ORIGINAL ARTICLE

Prognostic significance of expression patterns of c-erbB-2, p53, $p16^{INK4A}$, $p27^{KIP1}$, cyclin D1 and epidermal growth factor receptor in oesophageal adenocarcinoma: a tissue microarray study

R Langer, B H A Von Rahden, J Nahrig, C Von Weyhern, R Reiter, M Feith, H J Stein, J R Siewert, H Höfler, M Sarbia

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See end of article for authors' affiliations

Correspondence to: R Langer, Institute of Pathology, Technical University, Ismaninger Street 22, 81675 München, Germany; Rupert.Langer@lrz.tum.de

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Aims: To correlate immunohistochemical expression patterns and prognosis in oesophageal adenocarcinoma.

Methods: The expression of c-erbB-2, p53, $p16^{INK4A}$, $p27^{KIP1}$, cyclin D1 and epidermal growth factor receptor (EGFR) was studied in a series of 137 primarily resected oesophageal adenocarcinoma samples. The expression analysis on protein level was performed on routine paraffin wax-embedded material, with immunohistochemical staining of the samples, assembled on a tissue microarray. The results were correlated with clinicopathological features (pT, pN and G) and survival.

Results: 22 (16%) tumours showed an overexpression of the c-erbB-2 oncoprotein. Expression of EGFR was observed in 72 (55%) cases, accumulation of p53 in 68 (52%) cases and of cyclin D1 in 102 (77%) cases. Loss of $p16^{INK4A}$ expression was observed in 101 (76%) cases and low expression of $p27^{KIP1}$ in 91 (71%) cases. Expression of these proteins did not correlate with tumour stage, grade, Lauren's or World Health Organization classification or lymph node status. On univariate survival analysis, more advanced tumour stage ($p = 0.002$), lymph node involvement ($p = 0.003$), high tumour grade ($p = 0.017$) and lack of EGFR expression ($p = 0.034$) were found to be associated with poorer survival. On multiple regression analysis, only tumour stage ($p = 0.03$) and lymph node involvement ($p = 0.004$) were shown to have an association with the survival of the patient.

Conclusion: The immunohistochemical expression of c-erbB-2 oncoprotein, cylin D1, $p16^{INK4A}$, $p27^{KIP1}$, p53 and EGFR in most oesophageal adenocarcinomas suggests their implication in the pathogenesis of this entity. None of the molecular markers assessed, however, was of prognostic value. Identification of any marker superior to or even approaching the prognostic value of conventional histopathological markers (pT and pN) was therefore not possible.

The growing prevalence of Barrett's oesophagus and the incidence of oesophageal adenocarcinoma associated with Barrett's oesophagus have stimulated increasing interest in this disease. Consequently, many studies have investigated different genetic changes in relation to prognosis, but although several molecular markers in a few studies have shown prognostic correlations, no single molecular marker has provided consistent and independent prognostic information.[1–4] The tissue microarray (TMA) technology allows high-throughput analysis of molecular markers and thereby facilitates studies of multiple markers in large tumour series.[5–7] Our aim was to apply TMA to investigate the expression patterns of multiple markers and their prognostic correlations in oesophageal adenocarcinoma. By including 137 patients and investigating the expression of p53, $p16^{INK4A}$, $p27^{KIP1}$, cyclin D1, epidermal growth factor receptor (EGFR) and c-erbB-2, we carried out the largest immunohistochemical study on primary resected oesophageal adenocarcinoma samples so far.

MATERIAL AND METHODS

Patients and specimens

Paraffin wax-embedded tumour samples from 137 patients with oesophageal adenocarcinoma were investigated. According to Siewert and Stein's classification, all tumours were adenocarcinomas of the oesophagogastric junction type I.[8] Patients had been treated by radical surgical resection—either transthoracic or transhiatal oesophagectomy—between 1991 and 2002, so that there was a minimum follow up of 3 years for all patients. The mean number of lymph nodes sampled by lymphadenectomy was 20 (range 6–52). Mean survival was 45 months (range 3–164 months). None of the patients had received neoadjuvant chemotherapy or radiochemotherapy. The pT and the pN categories of the tumours were determined according to the current tumour-node-metastasis classification,[9] histological subtyping according to Lauren's classification and tumour grading according to the World Health Organization (WHO) classification.[10] All patients gave consent at the time of their operation. Table 1 shows the clinicopathological characteristics of the patients.

