p53 and EGFR Expression in Colorectal Cancer: A Reappraisal of 'Old' Tissue Markers in Patients with Long Follow-up

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Abstract. *Background: Extensive research into the biology of colorectal cancer has identified a plethora of molecular markers reputed to provide prognostic information. During the last two decades conflicting results have been drawn on the role of the p53 tumour suppressor gene and of the first identified member of the type receptor tyrosine kinase family, EGFR, on colorectal cancer prognosis. p53 Mutational status has been associated with both improved and reduced survival. EGFR has been associated with reduced length of survival, increasing Dukes' stage and lymph node metastases in several reports, but as many studies have reported no association with unfavourable prognostic parameters. The aim of this study was to evaluate the p53 and EGFR expression in patients with an at least 5-year follow-up. Patients and Methods: Paraffin-embedded material was retrospectively collected from 164 colorectal adenocarinoma (50 rectal) patients, who had been operated on between 1994 and 2003. The median follow-up was 5 years (range: 1-14). p53 and EGFR expression were evaluated by immunohistochemistry. Results: Positive p53 immunostaining and EGFR expression was observed in 63.4% and 43.9% of patients, respectively. p53 and EGFR positivity rates were significantly interrelated (p=0.004). No significant correlation was found with the examined clinicopathological parameters except for advanced T-stage, which demonstrated significant associations with p53 expression (p=0.004), EGFR expression (p=0.0001) and p53/EGFR coexpression (p=0.001). In univariate survival analysis (log rank test), stage (p=0.0001), lymphovascular invasion (p=0.005) and perineural infiltration (p=0.004) were associated with the overall cancer-specific survival, while a trend existed for EGFR (p=0.06) and p53/EGFR co-expression (p=0.07). On multivariate analysis, only stage was associated with increased risk of cancer death (Cox regression analysis p=0.0001, b-coefficient (SE): 1.898 (0.383). Conclusion: p53 and EGFR were overexpressed in this colorectal cancer patient population and were significantly associated with advanced T stage. In the context of new therapeutic strategies using EGFR-targeted therapies, although EGFR remains a controversial prognostic factor, this expression–stage association may play a crucial role in a decision to initiate an adjuvant treatment.*

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Worldwide, colorectal cancer (CRC) is the second most common malignancy in women (195,400 new cases) and the third in men (217,400 new cases), with estimated approximately 207,400 deaths for both genders in Europe; it remains the third most common cancer in the United States, with 153,760 estimated new cases and 52,180 deaths annually (1, 2). Despite significant advances in both surgical methodology and adjuvant therapy regimes, long-term survival for CRC patients remains in the range of 50-60%. Considerable interest has therefore focused on the identification of tumour-based markers that can more accurately predict the course of the disease, as well as help to determine optimal adjuvant therapy approaches.

One of the most intensively studied tumour markers is the p53 tumour suppressor gene. This gene encodes for a 53 kDa phosphoprotein and is frequently targeted for inactivation in a wide range of tumours (4). It is the target of point mutations and small deletions and insertions that lead to total or partial abolition of protein function. Inactivation is believed to abolish the ability of p53 to maintain genomic

integrity through regulation of various activities, including the control of cell cycle arrest, DNA repair and apoptosis. In some tumour types, notably breast cancer, mutation of the *p53* gene is consistently associated with shortened patient survival (4). p53 has also been the most investigated of all potential markers that may have prognostic or predictive value for patients with colon cancer. Mutations in *p53* have been found to occur in 40% to 60% of patients with colon cancer (4, 5). Given the logistical difficulties and resources associated with direct sequencing of the *p53* gene, most investigations have used immunohistochemistry as a means of detecting mutant p53, with the assumption that overexpression of p53 is often associated with a mutation, while the lack of expression is generally indicative of wild-type p53. The significance of p53 mutation as a prognostic marker for CRC is still a matter of controversy. Two groups have reported strong associations between p53 mutation and poorer prognosis in cohorts of more than 200 patients (4, 5), although both found that the association was confined to distal tumours. In investigations in which at least 100 patients with CRC have been studied, those in which monoclonal antibodies to p53 (PAB 1801/DO-7/D0-1) were used have generally demonstrated that mutation or overexpression of p53 is associated with a worse clinical outcome (6-9). However, this association has not been a constant finding; several investigations have found either the opposite or no association between the expression of p53 and clinical outcome (10-13). Thus, while the general impression is that the overexpression of p53 is associated with a less favorable clinical outcome for patients with CRC, investigations that demonstrate contrary associations indicate that the role of p53 as a prognostic marker requires additional investigation.

