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ORIGINAL ARTICLE

Activation of extracellular regulated kinases (ERK1/2) but not AKT predicts poor prognosis in colorectal carcinoma and is associated with *k-ras* mutations

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Abstract Signal transduction and modulation represent central mechanisms in cellular processes such as cell-cycle regulation, oncogenesis, and apoptosis. The aim of this study was to determine the prognostic relevance of two kinases important in the regulation of cell proliferation and apoptosis in 135 colorectal cancer cases: AKT and extracellular regulated kinases (ERK1/2). We investigated the relationship of phospho-ERK1/2 (pERK1/2) and phospho-AKT (pAKT) with associated parameters (EGFR, COX-2, cyclin-D1), proliferative activity (Ki-67), and apoptosis (TUNEL) using immunohistochemistry. Additionally, the *k-ras* gene was screened for mutations to determine its putative association with ERK1/2 activation. Activation of ERK1/2 but not AKT correlated statistically with the presence of *k-ras* mutations (P=0.015). Survival analysis of phospho-ERK1/2 immunoexpression showed a significant correlation with decreased overall survival (OS). The multivariate Cox regression analysis identified pERK1/2 as an independent prognostic parameter (P=0.005). Activation of ERK1/2 in colorectal cancer may indicate aggressive tumor behavior and may constitute an independent prognostic factor. Furthermore, our data suggest that mutations of the *k-ras* oncogene may induce activation of ERK1/2. We propose immunohistochemical determination of pERK1/2 status as a promising candidate for the identification of high-risk patients who would benefit from new anticancer drugs targeting the ERK pathway.

Keywords Colorectal cancer · Molecular oncology

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Introduction

Colorectal cancer ranks second in terms of both incidence and mortality in industrialized countries. An estimated 945,000 of new colorectal cancer cases occur worldwide each year, and colorectal cancer is responsible for some 492,000 deaths [38]. Colorectal carcinoma is traditionally managed by surgical resection. Chemotherapy has been used in the adjuvant setting for regional metastatic disease, whereas adjuvant chemotherapy for localized nonmetastatic disease remains controversial [43]. Besides the classic 5-fluorouracil (5-FU) adjuvant chemotherapy, further therapeutic options are desirable especially in 5-FU-resistant advanced disease. Recently, protein kinases have emerged as a group of molecular targets with the potential to be cancer specific, allowing the development of a new generation of chemotherapy agents which inhibit these kinases. Promising targets in this context are kinases involved in the MAPK and AKT/PI3K pathway [36].

The ERK signaling pathway is a component of the mitogen-activated protein kinases (MAPK) cascade and is activated by extracellular and intracellular, frequently mitogenic, ligands, including ras proteins and epidermal growth factor (EGF), resulting in increased cellular proliferation in vivo [29, 31] and cell differentiation [33]. ERK1/2, extracellular signal-related kinases—also referred to as

p44 and p42 MAP kinases—are ubiquitously expressed. Constitutive activation of MAP kinases was observed in several human cancer cell lines including colon cancer cell lines and various human cancer tissues [20]. In human colon cancers, infrequent activation of MAP kinases has been demonstrated [32]. A recent study revealed that MAP kinases are frequently expressed in human colorectal tumors in contrast to adjacent normal colonic mucosa [26]. However, up to date, the clinical relevance of activated MAP kinases in colorectal cancer remains unknown.

AKT/protein kinase B plays a critical role in controlling the balance between cell survival and apoptosis [15]. AKT phosphorylation is mediated by phosphatidylinositol-3-OH-kinase (PI3K) [14] in response to various growth/survival factors, including EGF, platelet-derived growth factor, interleukins, and oncogenic *ras* [5, 15, 16]. Upon activation, AKT promotes cell survival by inhibition of apoptosis [6, 11]. Besides its well-known antiapoptotic functions, AKT is involved in cell proliferation [12, 37, 47]. We recently demonstrated AKT phosphorylation as an independent prognostic parameter in breast cancer [34], whereas others found pAKT-associated decreased survival in malignant tumors, including hepatocellular carcinoma, leukemia, breast cancer, pancreatic cancer, and malignant melanoma [10, 28, 44, 45].