Preparation of TMAs

For each of the 137 carcinomas, one paraffin wax block was selected from the archives of the Institute of Pathology of the Technical University of Munich. An experienced pathologist (MS) marked the viable, representative areas of tumour specimens. Core needle biopsy specimens were retrieved from the original tumour blocks by using a manual arrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA) and

Abbreviations: EGFR, epidermal growth factor receptor; TMA, tissue microarray

Table 1 Clinicopathological features of 137 patients with Barrett's adenocarcinoma

Characteristics	Data, n (%)
Age (years)	
Mean	63
Range	33–83
Sex	
Female	12 (9)
Male	125 (91)
pT category	pT1
pT1	64 (47)
	pT1 (mucosa) 28 (20%)
	pT1 (submucosa) 36 (26%)
pT2	25 (18)
pT3	48 (35)
pN category	
pN0	82 (60)
pN1	55 (40)
Grade	
1	12 (9)
2	61 (45)
3	64 (46)
Lauren's classification	
Intestinal	119 (87)
Mixed	15 (11)
Diffuse	3 (2)

positioned in a recipient paraffin wax array block. We aimed to obtain at least three tissue cylinders per tumour with a diameter of 0.6 mm from each biopsy specimen.

Immunohistochemical study

Immunohistochemical analysis was carried out using an automated stainer (Ventana Benchmark, Tuscon, Arizona, USA). Fresh 2-µm sections from TMA blocks were transferred to glass slides, dewaxed and rehydrated. Antigen retrieval method (see table 2) (eg, microwave oven heating in citrate-buffered saline) was applied according to the manufacturer's recommendations. The TMA slides were cooled and incubated with the primary antibodies. All primary antibodies used were commercially available mouse monoclonal IgGs. Table 2 shows the detailed data on the antibodies. The reaction was developed with the labelled streptavidin-biotin-alkaline phosphatase system, with fast red used as the reaction indicator. After counterstaining with haematoxylin, slides were dehydrated in ascending concentrations of ethanol and mounted. For positive controls, we used tissues with known expression of the respective antigens (c-erbB-2: breast carcinoma; p53 and p16: colorectal carcinoma; p27: tonsils; cyclin D1: mantle cell lymphoma; EGFR: normal oesophageal squamous epithelium). For negative controls, we used irrelevant antibodies with the immunoglobulin isotype.

Scoring

Scoring for c-erbB-2 was carried out using the Hercep Test. This scoring method assesses both the percentage of cancer cells that show complete membrane staining, and the intensity of the staining as follows: no immunoreactivity or immunoreactivity in <10% of tumour cells: score 0; faint weak immunoreactivity in >10% of tumour cells, but only a portion of the membrane with positive staining: score 1+; weak to moderate complete membrane immunoreactivity in >10% of tumour cells: score 2+; moderate to strong complete immunoreactivity in >10% of tumour cells: scores of 3+. Scores 2+ and 3+ were considered to be a positive result for c-erbB-2 (HER2-neu) status.

EGFR membrane staining was quantified and graded as described recently:[11] no staining or membrane staining in <10% neoplastic cells: negative (score 0); complete or incomplete membranous staining in >10% neoplastic cells: positive (weak staining: score 1+; moderate staining: score 2+; strong staining: score 3+).

In cases of p53, cyclin D1 and $p16^{INK4A}$ staining, a visual grading system based on the number of positively stained nuclei of the malignant cells in each tissue was used: ⩾10% nuclei stained: positive, irrespective of staining intensity. Expression of $p27^{KIP1}$ was classified into low (<50% positive staining) and high (⩾50%).

Statistical analysis

Expression patterns for each marker were correlated with tumour pT category, pN category, grade, Lauren's classification and lymph node status, and with survival. For survival analysis, patients with positive resection margins, distant metastasis at the time of surgery or survival >1 month were excluded.

SPSS statistical software was used for statistical analysis. Associations in 2×2 tables were evaluated with Fisher's exact test. Survival analysis was carried out using Kaplan–Meier estimates, log rank tests and Cox's proportional hazards regression analysis. All tests were two sided, and significance level was set at 5%.

RESULTS

Neoplastic tissue was interpretable for all 137 cases. In all, 22 (16%) cases showed an overexpression of the c-erbB-2 oncoprotein. Expression of EGFR was observed in 72 (55%) cases, accumulation of p53 in 68 (52%) cases and of cyclin D1 in 102 (77%) cases. Loss of $p16^{INK4A}$ expression was observed in 101 (76%) cases. Loss of expression and low expression of $p27^{KIP1}$ was observed in 91 (71%) cases. Failure rates due to secondary technical issues, including sectioning artefacts, antibody gradients and obscuring debris were 4% (2.2–6.5; table 3).