The epidermal growth factor receptor (EGFR, cerbB-1) is the first identified member of the type I receptor tyrosine kinase family and is a major regulator of several distinct, diverse cellular pathways (14). The ability to activate multiple pathways results from the numerous activating ligands which can bind EGFR and also from the various fellow family members with which EGFR can form dimeric complexes (14). Activated EGFR has been shown to cause transformation *in vitro* (15) and a wide range of human tumours have been shown to overexpress EGFR *in vivo*, including breast (16), lung (17) and bladder (18) carcinomas. EGFR has been associated with decreased survival (19, 20) and increasing Dukes's stage and lymph node metastases in several reports (21, 22), but as many studies have reported no association with unfavourable prognostic parameters, such as the length of survival (23, 24) and Dukes stage (25, 26).

Those controversies prompted the evaluation of p53 and EGFR expression in a well-documented population of colorectal cancer patients with an at least 5-year follow-up and to analyze their relationship with the main clinico-histological prognostic factors and their respective impacts on patient prognosis and survival.

Patients and Methods

The study comprised 164 patients with colorectal adenocarcinomas which were surgically managed at the Authors' institutions between 1994 and 2003 and had adequate oncological follow-up data up to 2008. All resected CRCs were received fresh, fixed in 10% pH-neutral formalin and embedded in paraffin. All patients had adenocarcinomas and were staged according to the International Union Against Cancer TNM staging (UICC/TNM) system (27). Characteristics studied included age, sex, tumour site, degree of histological differentiation (well/moderate/poor), presence and number of invaded lymph nodes counted during the slide review, lymphovascular invasion and perineural infiltration (classified as present or absent) and the cancer-specific survival rate.

All histological slides were reviewed by two pathologists to confirm the diagnoses and evaluate the patterns, the intensity and the malignant cell positivity rate of p53 expression and EGFR reactivity. All cases were immunohistochemically stained using the standard three-step streptavidin–peroxidase technique, as previously described (28). The following primary antibodies were used: DO-1 for p53 (Oncogene Science, Uniondale, NY, USA; diluted 1:80) and the mouse monoclonal antibody (EGFR/113; Novocastra Laboratories Ltd, Newcastle, UK; diluted 1:20). Each run included, for each patient, non-immune mouse IgG used as the primary antibody for the negative controls and breast cancer sections positive for p53 and normal epidermis known to express EGFR served as the positive controls. Staining for all antibodies was assessed without foreknowledge of the clinical data by two observers, except for the TN stage. Whenever a difference of more than 10% between the two assessments was observed, slides were reviewed jointly and a consensus was reached. The evaluations agreed in more than 90% of the samples for all markers. A minimum of five randomly selected fields throughout the whole section at ×400 magnification were examined.

The assessment of p53 and EGFR was based on previously described guidelines (24, 29). As for p53 immunostaining assessment, if 10% or more of the malignant nuclei were stained, the slide was scored as positive, while if fewer than 10% of the nuclei were stained, the slide was scored as negative (29). The percentage of EGFR-labelled cells was graded as follows: grade 0, no positive cells; grade 1, 1-25% labelled tumour cells; grade 2, 25-50% labelled tumour cells; grade 3, >50% positive tumour cells. The intensity of peroxidase deposits, ranging from light beige to dark brown, was assessed visually as indicating the tumour cell membrane, cytoplasm, or both and was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). A composite score, potentially ranging from 0 to 9, was obtained by multiplying the grade by the intensity (24). Patients were analysed as a function of their EGFR expression: low <6 and high >6.

Statistical calculations were performed using the SPSS for Windows software package (SPSS Inc., Chicago, IL, USA). The χ^2 test was used for testing non-continuous categorical tumour variables (contigency tables). Survival was measured from the date of surgery to date of death or status at date of last follow-up (June 2008). These data were censored in patients known to have died from causes other than colonic cancer. Patients who died within 30

days of operation were excluded from the study. Kaplan-Meier curves were plotted and the log-rank test was used to compare survival with respect to p53 status, EGFR staining, TNM stage, tumour differentiation, lymphovascular invasion, perineural infiltration and patient age at operation in a univariate analysis (30). Stage I and stage II tumours were grouped together as were stage III and stage IV tumours. Similarly, well-differentiated tumours were grouped with moderately differentiated tumours and patients were categorized around median age at surgery. Those factors found to be statistically significant in univariate analysis were entered into a Cox regression model (categorical data) to determine the relative impact of each on survival (31). Two-sided *p*-values of <0.05 were considered statistically significant.

