibody and development in Novex AP substrate (Invitrogen).

3. Results

Biopsies for immunohistochemical analysis were selected on the basis of prior molecular testing. According to the test panel (Materials and Methods), 9 cases had *BRAF*V600E mutations (BRAF[m]), 14 cases had activating *KRAS* mutations (KRAS[m]) and 13 cases had neither *BRAF* nor *KRAS* mutations (BRAF/KRAS[n]). Patient data are summarized in the Table. Patients with the *BRAF*V600E mutation showed a preponderance of right-sided tumors (caecum, ascending and transverse colon) (78%), poor or mucinous differentiation (67%) and defect mismatch repair protein (MMRP) expression in 6 out of 9 tumors (67%). Defect expression of MLH1 and PMS2 was detected in 5 of the BRAF[m] tumors, which showed defect MMRP expression. This agrees with the previously reported frequent occurrence of epigenetic silencing of the MLH1 promoter by DNA hypermethylation in tumors with the V600E *BRAF* mutation and with the fact that such silencing is accompanied by defect PMS2 expression (reviewed by Kawakami
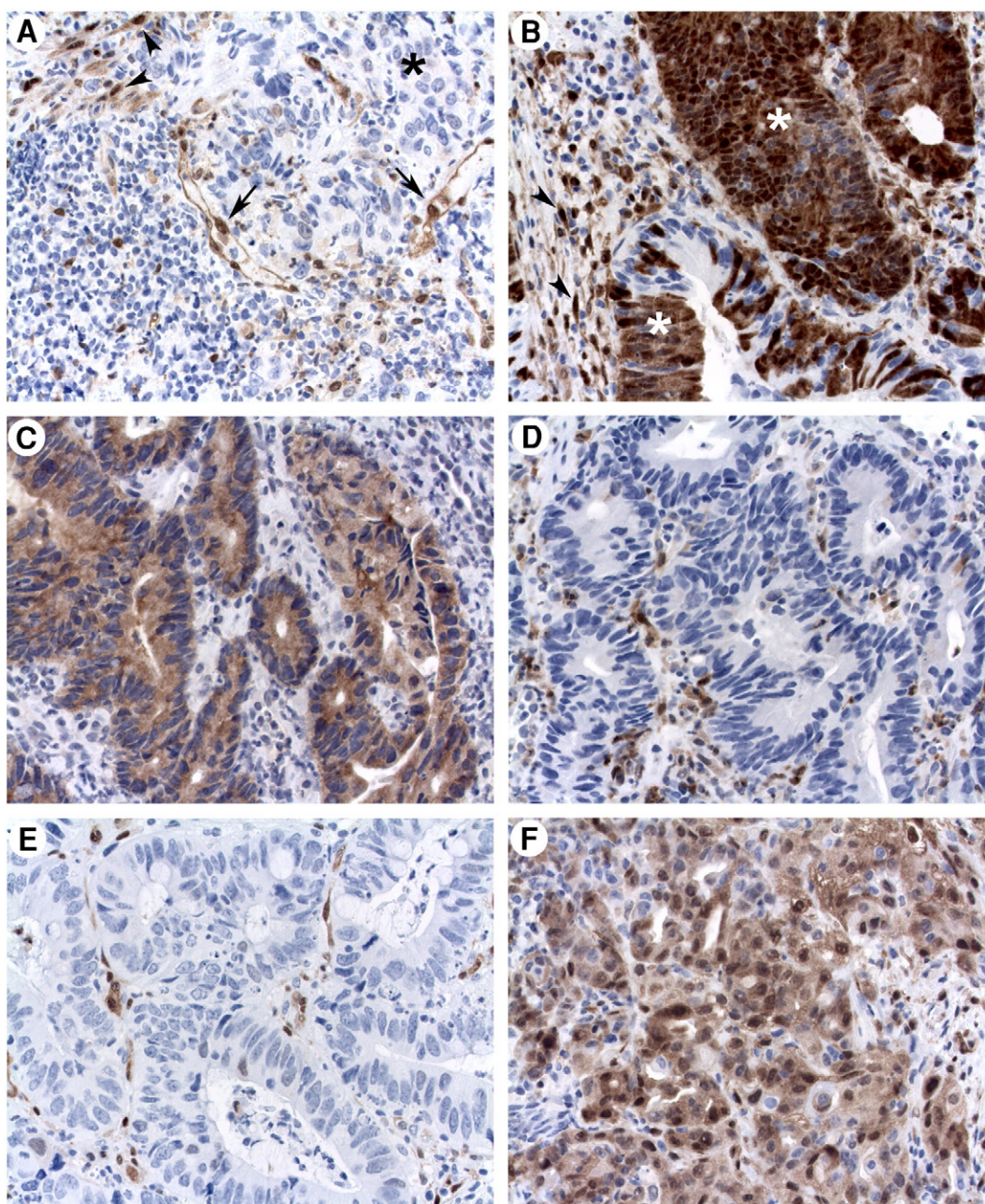
Fig. 2 CRC biopsies stained for pERK with the Milan8R antibody (A, B, D-F) or for BRAFV600E (C). A, BRAF/KRAS^n tumor (#1530) showing pERK staining of endothelial cells (exemplified by arrows) and myofibroblast-like cells (exemplified by an arrowhead) but little or no staining of cancer cells (exemplified by an asterisk) or of inflammatory cells (present in the lower left corner). B, BRAF/KRAS^n tumor (#1536) showing strong pERK staining of the majority of cancer cell nuclei (exemplified by asterisks) as well as of myofibroblast-like cell nuclei (exemplified by arrowheads). C and D, BRAF^m tumor (#1553) showing staining for the BRAFV600E mutant protein in all cancer cells, but not in stromal cells (C) whereas staining for pERK only occurs in stromal cells and is absent from the cancer cells in this field of vision (D). E, KRAS^m tumor (#1578) showing pERK staining of stromal cells but little or no staining of cancer cells. F, KRAS^m tumor (#1539) showing moderate staining of the majority of cancer cell nuclei.

