British Journal of Cancer (1996) 74, 86-91

Lack of prognostic significance of epidermal growth factor receptor and the oncoprotein p185^{HER-2} in patients with systemically untreated non-small-cell lung cancer: an immunohistochemical study on cryosections

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Summary The prognostic role of the epidermal growth factor receptor (EGFR) and the related receptor p185^{HER-2} in lung cancer is as yet undefined. We investigated the immunohistochemical expression of EGFR (monoclonal antibody R1; Amersham) and p185^{HER-2} (polyclonal antibody A485; Dako) in cryosections. A total of 186 unselected and systemically untreated patients with non-small-cell lung cancer (NSCLC) diagnosed and treated at Odense University Hospital, Denmark, were included. Median follow-up period was 66 months. EGFR and p185^{HER-2} was highly expressed in 55% and 26% of cases respectively. Expression of EGFR was independent of p185^{HER-2} expression. The expression of EGFR was higher in squamous cell carcinomas whereas the level of p185^{HER-2} staining was higher in adenocarcinomas. Expression of either or both receptors was not correlated with age, histological grading, stage and prognosis. We conclude that immunohistochemical detection of these growth factor receptors failed to demonstrate a prognostic significance in patients operated on for NSCLC.

Keywords: epidermal growth factor receptor; p185^{HER-2}; non-small-cell lung cancer

The improvement in survival for most cancer patients has only been modest and lung cancer is no exception to this rule. Surgical resection alone offers a chance of cure in early-stage non-small-cell lung cancer (NSCLC) (Mountain, 1994). However, some clinical trials indicate possible benefit from adjuvant (Souquet *et al.*, 1993) or neoadjuvant chemotherapy (Rosell *et al.*, 1994; Roth *et al.*, 1994). A prerequisite for further substantiation of these observations is, however, a proper prognostic classification of these patients. A whole cascade of tumour-biological characteristics related to growth, invasion and metastatic potential has been suggested for their possible prognostic value (Gazdar, 1994; Richardson and Johnson, 1993). The main criticisms of most prognostic studies are small sample size, non-homogeneous populations owing to selection bias and use of optimal cut-off values for prognostic variables without a prestated hypothesis (Altman *et al.*, 1994; Simon and Altman, 1994). In addition, various techniques have been employed without proper methodological validation. Not surprisingly, conflicting results have been obtained and it is still not possible to conclude which factors give valid prognostic information.

The protein product of the oncogene *HER*-1, the epidermal growth factor receptor (EGFR), is a 170 kilodalton (kDa) transmembrane protein, exhibiting an extracellular ligand-binding area, a transmembrane domain and an intracellular region with tyrosine kinase activity. The receptor is able to activate cytoplasmic signal proteins that trigger DNA synthesis associated with proliferation and differentiation (Prigent and Lemoine, 1992).

It has recently been shown that the *HER*-2 (or c-*erb*B-2) proto-oncogene encodes a 185 kDa glycoprotein (p185^{HER-2}), which has molecular homology with EGFR. Like EGFR, the p185^{HER-2} is a transmembrane receptor with tyrosine kinase activity (Prigent and Lemoine, 1992).

The type 1 (EGFR-related) family of growth factor receptors is important in the regulation of normal cells and in the carcinogenic process (Prigent and Lemoine, 1992), but whether expression of these receptors reflects prognosis remains to be established.

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Received 18 September 1995; revised 5 January 1996; accepted 15 January 1996

To determine whether immunohistochemical detection of EGFR and/or p185^{HER-2} in frozen tissue is of prognostic importance in patients with NSCLC, we conducted the present hypothesis-generating study in a homogeneously treated and unselected cohort of 186 patients. With the exception of two patients, cytotoxic therapy had not been given during the course of the disease.

