

# Combination of p53 expression and p21 loss has an independent prognostic impact on sporadic colorectal cancer

AURELIA NOSKE<sup>1</sup>, SYBILLE LIPKA<sup>2</sup>, JAN BUDCZIES<sup>1</sup>, KATHRIN MÜLLER<sup>2</sup>,  
CHRISTOPH LODDENKEMPER<sup>3</sup>, HEINZ JOHANNES BUHR<sup>2</sup> and MARTIN KRUSCHEWSKI<sup>2</sup>

<sup>1</sup>Institute of Pathology, Charité University Hospital Berlin, Campus Mitte; <sup>2</sup>Department of Surgery and

<sup>3</sup>Institute of Pathology, Charité University Hospital Berlin, Campus Benjamin Franklin, Berlin, Germany

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**Abstract.** There is no clear evidence on the prognostic and predictive value of abnormal p53 expression in colorectal cancer. The major downstream protein, p21, a cell cycle inhibitor, is transcriptionally regulated by p53. The prognostic impact of p21 expression in colorectal carcinomas is still under debate. In this study, we investigated the expression of p21 and p53 in a prospective cohort of 116 sporadic colorectal carcinomas at UICCII/III stage. We observed an expression of p21 in 26% and p53 in 63% of the carcinomas by immunohistochemistry. Patients with p21-negative colorectal carcinomas had a significant better recurrence-free and overall survival than patients with p21-positive carcinomas ( $p=0.02$  and  $p=0.005$ ). Expression of p53 was related to a better overall survival ( $p=0.048$ ). The combination of p21-negative/p53-positive expression was significantly related to better recurrence-free and overall survival ( $p=0.007$  and  $p=0.0001$ ) and gained independent prognostic significance (HR: 3.4,  $p=0.01$ ). Moreover, patients with combined p21/p53<sup>+</sup> expression had a remarkable benefit in overall survival after adjuvant chemotherapy as compared to the p21/p53<sup>-</sup> subgroup (HR: 3.6,  $p=0.027$ ). Our data suggest that the assessment of both p53 and p21 expression may provide prognostic information in colorectal cancer patients. This combination might be helpful to identify patients who could benefit from chemotherapy.

## Introduction

Colorectal cancer is the third most common cancer in both genders in the Western world with an estimated number of 112,340 new cases and 52,180 estimated deaths in the USA in 2007 (1). The five-year survival rates reported for patients

with stage II and III colorectal cancer vary between 92 and 67% (SEER Cancer Statistics). The pathologic tumor stage represents the most important prognostic factor and generally survival decreases with later stage cancers. The association of stage IIIa colon cancer with statistically significantly better survival than stage IIb (83 vs. 72%) in the AJCC sixth edition system may reflect current clinical practice, in which stage III patients receive chemotherapy but stage II patients generally do not (2). Patients with UICC stage III carcinomas will benefit from adjuvant chemotherapy in only 5-15% and the majority of patients who undergo adjuvant therapy will be overtreated (3,4). Colorectal cancer is considered relatively resistant to chemotherapy (5). Various chemotherapeutic approaches such as treatment with 5-fluorouracil (5-FU) have led only to a weak to moderate decline in mortality. Therefore, additional parameters such as molecular biomarkers are needed to identify patients who will really benefit from adjuvant anti-cancer treatment.

Alterations in different molecular signalling pathways are involved in initiation and progression of colon cancer, such as e.g. p53 ('suppressor-pathway'), mismatch repair genes ('mutator-pathway') and EGFR-KRAS-ERK-pathway. The activation status of certain molecules can influence patient prognosis. Thus, it has been shown, that patients with mismatch repair gene mutations or microsatellite instable (MSI high) colorectal carcinomas have a better overall survival than those without these genetic defects (6). Moreover, patients with MSI high colorectal carcinomas had no benefit from 5-FU-based adjuvant chemotherapy, whereas patients with MSI low/MSS tumors did benefit from this therapy (7). Currently, targeted therapy with an anti-EGFR-antibody is applied in metastasized colorectal cancer. Patients will have a benefit of anti-EGFR therapy if a mutation in the oncogene KRAS can be excluded (8).