Previous studies suggest that AKT is required in human colorectal carcinoma for suppression of cancer cell apoptosis and tumor progression [22] and that the PI3K signaling pathway is involved in the progression of colon adenocarcinoma [23].

To clarify the prognostic relevance of both of these important signaling pathways in colorectal carcinomas, we used tissue microarray (TMA) technology and evaluated pERK1/2 and pAKT immunoexpression in 135 colorectal tumor samples. Moreover, we investigated downstream targets (cyclin D1, cyclooxygenase-2) and upstream activators (EGFR) of these kinases to discover possible biological pathways. In addition, this study focuses on the oncogene *k-ras* and its impact on the ERK and AKT pathways. The *ras* gene product functions upstream of the MAPK pathway and is an important factor in the activation of ERK1/2 [3]. We screened for *k-ras* hot spot mutations in codons 12 and 13, which are frequently observed in colorectal cancer [42] and cause the ras protein to remain in an active state, continuously sending signals downstream [41].

Materials and methods

Patients

This study comprised 135 consecutive colorectal cancer patients, who underwent surgery in the years 1996–1998 according to the recommendations of the German Society of Surgery. Complete clinical records and follow-up information were available in all cases. The minimum follow-up period for patients still alive was 60 months. Surgical material was fixed in formalin and routinely processed. The tumors were classified according to the pTNM System (6th edition), and histopathological diagnosis was made according to the WHO classification of tumors of the rectum and colon [18]. Patients with UICC stages III and IV colorectal carcinoma received in the adjuvant setting a standardized chemotherapy. According to the recommendations of the German Surgical Oncology Working Group (CAO), patients with stages I and II colorectal carcinoma were not advised to any adjuvant treatment. The gender distribution was 68 men (50.4%) and 67 women (49.6%). Information about the tumor site was available for 129 cases, 78 tumors were located at the colon (60.5%), and 51 cases were at the rectum (39.5%). In 130 cases, detailed information about grading, lymph-node status, and distant metastasis was available; the T stage was available in 132 cases.

TMA construction

The most representative tumor area was carefully selected and marked on H&E (hematoxylin-and-eosin-stained) slide. In case of tumor heterogeneity, areas with lowest degree of differentiation were selected. The TMAs were constructed using a manual tissue-array instrument (Beecher Instruments, Silver Spring, MD, USA). Three 0.6-mm-thick tissue cores were taken from each colorectal specimen, and four composite TMA blocks with the primary tumors were designed [4]. One section from each TMA was stained with hematoxylin and eosin. Each block contained normal colon mucosa derived from resection margins of diverticulosis specimen.

Immunohistochemistry of TMA

The primary antibodies used as well as the technical details are summarized in Table 1. Immunohistochemistry was performed on 5-μm-thick paraffin sections. Dewaxed and rehydrated sections were incubated with hydrogen peroxide to block endogenous peroxidase. Immunostainings of phospho-AKT, EGFR, cyclin D1, COX-2, and Ki-67 were carried out with an automated staining device (DAKO Autostainer, Glostrup, Denmark). The staining pERK1/2 protocol for the automated staining device was not established at the time of publication, thus phospho-ERK staining was performed manually. After antigen retrieval antibody in a hot water bath demonstration was achieved using the alkaline phosphatase anti-alkaline phosphatase method (APAAP), the commercially available anti-mouse

Table 1 Antibodies used for immunohistochemistry

Antibody		Pretreatment (manual)	Incubation	Detection	Dilution	Company
Phospho-ERK1/2 [phospho-p44/42Map kinase (Erk1/2) threonine 202; tyrosine 204[a]]	Polyclonal	Hot water bath (95°C), pH 6.1, 45 min	Overnight at 4°C	APAAP	1:1,000	Cell Signaling Technology, Franfurt am Main, Germany
Phospho-AKT [phospho-AKT (1/2/3) serine 473]	Monoclonal	Hot water bath (95°C), pH 6.0, 20 min	30 min	Peroxydase-conjugated streptavidin; DAB	1:20	Cell Signaling Technology
EGFR	Monoclonal	TRS pH 6.1	30 min	EnVision, DakoCytomation	1:100	Zymed Laboratories, CA, USA
Ki-67	Monoclonal	TRS pH 6.1	30 min	EnVision, DakoCytomation	1:1,200	Biogenex, CA, USA
Cyclin-D1	Monoclonal	TRS pH 6.1	30 min	EnVision, DakoCytomation	1:20	Innovative Diagnostik-Systeme GmbH, Hamburg, Germany
COX-2	Monoclonal	TRS pH 6.1	30 min	EnVision, DakoCytomation	1:250	DCS, Hamburg, Germany