et al and Lipsyc et al [25,26]). Patients with KRAS^m also showed a preponderance of right-sided tumors (79%), but only 43% showed poor or mucinous differentiation, and only 1 (9%) showed defect MMRP (MSH2 and MSH6) expression (Table). Patients with BRAF/KRAS^n showed no right-sided preponderance (38%) and no defect MMRP expression.

Biopsies were stained for pERK using 2 different monoclonal antibodies (Milan8R and E10). Both antibodies detected staining of cancer cells and intermingling stromal cells (Figs. 1 B, 2A and B, D-F). Variable proportions of stromal and cancer cells were stained in individual tumors but all showed a uniform staining pattern throughout the block.

Staining was strongest in nuclei and weaker in the cytoplasm of both cancer and stromal cells (Figs. 1A and B, 2A and B, D-F). This concurs with data showing that most phophorylated ERK translocates to the nucleus [3]. Positive stromal cells, which intermingled with cancer cells, included endothelial cells and myofibroblast-like cells (Figs. 1B, 2A and B, D-F). In addition, intra- and extravascular inflammatory cells showed strong nuclear and weaker cytoplasmic staining for pERK in some (Fig. 1A), but not all (Fig. 2A), specimens.

In the mucosa adjacent to the tumors, staining for pERK varied considerably even within the same section. Thus, in some areas, staining of the surface epithelium was marked, whilst staining was absent from other nearby areas (Fig. 1C). Staining of endothelial cells and of myofibroblasts in the lamina propria was also observed (Fig. 1C). All staining detected in the tumor-adjacent mucosa occurred in both nuclei and cytoplasm but was strongest in nuclei.