Although the p53 expression is abnormal in >50% of the colorectal carcinomas, the prognostic role of the most intensively studied p53 tumor suppressor gene is contradictory. Patients whose tumors are associated with abnormal p53 protein have both reduced and improved outcome. The value in predicting response to chemotherapy is not well known. The p53 protein induces temporary cell cycle arrest, inhibits cell growth and contributes to apoptosis by regulation of p21. The p21/WAF1 cyclin-dependent kinase inhibitor blocks the transition from G1 to S-phase and inhibits cell proliferation.

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*Correspondence to:* Dr Martin Kruschewski, Department of Surgery, University Hospital Charité Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany  
E-mail: martin.kruschewski@charite.de

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Thus, p21 protein serves as a surrogate marker for p53 protein alterations. While there are several controversial studies on p53, the expression of p21 and its significance for the prognosis in colorectal cancer as well as predictive function on the outcome of adjuvant therapy is not well elucidated.

We carried out this study to investigate the expression of p53 and p21 in a prospective cohort of patients with colorectal carcinomas stage II/III and to evaluate the prognostic significance as well as the influence on the outcome of adjuvant 5-FU-based chemotherapy in colon cancer and radiochemotherapy in rectal cancer.

## Patients and methods

**Study population and tissue samples.** Immunohistochemical examination of p53 and p21 was performed retrospectively on tissue samples taken for routine diagnostic and therapeutic purposes. Curatively resected colorectal carcinomas of 116 patients who were diagnosed at the Institute of Pathology, Charité Hospital Berlin (Campus Benjamin Franklin) between 1995 and 2001 were included in the study. Only patients with primary colorectal adenocarcinomas and no other known malignancies and no preoperative radiochemotherapy were included. The tissue specimens consisted of 37 colon carcinomas and 79 rectum carcinomas as well as adjacent normal mucosa. Tissue samples were fixed in 4% neutral-buffered formaldehyde, embedded in paraffin and histopathological evaluation was performed on standard hematoxylin and eosin sections. The stage of tumors was assessed according to the UICC staging system. Tumor differentiation was determined according to the WHO guidelines. For all patients a minimum of 12 lymph nodes were investigated. Clinical follow-up data were available for all patients. The mean follow-up time of patients was 52 months.

Data on postoperative chemotherapy are known from 114 patients and on radiotherapy in 113 cases. Patients were treated according to the guidelines of the German Cancer Society. Thus, patients with colon cancer UICC stage III were administered chemotherapy (5-FU/folic acid). Postoperative radiochemotherapy (5-FU/folic acid and radiation doses of 45 Gy) was administered to patients with rectal carcinomas in UICC stage II and III.

**Immunohistochemistry.** Immunohistochemistry was performed according to standard procedures on paraffin sections containing both normal mucosa and the invasive tumor front. Briefly, slides were boiled in citrate buffer (pH 6.0) in a pressure cooker for 5 min and incubated with a monoclonal anti-p21<sup>WAF1/Cip1</sup> antibody (Clone SX118, Dako) at 1:50 for 1.5 h at room temperature as well as a monoclonal anti-p53 antibody (Clone DO-7, Dako) at 1:100 for 1 h at room temperature, followed by incubation with a biotinylated anti-mouse secondary antibody and the multilink biotin-streptavidin-amplified detection system (Biogenex, San Ramon, CA, USA). Staining was visualized using a fast-red chromogen system (Immunotech, Hamburg, Germany). Appropriate positive and negative controls were included in each run of immunostaining. The p21 and p53 immunostaining in tumor cells was evaluated independently by two authors (A.N. and S.L.), who were blinded to patient outcome.

Discordant cases were discussed on a multihead microscope until a final decision was reached. Expression of p21 and p53 was evaluated according to the percentage of positive tumor cell nuclei. The immunoreactivity of p21 was categorised in negative expression (<5% of the tumor cells) and positive expression (>5% of the tumor cells are positive). The percentage of p53-positive cells was scored as: 1 (0-10%); 2 (11-25%); 3 (26-50%); and 4 (>50%). For further analyses, a cutoff point of >25% positive cells was defined. All immunoreactive nuclei were regarded as positive irrespective of the staining intensity. Negative controls were performed by omitting the primary antibody.