[a] Manual staining

IgG detection kit (EnVision, DakoCytomation, Carpenteria, CA, USA) or peroxidase-conjugated streptavidin and diaminobenzidine as chromogen were applied. Replacement of the various primary antibodies by mouse immunoglobin served as negative controls. Positive controls were included in each staining series.

TUNEL

In situ DNA fragmentation was established using the TUNEL (terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling) technique in paraffin-embedded sections. We used the ApoTag™ Plus Peroxidase in Situ Apoptosis Detection Kit (Intergen, GA, USA). Staining procedures were performed following the manufacturer's recommendations. Incubation with 20 μg/ml proteinase K solution was modified to 10 min to achieve maximum staining results. Apoptotic count was performed using a light microscope, counting stained apoptotic tumor cells per 10 HPF (400× magnification, area of HPF 0.23 mm^2). Corresponding H&E sections were analyzed to avoid miscounting necrotic cells.

DNA extraction and analysis of *k-ras* mutation

Genomic DNA was extracted from routinely processed paraffin wax blocks, and the DNA was purified with the QIAmp DNA Mini Kit (Quiagen, Hilden, Germany). Further processing was performed according to the recommendations of the supplier. Exon 1 of *k-ras*, harboring the relevant codons 12 and 13, was amplified by polymerase chain reaction (PCR), followed by direct sequencing. The PCR reaction was carried out in a total volume of 50 μl with HotMaster Taq (Eppendorf, Hamburg, Germany) according to the recommendations of the supplier. Amplification was performed in a Primus 25 Thermocycler (MWG, Ebersberg, Germany) with 2 min of initial enzyme activation at 94°C followed by 40 cycles of denaturation at 94°C for 20 s, primer annealing at 55°C for 10 s, and extension at 65°C for 35 s. Sequences of both forward and reverse strands were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, CA, USA) using BigDye Terminator chemistry according to the recommendations of the supplier (Applied Biosystems, Foster City, CA, USA). The sequences of the PCR primers were: forward primer 5′-ATTATAAGGCCTGCTGAAAATGACTGA-3′ and reverse primer 5′-ATATGCATATTAAAACAAGATTTACCTCTA-3′.

Statistical analysis

All semiquantitative immunostainings (phospho-ERK1/2, phospho-AKT, and EGFR) were assessed by two of the authors (KJS, JB) in a blind-trial fashion without knowledge of the clinical outcome. In case of disagreement, slides were evaluated by both, and a final decision was made. All data were converted to a PC and statistically analyzed using SPSS Version 12 for Windows.

The kappa coefficient of interobserver agreement was calculated for semiquantitative pERK1/2, pAKT, and EGFR immunostaining results only; interpretation of the kappa value was performed using the commonly cited scale of Landis and Koch [24]. Interobserver agreement of

pERK1/2 and EGFR was almost perfect (κ=0.81 and 0.88, respectively). Interobserver agreement of pAKT immunostaining was substantial (κ=0.71). Relationships between ordinal parameters were investigated using two-tailed chi-square analysis (or Fisher's exact test where patient numbers were small). The relationship between categorical data (e.g., pERK1/2) and numeric data (e.g., number on Ki-67-positive tumor cells) was determined using the Kruskal–Wallis test. Overall survival (OS) curves were estimated using the Kaplan–Meier method, and any differences in the survival curves were compared by the log-rank test. For multivariate analysis, the Cox regression model was used. Overall, 95% confidence intervals were used throughout.