Of the two antibodies tested, the Milan8R antibody produced crisper staining than the E10 antibody. However, results obtained were closely similar (vide infra for quantitative results). Controls, including dephosphorylation prior to immunohistochemical staining for pERK or use of type-matched control IgG1 were negative. Western blotting of CRC cell lysates detected bands only at the positions of pERK1 and pERK2 (Fig. 3). With both antibodies, the pERK2 band predominated, as is the case in most cell types [27].

All specimens were examined by two observers (S.H. and L.I.L.), who were unaware of the mutation status. Cancer cell staining for pERK varied considerably between tumors whereas staining of intermingling stromal cells was constantly present. Quantitative results obtained with the Milan8R and the E10 antibodies showed a very strong correlation (Spearman $\rho = 0.822$, $P < .0001$). Interobserver agreement was highest for the Milan8R antibody ($\kappa = 0.73$) and somewhat poorer with the E10 antibody ($\kappa = 0.61$), which may relate to the more crisp and clear staining afforded by the former antibody. Staining for the mutated BRAFV600E protein revealed staining of over 90% of all cancer cells in all 9 BRAF^m tumors (Fig. 2C). There was no staining for BRAFV600E protein in stromal cells, nor was staining detected in tumors that were negative for BRAF^m by molecular testing. In 3 of the 9 BRAF^m tumors, less than 10% of the cancer cell nuclei stained for pERK (Fig. 2D), and in 2 of these, cancer cell nuclei were only weakly stained whilst intermediate nuclear staining intensity occurred in the third tumor. In all of these 3 BRAF^m tumors, strong staining of intermingling stromal cell nuclei was detected, as exemplified in Fig. 2D. In the remaining 6 BRAF^m tumors, 10%–60% of all cancer cells showed nuclear staining, which was of strong intensity in 2 and of intermediate intensity in 4. Of the KRAS^m cases, 4 tumors showed pERK staining in less than 10% of the cancer cell nuclei, and in 2 of these cases only weak nuclear staining occurred (Fig. 2E). The remaining 10 cases of KRAS^m tumors showed staining for pERK in 10%–60% of cancer cell nuclei (Fig. 2F) and in 5 of these cases such staining was strong. Of the BRAF/ KRAS^n cases, 3 tumors had less than 10% of cells showing
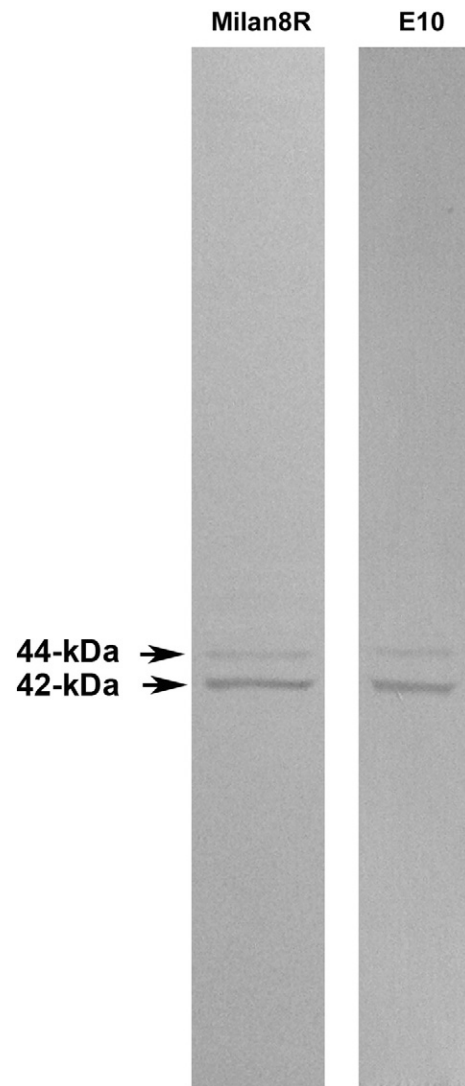