Results

The patient population consisted of 164 (112 male) patients [mean age (±SE): 64.9±8.75 years, median: 63.5, range 45–82] with stage T1, T2, T3 and T4 (12, 46, 80 and 26 patients, respectively) colorectal malignancies (70 N+ tumours). The majority (87.8%) were well and moderately differentiated. Fifty tumours were located in the rectum. Among the 26 patients (15.8%) with metastases at the time of diagnosis, all had liver metastases. Sixty patients received 5-fluorouracil-based adjuvant chemotherapy, while 20 rectal cancer patients were subjected to postoperative radiation treatment. None received neoadjuvant radiotherapy. No patients received a new therapeutic strategy such as EGFR-targeted therapy. The median follow-up was 5 years (range 1-14).

TNM stage was significantly related to lymphovascular invasion (p=0.0001) and perineural infiltration (p=0.005); poor differentiation was associated with the presence of perineural infiltration (p=0.022), and lymphovascular invasion with perineural infiltration itself (p=0.0001) (analytical data not shown).

p53 expression was recognized through a nuclear staining of positive cells. Normal epithelium was negative for p53. Overall, the extent of nuclear immunopositivity, regardless of staining intensity, ranged from 0% to 80% (mean±SE, 21.03%±1.98). Negative p53 expression was shown in 60 (36.5%) and positive in 104 (63.5%) samples (Table I). EGFR-positive expression was detected in 72 (43.9%) of the examined cases. p53 and EGFR positivity rates were significantly interrelated (p=0.004) (Table I). No significant correlation was found with the examined clinicopathological parameters except for advanced T-stage, which demonstrated significant associations with p53 expression (p=0.004), EGFR expression (p=0.0001) and p53/EGFR co-expression (p=0.001) (Table I).

In univariate survival analysis (log rank test), TNM stage (p=0.0001), lymphovascular invasion (p=0.005) and perineural infiltration (p=0.004) were associated with overall cancer-specific survival, while a trend existed for EGFR (p=0.06) and p53/EGFR coexpression (p=0.07) (Figure 1). When cases were subgrouped according to stage, location

Table I. *Correlation of p53 and EGFR expression and p53/EGFR coexpression with clinicopathological parameters.*

	p53 (low/high)	EGFR (low/high)	p53 & EGFR coexpression (absent/present)
Total number of patients	60/104	92/72	108/56
Age			
<70 years	40/80	64/56	74/46
>70 years	20/24	28/16	34/10
	p=0.2	p=0.288	p=0.066
Gender			
Male	46/66	62/50	74/38
Female	14/38	30/22	34/18
	p=0.085	p=0.866	p=0.533
Location			
Right (n=62)	30/32	34/28	40/22
Left (n=52)	16/36	22/30	32/20
Rectum (n=50)	14/36	36/14	36/14
	p=0.068	p=0.519	p=0.514
Stage			
I (n=24)	12/12	16/8	18/6
II (n=70)	28/42	46/24	52/18
III (n=62)	18/44	26/36	34/28
IV (n=8)	2/6	4/4	4/4
	p=0.552	p=0.217	p=0.299
Differentiation			
Well/moderate (n=144)	54/90	82/62	96/48
Low (n=20)	6/14	10/10	12/8
	p=0.645	p=0.678	p=0.677
Lymphovascular invasion			
Absent (n=86)	40/46	54/32	60/26
Present (n=78)	20/58	38/40	48/30
	p=0.05	p=0.2	p=0.433
Perineural infiltration			
Absent (n=128)	50/78	70/58	80/48
Present (n=36)	10/26	22/14	28/8
	p=0.380	p=0.628	p=0.227
T Stage			
T1 (n=12)	6/6	8/4	10/2
T2 (n=46)	30/16	38/8	40/6
T3 (n=80)	20/60	44/36	52/28
T4 (n=26)	4/22	2/24	6/20
	p=0.004	p=0.0001	p=0.001
Chemotherapy			
No	40/64	64/40	72/32
Yes	20/40	28/32	36/24
	p=0.614	p=0.074	p=0.237
EGFR expression			
Low	46/46		92/0
High	14/58		16/56
	p=0.004		p=0.0001
p53 expression			
Low			60/0
High			48/56
			p=0.0001