**Statistical analysis.** The statistical significance of the association between p21 and p53 status as well as clinico-pathological parameters was assessed by Fisher's exact test. The probability of overall survival as a function of time was determined by the Kaplan-Meier method. Differences in survival curves were compared by the log-rank test. Cox proportional hazard models were fitted in order to calculate hazard ratios and to carry out multivariate survival analyses. Generally, p-values <0.05 were considered significant, hazard ratios are reported with 95% confidence intervals. For the statistical evaluation the SPSS software Version 13.0 was used.

## Results

**Clinical and pathological characteristics of colorectal carcinomas.** Colorectal carcinoma specimens from 116 patients were investigated for immunoreactivity of p21 and p53. The mean age of patients at surgery was 64 years (range 38-89). Forty-eight patients were female and 68 male. The majority of tumors were diagnosed in tumor stage pT3 (89 cases, 76.6%). Most carcinomas (52.6%) were poorly differentiated. In total, 36 patients (31%) had no lymph node metastasis (pN0), whereas 80 patients (69%) had metastases in lymph nodes. Clinico-pathological features are summarized in Table I. Multivariate analysis revealed nodal- and metastasis status as the best discriminating factors regarding prognosis in our study cohort (Table II).

**Expression of p21 and p53 in colorectal cancer.** Immunohistochemical analysis of p21 was performed on 116 colorectal carcinomas (Fig. 1A). Positive expression of p21 was defined as a nuclear staining reaction in >5% of the tumor cells according to other publications (9). We observed no p21 expression in 8 carcinomas, a nuclear expression in <5% of the tumor cells in 77 carcinomas and a nuclear expression in >5% in 31 colorectal carcinomas. In adjacent colorectal mucosa a p21 expression was found only in the epithelium of the upper part and surface. For p53, expression analysis was performed on 114 colorectal carcinomas (Fig. 1B). Positive expression of p53 was defined as a nuclear staining of >25% tumor cells. According to the scoring system, we found a weak expression (0-10% of the cells) in 30 tumors, a moderate expression (11-25% of the cells) in 12 cases and a strong nuclear immunoreaction (>25% of the cells) in 72 colorectal carcinomas. In adjacent normal mucosa no p53 expression was observed.

SPANDIDOS PUBLICATIONS clinicopathological characteristics of 116 patients with cancer.

Characteristic	All cases n (%)
pT	
pT1	2 (1.7)
pT2	6 (5.2)
pT3	89 (76.7)
pT4	19 (16.4)
pN	
pN0	36 (31)
pN1	44 (37.9)
pN2	27 (23.3)
pN3	9 (7.8)
Histological grade	
G1	1 (0.9)
G2	54 (46.6)
G3	61 (52.6)
UICC stage	
II	36 (31)
III	80 (69)
Location	
Colon	37 (31.9)
Rectum	79 (68.1)

Table II. Cox regression analysis under inclusion of clinico-pathological factors (n=114).

Parameter	Overall survival	
	HR (95% CI)	P-value
Tumor stage		
pT1/pT2	1.000	
pT3/pT4	1.63 (0.49-5.37)	0.425
Lymph node status		
pN0/pN1	1.000	
pN2/pN3	2.71 (1.45-5.08)	0.002
Histologic grade		
G1/G2	1.000	
G3	1.11 (0.59-2.11)	0.746
Metastasis status		
M0	1.000	
M1	3.37 (1.87-6.08)	<0.001
Age at diagnosis per year	1.05 (1.02-1.09)	0.001
Expression		
p21-/p53+	1.000	
p21+/p53-, p21+/p53+, p21-/p53-	3.4 (1.63-7.24)	0.001

HR, risk ratio and 95% CI, 95% confidence interval.

*Relationship between p21, p53 and clinicopathological features.* There was no relation between p21 and p53 expression ( $p=0.19$ ). Colorectal carcinomas of patients without p21 expression were significantly associated with rectal tumor localization compared to tumors localized in colon ( $p=0.003$ ). About half of the colon carcinomas (46%) were p21-positive compared to only 18% of the rectum carcinomas. There was no significant relationship to any other clinicopathological parameter such as gender, age, tumor stage, tumor differentiation and nodal status. Concerning p53 expression, we did not find any significant association with clinicopathological parameters.