Results

Immunohistochemical analysis of phospho-ERK1/2 and phospho-AKT

Phospho-ERK1/2 immunohistochemistry of the complete series revealed strong nuclear immunostaining and partly cytoplasmic immunostaining, while nonneoplastic tissue revealed a positive staining, exclusively located at the surface epithelium. Tumors lacking pERK1/2 staining were classified as negative. In all, 115 (85.2%) tumors were classified as negative, and 20 (14.8%) were classified as positive. We refrained from classifying pERK1/2 staining into three categories (negative, moderate, and strong) because further stratification did not provide additional information in survival analysis.

Phospho-AKT immunohistochemistry was available for 133 cases. Loss of discs from slide occurred in two cases. Immunostaining of colorectal tumor tissue revealed a specific cytoplasmic staining, whereas normal mucosal tissue exhibited no staining. Tumors were classified according to their cytoplasmic staining intensity: negative, moderate, and strong. In all, 28 (20.7%) tumors were classified as negative, 77 (57%) as moderate, and 28 (20.7%) as strong. No correlation was observed between pAKT and COX-2 (P=0.185), pERK1/2 (P=0.418), EGFR (P=0.526), cyclin-D1 (P=0.644) or growth fraction (GF) (P=0.299) and apoptosis (P=0.111), respectively.

The relationship of pERK1/2 and pAKT with relevant clinicopathological data is summarized in Table 2. Representative immunohistochemical staining results of pERK1/2 and pAKT are shown in Fig. 1.

Correlation between phospho-ERK1/2 and pAKT immunostaining with survival

In all, 60 patients died of colorectal cancer, and nine were excluded from survival-related statistics, having died from either benign or other malignant neoplasm, for example leukemia after radiation therapy. In the remaining 126 cases, 47 out of 106 (44.3%) patients with a tumor showing negative pERK1/2 immunoreactivity died from colorectal cancer. In contrast, 13 out of 20 (65%) patients with positive pERK1/2 immunoreactivity died from colorectal cancer. OS was inversely associated with a positive pERK1/2 staining status. The impact of pERK1/2 expression and clinicopathological features on patient survival

Table 2 Expression of pERK1/2 and pAKT in relation to clinicopathological data

	N	pERK1/2			pAKT (serine 473)			
		Negative	Positive	*P*-value	Negative	Moderate	Strong	*P*-value
Grade[a]				0.885				0.99
1	31	27 (87.1)	4 (12.9)		3 (10)	22 (73.3)	5 (16.7)	
2	72	60 (83.3)	12 (16.7)		14 (19.7)	40 (56.3)	17 (23.9)	
3	27	23 (85.2)	4 (14.8)		10 (37)	12 (44.4)	5 (18.5)	
T stage[b]				0.231				0.041[c]
pT1	5	3 (60)	2 (40)		1 (20)	2 (40)	2 (40)	
pT2	14	12 (85.7)	2 (14.3)		2 (14.3)	6 (42.9)	6 (42.9)	
pT3	80	71 (88.8)	9 (11.2)		13 (16.5)	53 (67.1)	13 (16.5)	
pT4	33	26 (78.8)	7 (21.2)		12 (21.5)	14 (43.8)	5 (20.8)	
Lymph-node status[a]				0.143				0.443
pN0	63	56 (88.9)	7 (11.1)		10 (16.4)	38 (62.3)	13 (21.3)	
pN+	67	54 (80.6)	13 (19.4)		17 (25.4)	36 (53.7)	14 (20.9)	
Distant metastases[a]				0.534				0.231
pM0	99	84 (84.8)	15 (15.2)		18 (18.6)	58 (59.8)	21 (21.6)	
pM+	31	26 (83.9)	5 (16.1)		10 (32.3)	15 (48.4)	6 (19.4)	

[a] Data available for 130 cases
[b] Data available for 132 cases
[c] Statistically significant