Table 2 Antibodies used in this study

Marker	Clone	Company	Dilution	Buffer, antigen retrieval
c-erbB-2	c-erbB-2	Dako, Hamburg, Germany	1:300	Citrate, pH6; heating 7 min
EGFR	31 G7	Cytomed, Baden–Baden, Germany	1:60	Protease 20 min
p53	DO-7	Dako, Hamburg, Germany	1:200	Citrate, pH6; heating 7 min
cyclin D1	SP4	Dcs-diagnostics, Hamburg, Germany	1:50	EDTA; heating 30 min
p16	I6 PO7	LabVision/NeoMarkers, Fremont, California, USA	1:100	Citrate, pH6; heating 7 min
p27	F-8	BD Transduction Laboratories, Lexington, Kentucky, USA	1:10	Citrate, pH6; heating 7 min

EGFR, epidermal growth factor receptor.

Table 3 Immunohistochemical expression patterns of c-erbB-2, p53, $p16^{INK4A}$, $p27^{KIP1}$, cyclin D1 and epidermal growth factor receptor in 137 patients with Barrett's adenocarcinomas

Marker	Staining pattern	Fraction of staining patterns (%)
p53	Positive	68/132 (52)
	Negative	64/132 (48)
Cyclin D1	Positive (accumulation)	102/133 (77)
	Negative	31/133 (23)
$p16^{INK4A}$	Negative (loss)	101/133 (76)
	Positive (present)	32/133 (24)
$p27^{KIP1}$	Negative (loss)	91/128 (71)
	Positive (present)	37/128 (29)
EGFR	Negative	61/132 (45)
	Positive	72/132 (55)
	Score 1+	45/132 (34))
	Score 2+	21/132 (16)
	Score 3+	6/132 (5)
c-erbB-2	Negative	95/134 (71)
	Dako score 1+	17/134 (13)
	Overexpression	22/134 (16)
	Dako score 2+	9/134 (7)
	Dako score 3+	13/134 (10)

Associations between dichotomised staining patterns

We found a significant association between the expression patterns for c-erbB-2 and p53, where there was a positive correlation between c-erbB-2 overexpression and p53 accumulation: 16 of the 22 (73%) tumours with c-erbB-2 overexpression also showed an accumulation of p53 whereas 57 of 63 (90%) specimens with negative staining for p53 also failed to show c-erbB-2 overexpression ($p = 0.026$; Fisher's exact test).

We found no significant correlation between the expression patterns of p53, cyclin D1, $p16^{INK4A}$, $p27^{KIP1}$, EGFR and c-erbB-2 and clinicopathological features (pT category, grade, Lauren's classification and lymph node status).

Prognostic correlations

Univariate analysis using time of death as the clinical end point showed significantly increased hazard ratios (HR) for pT category (pT1 *v* pT2 *v* pT3; HR 1.97; CI 1.30 to 2.98; $p = 0.002$), tumour differentiation grade (HR 2.4; CI 1.28 to 4.53; $p = 0.007$) and lymph node involvement (HR 3.67; CI 1.74 to 7.8; $p = 0.0006$). Negative or reduced membranous staining for EGFR was significantly associated with shortened survival (HR 0.99; CI 0.98 to 1.0; $p = 0.039$) as well. Expressions of p53, cyclin D1, $p16^{INK4A}$, $p27^{KIP1}$ and c-erbB-2 failed to show any significant correlation with survival.

By using multiple regression analysis including pT category, tumour differentiation grade, lymph node involvement and expression of p53, cyclin D1, $p16^{INK4A}$, $p27^{KIP1}$, EGFR and c-erbB-2, we found that only pT category (HR 2.33; CI 1.05 to 5.16; $p = 0.03$) and lymph node involvement (HR 2.89; CI 1.40 to 5.95; $p = 0.004$) showed an association with the survival of the patient.

DISCUSSION

Changes in the p53 gene are early and frequent events[1] associated with the progression of Barrett's oesophagus to adenocarcinoma, and may therefore be a crucial factor with respect to Barrett's carcinogenesis. In about 40%[12] and up to 87%[13] of oesophageal adenocarcinomas, overexpression of p53 is detectable by immunostaining. In the present study, 52% of the tumours showed immunostaining for p53. Several investigators have studied correlations between p53 status and prognosis, with partly contradictory results. Mutational status in p53 was reported to be an independent prognostic factor for patients with R0 resected oesophageal adenocarcinomas. Owing to deletion-type mutations, however, the correlation between increased immunoreactivity and the presence of mutations is imperfect; and false negative results with p53 immunohistochemistry have to be expected in 20–33% of patients.[14 15] In addition, increased p53 expression may also be induced by non-mutational mechanisms.[16] With regard to the impact of p53 protein overexpression on survival of patients with oesophageal adenocarcinoma, there are several studies that show trends or significant results towards reduced survival in a limited number of patients.[12 17] Most larger studies,[18–20] including the present one, however, have not supported any prognostic impact with immunostaining for p53. This is a strong argument against a prognostic role for p53 immunostaining in oesophageal adenocarcinoma.