Fig. 3 Full-length Western blots of CaCo-2 cell lysates stained with the pERK monoclonal Milan8R and E10 antibodies. Staining only occurs in two bands present at the positions of pERK1 (44-kDa) and pERK2 (42-kDa). Staining of the pERK2 band is strongest, which is the case in most tissues.

pERK staining in cancer cell nuclei (Fig. 2A), whilst 10 cases showed staining in 10%–60% of the nuclei (Figs. 1B and 2B). In 5 of the latter cases, nuclear staining for pERK was strong (Fig. 1B). In all cases, pERK staining also occurred in adjacent stromal cells, which intermingled with the cancer cells (Figs. 1B and 2A and B, D-F). When the numbers of stained cancer cell nuclei were multiplied with the staining intensities, weak staining (≤ 1) was detected in 2 tumors of each category (exemplified in Fig. 2A, D and E), whilst strong staining (> 3) was detected in 2 (22%) BRAF^m tumors, 5 (36%) KRAS^m tumors and 4 (31%) BRAF/KRAS^n tumors (exemplified in Figs. 1B, 2B and F). There were no significant differences between the BRAF^m, KRAS^m and BRAF/KRAS^n tumor groups with respect to mean staining for pERK (Fig. 4).

Since differences in expression levels of total ERK1 and 2 protein potentially could explain why not all BRAF^m and
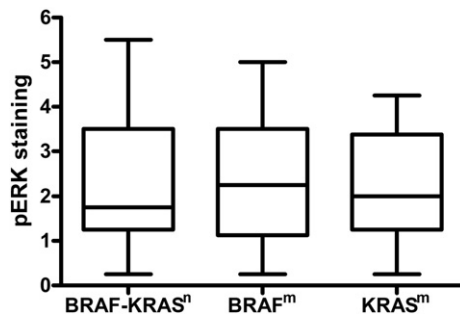
Fig. 4 Box-and-whiskers plots showing that pERK staining (cell number scores multiplied with staining intensity scores) of cancer cell nuclei does not vary with the mutational status. Horizontal lines indicate medians, boxes indicate interquartile ranges and whiskers indicate total ranges. A Kruskal-Wallis test of all three groups reveal no significant differences ($P = .996$). Additionally, no significant differences occurred between the individual groups (Mann–Whitney U test: BRAF^m *vs* BRAF/KRAS^n: $P = .947$; KRAS^m *vs* BRAF/KRAS^n: $P = .981$; BRAF^m *vs.* KRAS^m: $P = 1.000$).

KRAS^m tumors showed evidence of increased ERK activation, we stained all tumors with an antibody that did not discriminate between phosphorylated and non-phosphorylated ERK1/2. All tumors showed cytoplasmic staining of cancer cells. In addition, some tumors showed nuclear staining of cancer cells, whilst stromal cells (endothelial cells and myofibroblast-like cells) showed cytoplasmic and variable nuclear staining. Variable degrees of staining were also observed in nuclei and cytoplasm of inflammatory cells. Blind-coded specimens were scored for nuclear and cytoplasmic staining of cancer cells. There were no significant differences in total ERK1/2 protein staining of either cancer cell cytoplasm or nuclei between the BRAF^m, KRAS^m and BRAF/KRAS^n tumor groups (data not shown). Importantly, the cancer cells, regardless of whether or not they contained pERK, expressed ERK1/2 protein in the cytoplasm. Moreover, scorings showed that levels of cytoplasmic ERK1/2 protein did not differ between cancer cells that contained high and low levels of pERK (Fig. 5). This was also evident from studies of adjacent sections stained for pERK and for ERK1/2 protein (Fig. 6A and B). These results definitely prove that deficient ERK phosphorylation in some of the cancers does not reflect deficient ERK1/2 protein expression. In agreement with the fact that phosphorylation of ERK1/2 is needed for its translocation into the nucleus [3], tumors having cancer cells with pERK-positive nuclei contained comparable numbers of nuclei staining for ERK1/2 protein (Figs. 5B, 6C and D).