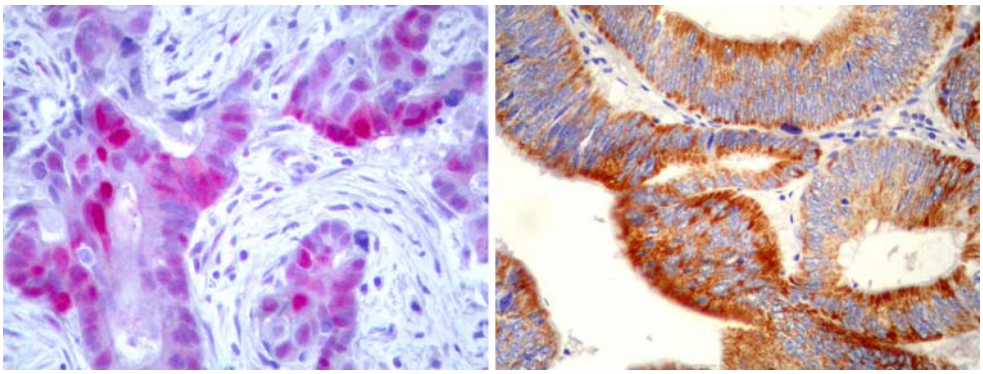

Fig. 1 Light micrograph displaying strong phospho-ERK1/2 (*left*) and strong phospho-AKT (*right*) in colorectal cancer as analyzed by immunohistochemistry

was assessed using univariate Kaplan–Meier survival analysis. Overall disease-specific survival (OS) was inversely associated with different pERK1/2 staining results (Fig. 2a). The 13 patients who had died from ERK1/2-positive colorectal cancer exhibited mainly advanced tumors with local or distant metastases. In detail, all 13 tumors were classified as T3 or T4; 12 tumors (92.3%) exhibited local and 5 tumors (38.5%) presented distant metastases. In contrast, only three (42.9%) of the seven ERK1/2-positive survivors showed T3 or T4 tumors; six (85.7%) were free of local lymph-node metastases, and none developed distant metastases. A comparable distribution was observed in the group of 46 nonsurviving patients with ERK1/2-negative tumors. Nearly all (44; 95.7%) were classified as T3 or T4 tumors; 29 (65.9%) exhibited local and 19 (42.2%) distant metastases. In contrast, 47 (82.5%) of the 57 ERK1/2-negative survivors showed T3 or T4 tumors; 35 (61.4%) were free of local lymph-node metastases, and nearly all patients lacked distant metastases (52; 92.9%). The main difference between ERK1/2-negative nonsurvivors and ERK1/2-positive nonsurvivors is the extent of survival. Whereas the 13 ERK1/2-positive nonsurvivors showed a mean survival time of 16 months until death, the mean survival time of ERK1/2-negative nonsurvivors increased to 23.7 months.

The unfavorable prognostic effect of tumors with elevated pERK1/2 levels increased in the subgroup of lymph-node-positive cases (Fig. 2b), whereas in the subgroup of lymph-node-negative cases, no significance could be detected in univariate Kaplan–Meier analysis. Results of the univariate survival analysis of the remaining parameters are shown in Table 3.

OS and relevant clinicopathological parameters (pERK, histological grading, TNM, lymph-node metastases, and distant metastases) were subjected to multivariate analysis. As a result pERK1/2, tumor size, lymph-node metastasis, and distant metastasis were statistically associated with OS (Table 4).

Correlation of pERK1/2 with COX-2, EGFR, GF, and apoptosis

All cancer specimens, including the cases excluded from survival analysis, were subjected to cyclin-D1, COX-2, EGFR, Ki67, and TUNEL analysis.

A total of 132 tumors were analyzed for COX-2 protein expression; the remaining showed loss of discs. Tumor cells