Cell cycle regulatory proteins that are implicated in the carcinogenesis of oesophageal adenocarcinoma include $p16^{INK4A}$ and cyclin D1. Inactivation due to deletion, mutation or promoter hypermethylation of $p16^{INK4A}$ occurs during progression of Barrett's oesophagus to oesophageal cancer.[21] Loss or inactivation of $p16^{INK4A}$ occurred in 100 (77%) cases, so this observation was concordant with previously reported results of studies investigating the role of $p16^{INK4A}$ in oesophageal adenocarcinoma.[22] On the other hand, increased expression of cyclin D1 often occurs in adenocarcinomas of the oesophagus.[23] In our study, overexpression of cyclin D1 was observed in 76% of cases. We could not show a correlation between the expression of cyclin D1 and $p16^{INK4A}$, as previously reported.[24] From the clinical point of view, no association between the expression of cyclin D1 and $p16^{INK4A}$ and the survival of the patients was noted, and these results are concordant to previously published studies.[24]

In contrast with other tumour entities, few data exist on the role of the tumour suppressor gene $p27^{KIP1}$ in the biology of oesophageal adenocarcinoma.[25] Loss of staining or reduced staining of $p27^{KIP1}$ is reported in 83% of oesophageal adenocarcinoma cases and is correlated with tumour stage, depth of invasion, lymph node involvement and a decreased survival rate. These findings, however, were obtained from a single collective of 54 patients.[26] In our study, low expression of $p27^{KIP1}$, indicating genetic change or inactivation, was observed in 71% of the cases. A correlation with clinicopathological features could not be shown. Furthermore, we found no correlation between the expression of $p27^{KIP1}$ and survival of patients. Thus, $p27^{KIP1}$ may not be a useful marker to estimate prognosis in patients with oesophageal adenocarcinoma.

EGFR activation results in a cascade of cellular responses occurring in cell division, proliferation, differentiation, apoptosis and angiogenesis. The prognostic significance of EGFR expression has been established for various tumour entities, but only a few studies have assessed its prognostic role in oesophageal adenocarcinoma.[27–29] Our findings on EGFR expression in 55% of the cases is well in accordance with findings by other authors. In addition, in our study lack of EGFR expression tended to be associated with shortened survival, and this observation contradicts previously reported data.[28 29] Study populations in these investigations, however, were non-homogeneous and the sample size was much smaller than the collective of the present one. Thus, further studies may be needed to validate the prognostic role of EGFR expression in oesophageal adenocarcinoma, particularly against the background of the introduction of new EGFR-targeted drugs in the treatment of oesophageal cancer.

Currently available data are also conflicting on the expression of the c-erbB-2 oncoprotein as a significant prognostic marker in adenocarcinoma of the oesophagus.[30]

In most cases, gene amplification is correlated with protein overexpression and is a late event in the carcinogenesis of oesophageal adenocarcinoma.[31] Overexpression of the c-erbB-2 oncoprotein is estimated to occur in about 25% of oesophageal adenocarcinoma cases. In our study, c-erbB-2 overexpression was observed in 16% (according to the Hercep Test Scoring System) of cases, and c-erbB-2 overexpression was markedly associated with p53 accumulation. From the clinical point of view, most previously performed studies indicate that c-erbB-2 expression has considerable prognostic value. Studies that found an association with prognosis, however, were smaller[32 33] or included patients with different treatments—for example, neoadjuvant treatment[19]—although larger studies on patients with primarily resected tumours, including the present one, failed to show any association of c-erbB-2 expression with prognosis in patients.

In summary, the immunohistochemical evidence of p53, $p16^{INK4A}$, $p27^{KIP1}$, cyclin D1, c-erbB-2 oncoprotein and EGFR in most oesophageal adenocarcinomas suggests their implication in the pathogenesis of this entity.[1 2] It was disappointing, however, that none of the molecular markers analysed were of prognostic value and we could not identify any marker superior to or even approaching the prognostic value of conventional histopathological markers (pT and pN).

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Authors' affiliations

R Langer, J Nahrig, C Von Weyhern, R Reiter, H Höfler, M Sarbia, Institut für Pathologie, Technical University, München, Germany
B H A Von Rahden, M Feith, H J Stein, J R Siewert, Chirurgische Klinik und Poliklinik, Technical University

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