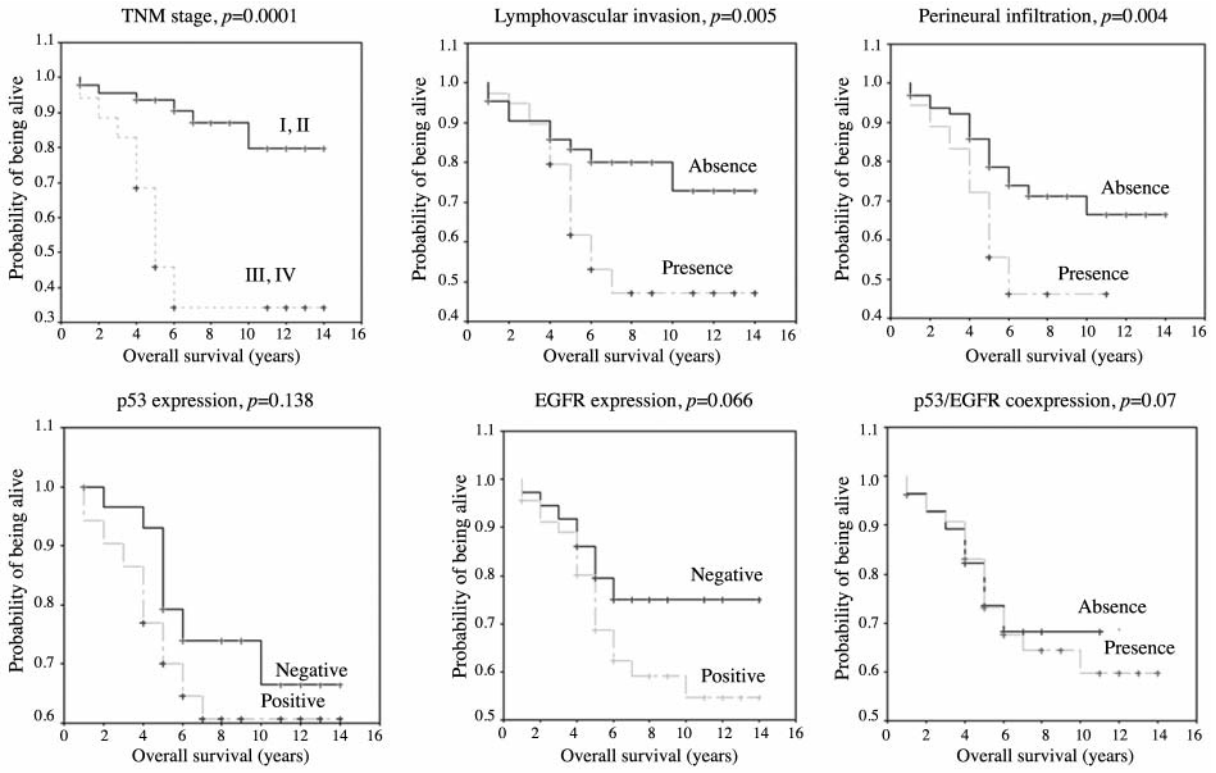

Figure 1. *Kaplan-Meier curves stratified for various parameters.*

and use of chemotherapy, no significant associations were noted between p53- and EGFR-positive expression and patients' survival (analytical data not shown). On multivariate analysis, only stage was associated with increased risk of cancer death [Cox regression analysis p=0.0001, b-coefficient (SE): 1.898 (0.383)].

Discussion

Over the past two decades, p53 and EGFR have been two of the most studied presumptive prognostic markers in colorectal cancer. The lack of a clear consensus in the literature on p53 prognostic significance may be because of the use of heterogeneous study populations, different antibodies, differences in immunohistochemical methods, variations in cut-off values, patient stages included and duration of follow-up (6-13, 29, 32, 33). Allegra *et al*. recently published a large immunohistochemistry (IHC)-based study showing that patients with Dukes' stage B and C colon cancer expressing mutant p53 protein did significantly worse than patients with p53-normal tumours (29). Several previously published series have supported the deleterious effect of p53 overexpression on prognosis (4-9). This association has not been found to be universally true, as several investigations, including the present, failed to demonstrate a significant association between p53 overexpression and clinical outcome in patients with colorectal cancer (10-13, 32). In addition, Soong *et al*. correlated *p53* mutation with improved clinical outcome in distal colonic carcinomas and then in a larger study reported that *p53* mutation was not a useful prognostic marker for colorectal disease (33). The largest population-based study to date, which looked at 1,464 patients (SSCP analysis, codons 5-8), concluded that *p53* mutation was not an independent predicator of prognosis; however, mutations in proximal tumours were associated with a significantly worse five-year survival than those with wild-type p53 (34). Similarly Manne *et al*. showed nuclear accumulation of p53 to be a poor prognostic indicator specifically for tumours of the proximal colon (35). Site-specific relations of p53 expression and prognosis were not supported in the presented study.