*Relationship between p21, p53 and survival.* In univariate Kaplan-Meier analysis, patients with p21-negative carcinomas had a significantly better recurrence-free ( $p=0.02$ ) and overall survival ( $p=0.005$ ) than patients with p21-positive carcinomas (Fig. 2A and B). Patients with carcinomas, localized in colon and negative p21 expression had a better recurrence-free ( $p=0.046$ ) and overall survival ( $p=0.002$ ) as compared to patients with positive p21 expression. In univariate analysis, patients with p53-positive carcinomas had a significant better overall survival than patients without p53 expression ( $p=0.048$ ). No differences were found concerning the progression-free survival ( $p=0.161$ ). Patients with p53-positive rectal carcinomas had a better overall survival as compared to patients without p53 expression ( $p=0.002$ ).

*Relationship between p21, p53, adjuvant therapy and patient prognosis.* Patients with p21-negative colorectal carcinomas and adjuvant therapy had a significantly better overall survival than patients without therapy ( $p=0.017$ ). Patients without therapy were exposed to a significantly higher risk,  $HR = 2.7$  (CI 1.2-6.4). The hazard ratio adjusted for patient age, tumor stage, lymph node and metastasis status and histological grade was  $HR_{adj} = 1.83$  (CI 0.63-5.4,  $p=0.271$ ). There was no significant impact on the recurrence-free survival ( $p=0.239$ ). Concerning the p53 expression, no differences in recurrence-free and overall survival were observed in patients who received adjuvant therapy ( $p>0.05$ ).

*Association of combined p21/p53 expression and prognosis.* Since the regulation of p21 is partly mediated by p53, we analyzed the combination of p21 and p53 expression with patient survival. Thus, we found a significantly better recurrence-free ( $p=0.007$ ) and overall survival ( $p<0.0001$ ) in p21-negative/ p53-positive carcinomas as compared to other subgroups such as p21+/p53-, p21+/p53+ and p21-/p53- (Fig. 3A and B). Cox analysis resulted in a higher risk for the latter patient group with  $HR = 3.6$  (CI 1.8-7.3). When adjusted for patient age, tumor stage, lymph node and metastasis status and histological grade the hazard ratio remained significantly greater than one,  $HR_{adj} = 3.4$  (CI 1.6-7.2,  $p=0.001$ ).

In this study, we have shown a significantly better overall survival after adjuvant therapy in patients with p21-negative carcinomas. Therefore, we investigated the role of the p53 expression status on the effects of postoperative therapy. We observed a better overall survival in patients with p21-/p53+ carcinomas as compared to p21-/p53- carcinomas (log-rank,