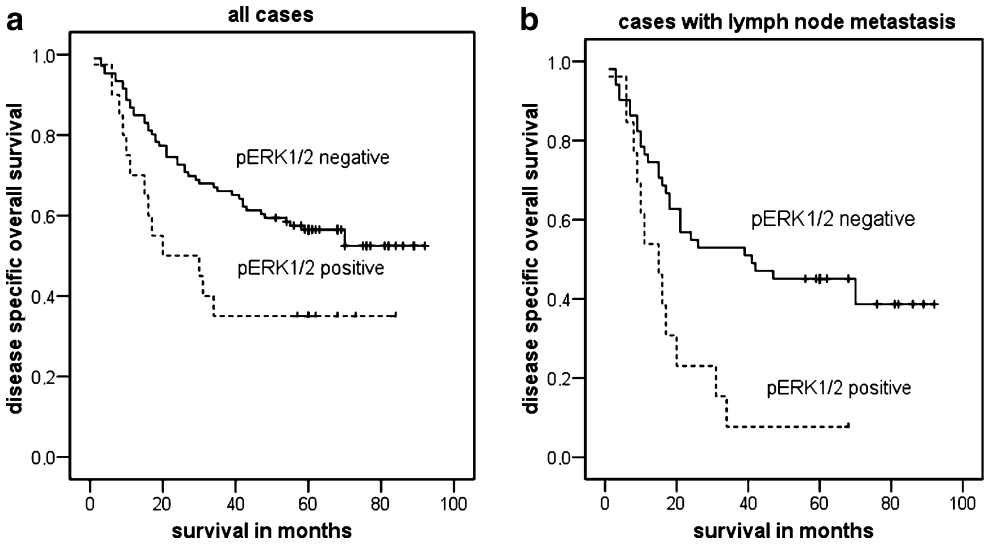

Fig. 2 Kaplan–Meier survival plot (*left*) for disease-specific overall survival in the complete series of 126 colorectal cancers in relation to pERK immunostaining intensity. Log-rank test: $P=0.0299$. Kaplan–Meier survival plot for disease-specific overall survival in colorectal cancer with lymph-node metastasis (*right*). Log-rank test: $P=0.0032$

Table 3 Univariate analysis (Kaplan–Meier, log-rank test) for prognostic significance of pERK1/2, pAKT, histological grading, tumor size, lymph-node metastases, distant metastases, COX-2, cyclin D1, EGFR, and *k-ras*

Parameter	*P*-value
Phospho-ERK1/2 (negative/positive)	0.0299[a]
Phospho-AKT (negative/weak/strong expression)	Not significant
Histological grading (G1–G3)	0.051[a]
Tumor stage (pT1-4)	0.000[a]
Lymph-node metastases (pN0 vs pN+)	0.000[a]
Distant metastases (M0 vs M1)	0.000[a]
COX-2	Not significant
Cyclin-D1	Not significant
EGFR	Not significant
k-ras (mutated vs wild-type)	Not significant

[a] Statistically significant

exhibited a specific cytoplasmic staining pattern. Classification was performed according to the amount of positively stained tumor cells: negative (0–10%) and positive (>10%). In all, 99 (75%) tumors were classified as positive and 33 (25%) as negative. No significant relationship of elevated pERK1/2 with COX-2 expression was observed. COX-2 expression was not associated with EGFR expression, cyclin-D1 immunoreactivity, GF, or apoptotic rate.

EGFR expression was analyzed in 128 tumor samples. Tumor cells revealed a specific cytoplasmic immunoreaction. Classification was performed according to the guidelines of PharmDx™ (DakoCytomation). Tumor samples lacking an immunostaining were classified as negative (0), whereas the remaining were classified as positive. In all, 83 (64.85%) tumors were classified as positive and 45 (35.2%) as negative. EGFR staining intensity was not related to pERK1/2, pAKT, and COX-2 immunostaining results. EGFR-positive tumors revealed a higher amount of proliferating tumor Ki-67-positive cells ($P<0.001$) and an increased apoptotic rate ($P=0.016$).

Table 4 Results of multivariate analysis (Cox regression) to determine the independent prognostic value of different variables in relation to overall survival ($n=126$)

Covariate	Relative risk (e^{β})	95% CI	*P*-value
pERK1/2 (negative vs positive)	2.603	1.336–5.073	0.005[a]
Histological grade (1, 2, 3)	1.027	0.663–1.592	0.904
Tumor stage according to TNM (1, 2, 3, 4)	3.812	2.212–6.571	0.000[a]
Lymph nodes (pN0 vs pN+)	1.798	0.939–3.444	0.030[a]
Distant metastases (M0 vs M1)	3.584	1.962–6.546	0.000[a]

[a] Statistically significant

The GF was defined as the percentage of Ki67-positive nuclei per 300 tumor cells. Statistical analysis showed no significant relationship of growth fraction with pERK1/2, pAKT, or COX-2 immunostaining. The amount of Ki-67-positive tumor cells correlated directly with the percentage of cyclin-D1-positive tumor cells ($P=0.033$). As mentioned above, the growth fraction was significantly increased in EGFR-positive tumors.