4. Discussion

We have previously shown that immunohistochemical staining for pERK can be highly optimized by alkaline demasking [9]. This results in much more intense staining than citrate buffer demasking, which is recommended by the
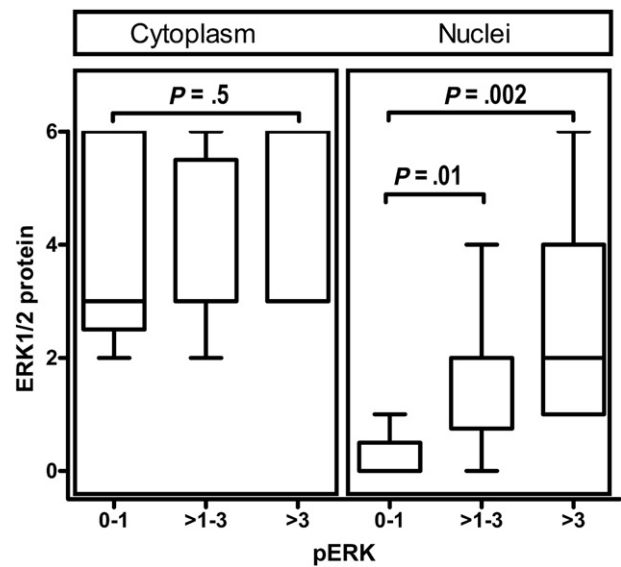

Fig. 5 Box-and-whiskers plot of cytoplasmic (left) and nuclear (right) staining scores for ERK1/2 protein versus nuclear staining scores for pERK. Note that also cancer cells, which lack significant nuclear pERK staining, contain substantial cytoplasmic stores of ERK1/2 protein, whereas nuclear staining scores for ERK1/2 protein vary in parallel with nuclear staining scores for pERK. The P values refer to Mann–Whitney U tests.