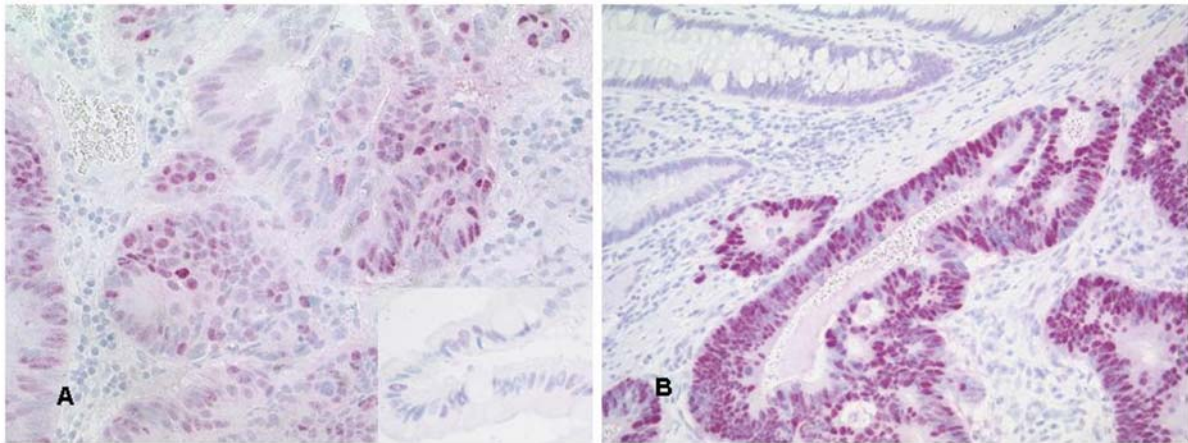


Figure 1. Expression of p21 and p53 in colorectal cancer. Nuclear expression of p21 in a colorectal adenocarcinoma. The insert shows superficial epithelial cells from adjacent normal mucosa with a weak nuclear staining (A). Strong nuclear expression of p53 in >50% of an adenocarcinoma of the colon (right side) and adjacent normal colon mucosa without p53 expression on the left side (B).

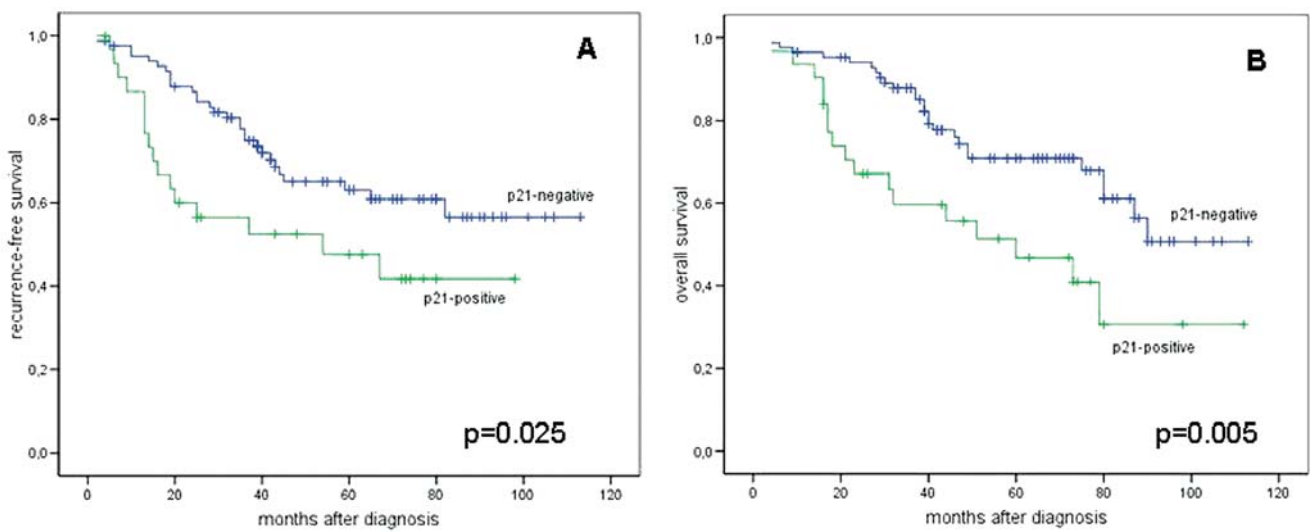


Figure 2. Univariate Kaplan-Meier survival analyses. Expression of p21 is related to better recurrence-free survival (A) and overall survival (B).