There was no relationship between the different pERK1/2 or pAKT immunostaining and the number of apoptotic tumor cells as determined by TUNEL. Statistical analysis revealed no association of the rate of apoptotic cells with COX-2 or cyclin-D1 staining. As mentioned above, EGFR-positive tumors revealed higher amounts of apoptotic cells.

Correlation of pERK1/2 with cyclin-D1

A total of 123 tumors was analyzed for cyclin-D1 protein expression. In 12 cases, the loss of discs from slides occurred. Tumor cells revealed a strong and specific nuclear staining pattern. Tumors were classified according to their amount of stained nuclei: negative (0–10%), weakly positive (11–30%), and strongly positive (>30%). Thirty-eight (30.9%) tumors were classified as negative, 61 (49.6%) tumors as weakly positive, and 24 (19.5%) tumors as strongly positive.

Tumors classified as pERK1/2 negative showed a higher proportion (20.5%) of cyclin-D1-positive tumor cells (mean value), whereas cases with elevated levels of pERK1/2 exhibited a lower proportion (12.7%) of cyclin-D1-positive tumor cells. All cases with a strong expression of cyclin-D1 (>30%) were classified as pERK1/2 negative. Statistical analysis indicates that tumor samples with elevated pERK1/2 expression exhibit significantly lower amounts of cyclin-D1-positive tumor cells (0.042, chi-square analysis).

Mutation of *k-ras* genes

In the present study, mutations were detected in codons 12 and 13 in 36 out of 134 cases (26.9%). Twenty-five mutations were G→A transversions, three mutations were G→T transversions, and eight mutations were G→C transversions. Chi-square analysis showed that tumors with elevated pERK1/2 levels more frequently presented *k-ras* mutations ($P=0.015$). Ten of the 20 (50%) tumor samples with elevated phospho-ERK1/2 levels exhibited a *k-ras* point mutation at codon 12 or 13 in contrast to only 26 of 114 (22.8%) in the group of tumor levels with low pERK1/2 levels. A strong pAKT expression was more frequently found in patients with *k-ras* mutations. In the subgroup of 98 tumors with wild-type *k-ras*, only 19 cases (19.4%)

showed a strong pAKT expression in contrast to nine (26.5%) cases in the subgroup of 34 tumors with mutated *k-ras*. On the other hand, the percentage of pAKT-negative classified tumors was higher in patients with wild-type *k-ras* (22 of 98; 22.4%) in contrast to patients with mutated *k-ras* (6 of 34; 17.6%). Chi-square analysis revealed no statistical significance between the presence of *k-ras* mutations and the expression of pAKT (P=0.639).

Discussion

This study demonstrates for the first time that phosphorylation of ERK1/2 but not AKT predicts poor patient prognosis in colorectal cancers independent of tumor stage, histological grading, and local or distant metastasis. The statistical power of this study is, unfortunately, reduced due to the relatively small number of pERK1/2 positive classified colorectal cancers. Therefore, it is necessary to confirm these promising results in larger cohorts. A detailed analysis of the nonsurviving patients with different pERK1/2 levels revealed that both ERK1/2-positive and ERK1/2-negative nonsurvivors exhibited advanced colorectal cancers with large tumor size and presence of local and/or distant metastasis. However, patients dying from pERK1/2-positive colorectal cancer displayed markedly decreased survival times in contrast to pERK1/2 negative nonsurvivors. Thus, raised pERK1/2 levels seem to enhance aggressiveness of advanced cancer disease rather than initiate or promote tumor growth or metastasis. Analysis of putative upstream mechanisms revealed an association of *k-ras* mutations with ERK1/2 phosphorylation, suggesting a biological link between *k-ras* mutations and activation of this MAPK pathway in colorectal cancer. Our results support the notion that *k-ras* mutations also contribute to activation of the AKT pathway; however, due to the relatively small number of tumors with *k-ras* mutations, significance was not reached. Mutations of *k-ras* are found in approximately 36% of colorectal cancers and are considered to constitute a crucial step in colorectal carcinogenesis [1]. Because *k-ras* mutations occur frequently in colorectal cancer and the RAS/RAF proteins are well-known activators of ERK1/2 [1], phosphorylation of these kinases is likely to reflect the oncogenic potential of *k-ras*. A recent study found *k-ras* mutations to be associated with poor survival in a cohort of 107 colorectal cancers [9], a finding that cannot be confirmed in our study. Although the frequency of *k-ras* mutations in our study (26.7%) was comparable to that described by Conlin et al. (27%), we cannot explain the diverse results. Thus, the prognostic value of *k-ras* remains to be clarified in further studies.