antibody vendors and which has been used in most previous studies [16,18–20]. Additionally, endoscopic biopsies that immediately were fixed upon removal were used. This is important because variations in ischemia time may compromise staining of these highly labile phosphoepitopes [9,16,21–23]. Moreover, staining of stromal cells served as a valuable internal positive control and was uniform throughout the blocks with no evidence of a gradient. The staining of stromal cells in CRC was not unexpected because ERK activation is important both to angiogenesis and fibroblast proliferation [4,5] – phenomena that characterize most cancers. Staining of tumor-adjacent mucosa was very variable, even within the same biopsy, and was therefore not useful as an internal positive control. This variability may conceivably relate to effects like pressure or other influences of the tumor on adjacent mucosa, to differences in patient age, to the degree of concomitant inflammation and/or to different types of enemas administered prior to endoscopy. Two different monoclonal pERK antibodies produced identical results but the Milan8R antibody was preferred because it produced more crisp staining than the E10 antibody. Interobserver agreement was better with the Milan8R antibody than with the E10 antibody, which may reflect the clearer staining afforded by this antibody. Nevertheless, there was a very strong correlation between results obtained by both antibodies. Three controls for the specificity of the staining were performed. Firstly, the primary pERK antibody was substituted for a type-matched IgG1 monoclonal of irrelevant specificity, and this resulted in no staining. Secondly, in sections that were dephosphorylated prior to immunohistochemistry for pERK no staining occurred. Thirdly, we used
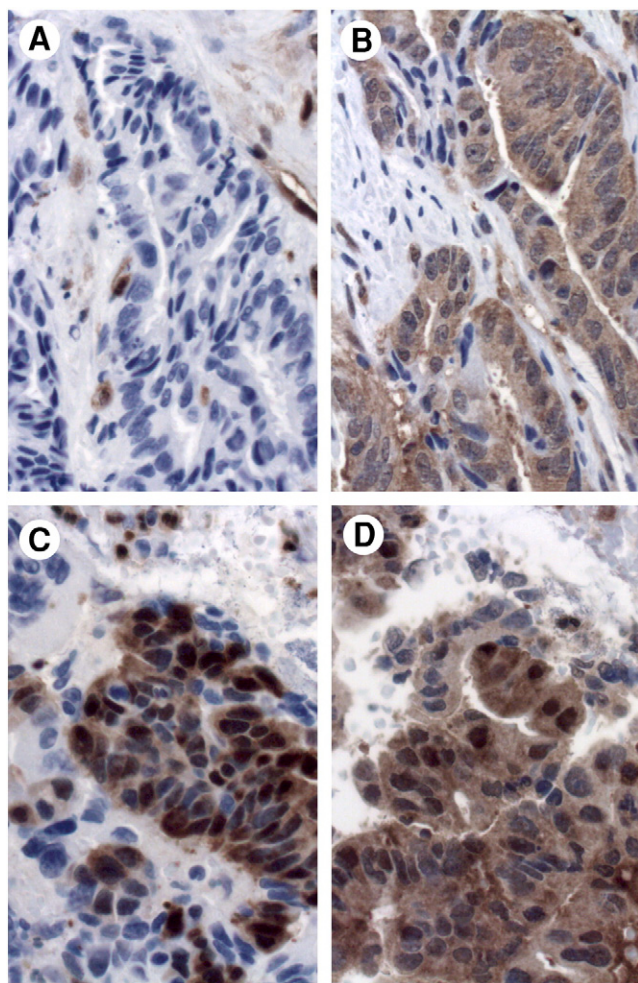
Fig. 6 Adjacent sections of tumors stained for pERK (Milan8R) and total ERK1/2 protein. A and B, BRAF^m tumor (#1521), showing little or no pERK staining in cancer cells but uniform cytoplasmic staining for ERK1/2 protein. Note also positive staining for both pERK and ERK1/2 protein in stromal cells. C and D, KRAS^m tumor (#1510), showing strong nuclear and weaker cytoplasmic staining for both pERK and for ERK1/2 protein.

Western blots of cultured CRC cells to avoid contributions from intermingling stromal cells, and such blots revealed two bands in the positions expected for pERK1 and pERK2 and no additional bands.

The purpose of the present study was to examine whether the optimized procedure for pERK immunohistochemistry could detect differences in ERK activation between CRCs with or without activating mutations in the *KRAS* or *BRAF* genes. Thus, previous studies on CRC have yielded somewhat conflicting data [18–20], whereas studies of melanomas have shown little correlation between expression of the predominant *BRAF* mutation (*BRAF*V600E) and ERK activation [16]. Previous studies have also demonstrated cell heterogeneity with respect to expression of this *BRAF* mutation in some melanomas [17]. Potentially, such heterogeneity could translate into heterogeneous ERK activation. We therefore also stained the CRC tumors with an antibody detecting the

mutated BRAFV600E protein and examined blind-coded specimens. Staining for BRAFV600E did, in agreement with previous studies [13–15], correlate perfectly with the results of molecular testing. Moreover, at least 90% of all cancer cells stained for the mutated BRAFV600E protein in all 9 BRAF^m tumors examined. In contrast, pERK staining was present in less than 10% of all cancer cells in 3 of these tumors. However, even in these 3 cases, strong pERK staining was present in nuclei of intermingling stromal cells. Thus, we obtained no evidence for heterogeneous expression of BRAFV600E in CRC and conclude that BRAFV600E expression and ERK activation do not correlate. Similarly, less than 10% of cancer cell nuclei in 4 KRAS^m tumors and in 3 BRAF/KRAS^n tumors stained for pERK even under these highly optimized conditions. Although the number of investigated tumors is comparatively small (n = 36), the essential point is that several tumors with documented *BRAF* (n = 3) or *KRAS* (n = 4) mutations showed staining for pERK in only few of all cancer cell nuclei and that this staining, moreover, was weak in 2 of the BRAF^m and in 2 of the KRAS^m tumors. Inclusion of more patients would not change this evident discrepancy between activating mutations in the pathway and ERK phosphorylation.