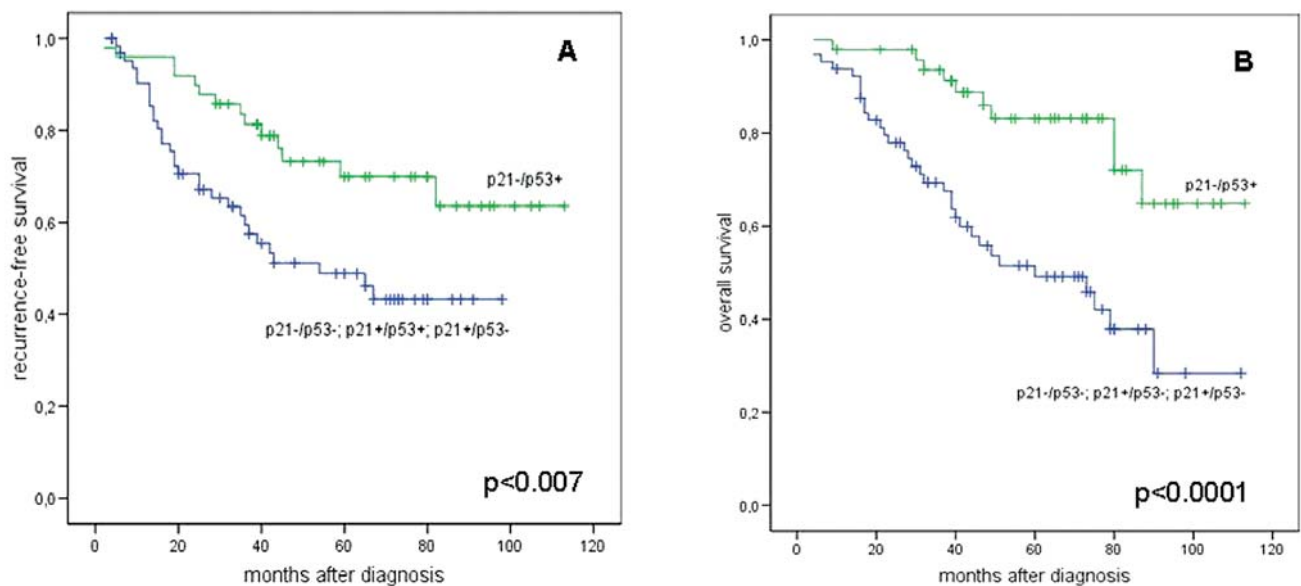


Figure 3. Univariate Kaplan-Meier survival analyses. The combination of p21-/p53+ expression is associated with better recurrence-free (A) and overall survival (B).



The p53-negative patients were exposed to higher  $\text{HR}_{\text{adj}} = 3.5$  (CI 1.2-9.8) and  $\text{HR}_{\text{adj}} = 3.6$  (CI 1.2-11.6) when corrected for patient age, tumor stage, lymph node and metastasis status and histological grade.

## Discussion

In this study, we hypothesized that alterations in p53 and p21 expression may provide prognostic information on colorectal cancer patients, particularly with regard to the adjuvant therapy.

*p53 expression and relation to clinicopathological factors and patient outcome.* We observed an expression of p53 in 63% of the colorectal carcinomas which is in line with previous studies, where a range of 40 to 81% p53 positivity was found (10-13). This wide range depends on different techniques used in these studies, such as different antibodies, scoring systems, cutoff-values and study populations. Inactive and mutated p53 protein accumulates in the nucleus and can be detected by immunohistochemistry. Antibodies used for immunohistochemistry can detect both the wild-type and the mutated p53 protein. Since, the wild-type occurs only at low detection levels and has a short half-life, it is widely accepted that most commercial antibodies are suitable for detection of abnormal p53. Since, this method has been shown to have both false positives and false negatives for the assessment of TP53 mutations (14), a higher threshold of p53 positivity was suggested to use for prediction of TP53 gene mutations (15).

We did not find any relationship to clinicopathological features as other authors did (12,16-18). In contrast, some studies reported a close correlation between p53 overexpression and poor prognostic markers such as lymph node metastasis and invasion depth (19-21). Whereas others found a relation to better differentiated tumors and location in the distal colon and rectum (22).

In our study, patients with p53 expression had a better overall survival ( $p=0.048$ ) which is in line with previous studies (9,22,23). These studies partly include large patient cohorts and long follow-up periods. A trend for improved overall survival for patients with p53 mutations in distal colonic carcinomas analyzed by single-strand conformation polymorphism analysis (SSCP) was also shown (23). Other studies reported an unfavourable prognosis in patients with p53-positive carcinomas (12,13,24-28), but only few multivariate analyses yielded a significant result in these studies (25). However, other authors did not found a correlation of p53 and prognosis (11,16,29,30). Additionally, we observed that patients with p53-positive carcinomas tended to have a better recurrence-free survival. In contrast, other studies found an association of p53 positivity and higher risk of tumor relapse as well as no relation to tumor recurrence (12,13,27,29,31). The discrepancies between patient outcomes may depend on the tumor location (proximal vs. distal), p53 mutation site, tumor type and response to adjuvant therapy.