The introduction and the routine utilisation of adjuvant chemotherapy with curative or palliative intent for Dukes' C and D CRC, respectively, may be an additional confounding factor. Two studies have reported a worse outcome for patients receiving chemotherapy if they had *p53* mutation (36, 37). The lack of a p53 value in terms of prognosis was irrespective of the use of chemotherapy in the present study. However, the speculation that *p53* mutation may be able to

identify patients who do not respond as well to chemotherapy as those with the wild-type gene cannot totally be ruled out until further evaluation of the predictive significance of this molecular alteration from matched treated and untreated patient groups in large scale clinical trials takes place.

EGFR has been found to be elevated in CRCs, with expression rates ranging from 25% to 77% (19, 24, 25). This heterogeneity of expression is attributed to the different detection techniques, although most are based on quantitative immunohistochemical labelling with monoclonal antibodies (19). Despite these known reproducibility and validation difficulties, immunohistochemical testing remains one of the most common and accurate methods used to assess EGFR expression (24). The presented observations confirm EGFR expression in colorectal adenocarcinomas, consistent with high EGFR expression in 43% of the tumours examined. In the present study, an EGFR immunohistochemistry composite score (positivity multiplied by intensity) was used, previously employed in large studies on CRC evaluating EGFR expression (19, 24). Consistent with the results of the main studies, no differences in EGFR expression were found among the different tumour sites (19, 24, 38). Only one study has demonstrated higher EGFR expression in cancer of the distal colon than the rectum (39).

When included along with known clinicopathological prognostic factors, the only significant association shown along with the p53 overexpression was between EGFR positivity and advanced tumour (T) stage. Nevertheless, as also occurred with p53, it was not possible to demonstrate EGFR as an independent prognostic variable. This is in line with previous studies that did not demonstrate any influence of EGFR expression on patient survival (23, 26, 38). Nevertheless, although not confirmed by this study, patients with EGFR overexpression seem to have a higher risk of generating liver metastases (40).

Despite the fact that EGFR is not considered as a major prognostic factor to suggest chemotherapy as adjuvant treatment for CRC patients, its major overexpression in stage T3/4 disease, irrespective of the nodal status of the cancer, suggests the potential implication of EGFR-targeted therapies in such a setting.

Possible associations between EGFR expression and other clinicopathological parameters in CRC patients remain unclear. EGFR expression was reported to be correlated with more aggressive disease, increased risk of metastasis, advanced tumour stage, significantly more reactivity in more deeply invaded regions compared with superficial tumours and higher rates of mesenteric lymph node involvement (19, 40).

According to the major studies published to date, no significant association was found between histological grade and EGFR expression (19, 25, 41, 42). Few studies reported a relationship between histological grade and EGFR overexpression (21, 23). However, in these studies, moderately and poorly differentiated tumours predominated, whereas the present analysis was based on mostly well and moderately differentiated adenocarcinomas.

One of the predominant findings in this paper demonstrate a significant association between high EGFR expression and advanced T3, and 4 stage, highlighting a relationship between EGFR overexpression and tumour invasion. This is in partial accordance with a recent study by Spano *et al*. which showed that EGFR expression was significantly overexpressed in TNM tumour stage T3 (24). This finding is in agreement with an earlier study by Goldstein and Armin, who demonstrated that EGFR immunoreactivity was significantly higher in the deepest regions of the tumours, as compared to the superficial or luminal zones (19). This finding along with the similar p53 overexpression in the T3/4 tumours, suggest that p53 and EGFR alterations in carcinoma cells might provide the cells with a certain growth advantage in terms of depth invasion and that may represent a rather late event in CRC histogenesis. Consistent with previous reports, these observations confirm the assumption that the molecular profile of colon mucosal cells is altered from early to late stages (29, 43, 44). Accordingly, in this series, the molecular markers showed a strong correlation with each other but no correlation with other clinicopathological factors, particularly signs of more aggressive colon cancer behavior, bearing out the hypothesis that molecular alterations could be primarily implied in colon carcinogenesis and fundamental aspects of tumour progression.

Assessment of a combination of molecular markers can be potentially useful to define a higher-risk group of CRC patients who may benefit from adjuvant conventional chemotherapy, or new treatments such as anti-EGFR targeted regimens, which are already in clinical use (45, 46). The search for molecular markers to predict prognosis and identify target populations for specific treatment regimes remains a major focus of CRC research. Despite the discordant results, knowledge of molecular features that determine the behavior of individual colon tumours represents a fundamental step to identify high-risk categories of patients and, consequently, to allow modulation of patient-tailored cancer treatment options.

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