A caveat of this, and previous, studies of correlations between *RAS* mutations and ERK activation is that not all possible activating mutations in the *RAS* genes could be tested for (cf. new guidelines discussed by Atreya et al [12]). Thus, it cannot be formally excluded that an occasional patient in the BRAF/KRAS^n group could harbor RAS mutations not tested for (for example in *KRAS* codons 117 or 146 of exon 4 or in *NRAS*). However, *NRAS* mutations only occur in only about 3%–6% of CRC tumors, and mutations in *KRAS* codons 117 or 146 of exon 4 occur in 6%–7% [12,25]. Again, however, the essential point raised by our results is that tumors proven to have activating *BRAF* or *KRAS* mutations do not necessarily show uniformly high ERK activation. Thus, the fact that all BRAFV600E-mutated tumors expressed the mutant protein in over 90% of the cells, whilst pERK was detectable in less than 10% of all cancer cells in 3 BRAF^m cases drives home the fact that there is no obligate correlation between *BRAF* mutations and ERK activation. Since similar findings were made with the KRAS-mutated group we may infer a similar lack of correlation here, but with the proviso that no immunohistochemical method for localizing the multiple forms of mutated RAS proteins exists.

Thus, even with an optimized method, pERK staining cannot be used as a surrogate marker for activating *BRAF* or *KRAS* mutations in CRC. This concurs with previous findings using the less efficient citrate demasking method in CRC [18,19] or in melanomas [16]. Moreover, studies of transgenic mouse models concur with this by showing no obligatory association between activating *KRAS* mutations and ERK activation (reviewed by Deschênes-Simard et al [28]). Our staining results for total ERK protein clearly demonstrate that the failure of some BRAF^m and KRAS^m tumors to show significant ERK phosphorylation is not due to defect expression of ERK protein. Thus, cytoplasmic staining for total ERK protein did

not differ between tumors showing weak or strong pERK staining (or between mutated and BRAF/KRAS^n tumors). In contrast, nuclear staining for total ERK showed excellent correlation to nuclear staining for pERK. This reflects the fact that ERK can only be imported into the nucleus when phosphorylated [3] and adds further credence to the validity of the immunohistochemical method for pERK.

Mechanisms which regulate the magnitude and duration of ERK activation may conceivably explain why pERK is only detectable at a low level in some of the BRAF^m or KRAS^m tumors. Thus, ERK phosphorylation is typically transient because several mechanims limit its duration (reviewed by Larsson and Holck [2]). These mechanisms profoundly affect the biological outcome of ERK activation because transient activation stimulates cell proliferation, whilst prolonged activation induces differentiation and senescence [28]. This has been noted to occur also in the context of *BRAF* mutations [29]. Downregulating mechanisms include MAP kinase phosphatases (MKPs, also referred to as dually specific phosphatases: DUSPs), which dephosphorylate pERK [30]. Expression levels and stabilities of MKPs are tightly regulated, both by pERK [31,32] and by the stress-activated protein kinases p38 and Jun N-terminal kinase [32,33]. Additional regulating mechanisms exist, including interactions with the AKT–mTOR pathway, with RAF kinase inhibitors, with Sprouty and with scaffolding proteins like IQGAP1 [16,34–36]. Interestingly, expression levels of MKP-1, MKP-2 and IQGAP1 vary between tumors and correlate with tumor behavior [36–38]. Further studies are needed to determine whether these mechanisms contribute to the discrepancies between *BRAF* and *KRAS* mutations and ERK activation in CRC.

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