Although there have been previous studies describing elevated pERK1/2 levels in colorectal cell lines [21] and human colorectal cancer tissue [26], the prognostic significance of ERK1/2 activation has not been examined yet. Only few studies focused on the prognostic significance of pERK1/2 in human cancer arriving at contradictory data. Whereas activation of the ERK1/2 pathway in mucoepidermoid carcinoma of the salivary gland was associated with aggressive tumor behavior [19], other studies report pERK1/2 to be a favorable prognostic marker in breast cancer [19, 27] and serous ovarian cancer [17]. We found pERK1/2 not to be related to prognosis in nodal-negative breast cancer [34]. Apparently, activation of the ERK1/2 pathway can initiate cellular processes, resulting in either favorable or worse clinical outcome depending on the tumor type and tumor localization. Keeping the complex signaling network in mind, this is not surprising. In fact, ras/ERK-transduced signals can induce adverse effects, depending on intensity and duration of the signal: a short duration of ERK activation favors a mitogenic response, whereas a long duration primarily stimulates differentiation [46]. To disclose putative pathways contributing to the increased tumor aggressiveness and reduced survival of patients with pERK1/2-positive colorectal cancer, we investigated factors associated with ERK activation and ERK1/2-mediated downstream signaling effects. One of the upstream events which potentially contributes to MAPK activation is the overexpression of EGF receptor [8], but we did not find any linkage of EGFR with pERK1/2.

Within several downstream signaling effects, the ERK pathway is involved in cell proliferation, apoptosis and tumor invasion, and metastasis [13]. Important factors that influence cell proliferation are cyclin-D1 [25] and $p21^{Waf1}$ [13]. In this study, however, we neither were able to demonstrate an increase of cyclin-D1 protein expression nor proliferative activity measured by Ki-67 immunohistochemistry. Surprisingly, cyclin-D1 expression was lower in tumors with elevated pERK1/2, a fact which we cannot sufficiently explain at this time. In addition, we could not detect an association of ERK1/2 activation and tumor-cell apoptosis as measured by TUNEL. Thus, further studies are needed to elucidate the exact mechanism of ERK1/2-induced tumor aggressiveness. Maybe, the consequence of ERK pathway activation on matrix metalloproteinases or vascular endothelial growth factor might contribute to a more aggressive phenotype [13]. Thus, our results suggest that at least in this cohort, the effect of ERK1/2 signaling in tumor invasion and metastasis might be more relevant than its influence on proliferation and apoptosis, but this remains speculative.

Because a study on breast cancer cell lines identified the MAPK pathway as an upstream activator of *COX-2* gene expression [39], we determined the COX-2 protein expression in our cohort. However, COX-2 protein seems not to be linked with ERK1/2 activation.

Among the tumor-cell survival pathways, those mediated by the AKT kinase are the most critical [5, 7]. In human malignancies, AKT is activated in a variety of carcinomas including prostate, ovary, and breast cancer [2, 34, 40]. The prognostic significance of activated AKT (pAKT) in colorectal cancer has not been investigated yet although first studies suggest that AKT is involved in colorectal carcinogenesis [23]. Our data indicate that AKT does not serve as a prognostic parameter in colorectal cancer.

In our study, the clinical impact of ERK1/2 activation was strongest in the subgroup of cancer patients with lymph-node metastases. ERK1/2 activation in these patients might offer a new approach of biochemical therapy due to the existence of ERK-targeted, chemopreventive and chemotherapeutic anticancer drugs. Such elegant drugs are currently being developed [30, 35]. The detection of phosphorylated ERK1/2 in cancer tissue could be a straightforward way to select patients for these treatments.

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