*p21 expression and association with clinicopathological factors and patient outcome.* In the present study, we observed a positive p21 expression in 26% of the colorectal carcinomas. Similar results were found by Fu *et al* (32) in rectal carcinomas. While other studies reported on p21 expression in 36 to 68% (27,30,33,34). The different expression levels may depend on

the different methods used, e.g. variable scoring systems. Further, we found a significant association of p21-negative carcinomas and rectal localization. Ricciardiello *et al* (10) reported an enhanced p21 expression in the right colon. Apart from these studies, data on p21 and its relationship to clinical and pathological findings are rare. Here, we observed a significantly better overall- and recurrence-free survival in patients with negative p21 colorectal carcinomas. In contrast, a negative p21 status was associated with higher risk of tumor relapse, poorer clinical course and lymph node and liver metastases in colorectal cancer (27,35,36). Positive p21 expression correlated significantly with better recurrence-free survival and was shown to be an independent prognostic factor in colorectal cancer (27,33). In rectal carcinomas, no association between p21 expression and clinicopathological features or survival was found (37).

Data on the impact of p21 expression on patient outcome in different cancer types is variable. Thus, expression of p21 has been correlated directly or inversely or not at all in survival of patients with invasive breast carcinomas (Cornfield *et al*) (38). The wide variety of stimuli that effect p21 expression through both p53-mediated and non-p53 pathways may account for the disparate observations.

*Impact of p53 and p21 expression as well as adjuvant therapy on prognosis.* In our study, patients with p53-positive carcinomas had a better (not significant) survival independent of adjuvant chemotherapy than p53-negative cases. Patients with left-sided carcinomas, Dukes C stage and normal p53 appear to benefit from adjuvant therapy rather than those with p53 mutation (23). Patients with stage III colon cancer and no p53 expression benefit from adjuvant 5-FU/levamisole, whereas those with p53 overexpression do not (39). Previous studies have shown that the presence of p53 mutation failed to predict which patients would benefit from 5-FU-based adjuvant therapy (40). Abnormal p53 appears to be of no value in predicting response to chemotherapy alone, since the effects of p53 on the response to cytotoxic drugs, radiation and chemoradiation are complex (40). No significant association between p53 status and adjuvant chemotherapy was found in several colorectal cancer studies (16,41). In this study, we demonstrated to our knowledge for the first time a significant better overall survival in patients with p21-negative carcinomas after adjuvant therapy. Similarly, negative p21 expression was associated with better recurrence-free survival after neoadjuvant treated in rectal carcinomas (42).

*Relation of p53 and p21.* In this study, we did not observe an association between p53 and p21 expression which is in line with other studies (43). In contrast, colorectal carcinomas with loss of p21 (50% of 737 cases) exhibited a significantly higher frequency of p53 expression in a large prospective study. Both p21 negativity and p53 positivity were inversely associated with high microsatellite instability (MSI-H), CpG island methylator phenotype (CIMP-high) and BRAF mutations (15). Other authors reported an inverse correlation between these proteins (10,27).

*Combination of p53+/p21- and survival.* Further, we observed a better progression-free and overall survival in patients with

p21-negative/p53-positive colorectal carcinomas as compared to other subgroups. Moreover, the p53<sup>+</sup>/p21<sup>-</sup> subgroup showed a benefit in overall survival after adjuvant chemotherapy as compared to the p53<sup>-</sup>/p21<sup>-</sup> subgroup. Interestingly, patients with p53-negative tumors were exposed to higher risk (HR = 3.5 (CI 1.2-9.8) and HR<sub>adj</sub> = 3.6 (CI 1.2-11.6). It is conceivable that the p53 expression may result in activation of p21 and contribute to cell cycle arrest and apoptosis. Thus, this combined expression status may be the reason for the better outcome in a subgroup of patients with colorectal carcinomas.

Our data suggest that the analysis of p53 expression in combination with other molecular parameters such as p21 might be helpful in assessing patient prognosis in colorectal cancer. The combination of p53<sup>+</sup>/p21<sup>-</sup> may also serve as a biomarker for identification of patients who would have a benefit from adjuvant chemotherapy.

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