Materials and methods

Patients and tumour samples

A total of 186 patients with NSCLC were followed for a median of 66 (40-119) months. The 131 men and 55 women had a median age of 61 (42-79) years at the time of diagnosis. Characteristics of patients were collected from their records (Table I).

All the patients were treated surgically at the Department of Thoracic Surgery at Odense University Hospital, Denmark, from 1984 to 1991. Pulmonary resection was accompanied by intraoperative evaluation of tumour extension with biopsy of suspicious areas and lymph nodes; complete mediastinal lymph node dissection or systematic lymph node sampling was not performed. The stage of the primary tumour (Table I) was determined retrospectively, including a review of the surgical and pathological reports, according to the new International Staging System for lung cancer (Mountain, 1994).

The surgical procedure was considered radical in 152 patients (microscopic radical, 104; macroscopic radical, 48), whereas macroscopic tumour tissue was left in 34 patients. Post-operative adjuvant therapy was not part of the treatment strategy. At the discretion of the treating physician, two patients out of these 186 patients with NSCLC received cytotoxic therapy during the course of their disease. No patient received post-operative adjuvant radiotherapy.

Tissue preparation

Lung tissue was received unfixed in the pathology laboratory immediately after surgical removal. One piece of tumour measuring approximately 1 cm^3 was cut out and divided. One part was placed in a cryoconservation tube, snap frozen at

Table I Characteristics of 186 patients with NSCLC

Parameter		n
Total		186
Follow-up (months)		
Median	66	
Range	40–119	
Age (years)		
Median	61	
Range	42–79	
Male		131
Female		55
Histological classification		
Squamous cell carcinoma		102
Adenocarcinoma		59
Large cell carcinoma		25
Histological grading		
Highly differentiated		15
Moderately differentiated		46
Lowly differentiated		100
Undifferentiated		25
Stage		
I		86
II		48
IIIa		41
IIIb		3
IV		8
Adjuvant therapy		
Radiotherapy		0
Cytotoxic therapy		0
Surgical procedure		
Pneumonectomy		48
Lobectomy		115
Segment resection		21
Exploratory thoracotomy		2
Radical surgery		
Microscopic		104
Macroscopic		48
None		34

−80°C and stored until further examination. The other part was formalin fixed and embedded in paraffin.

Histological classification

Haematoxylin–eosin (H&E)-stained sections from formalin-fixed paraffin-embedded tumour specimens were reviewed by one experienced pathologist (PPC). The morphological examination, classification and grading of tumours were performed according to WHO (1981).

Immunohistochemical analysis

Before we started immunohistochemical staining of the entire material we performed a series of experiments to select the best antibodies, optimal concentrations and incubation time of reagents. We examined multitissue blocks containing unfixed normal tissue and tumours with a known (low and high) expression of EGFR and p185^{HER-2}. Several antibodies produced convincing results but the best signal–noise ratio was obtained with R1 (Amersham, UK) and A485 (Dako, Denmark) respectively.

EGFR and p185^{HER-2} immunostaining was performed on cryostat sections, approximately 5 μm thick, using the peroxidase-labelled streptavidin–biotin (LSAB) technique. Frozen sections were air dried and fixed in acetone for 10 min (EGFR), or in 4% neutral buffered formaldehyde for 2 min (p185^{HER-2}). Tissue sections were then washed twice with Tris-buffered saline (TBS) for 2 min each and incubated with blocking serum [bovine serum albumin (BSA) 2% (Sigma A-7906) in TBS, pH 7.4] for 10 min. Owing to very low background staining, cryostat sections did not require blocking of endogenous peroxidase. All the incubations were performed at room temperature in wet chambers. The blocking serum was drained off and sections were incubated with the primary antibody for 30 min; the optimal dilution had been determined previously (EGFR, 1:100 in 1% BSA/TBS with 15 mM sodium azide, and p185^{HER-2}, 1:300 in 1% BSA/TBS with 15 mM sodium azide). For detection of EGFR, we used the murine monoclonal antibody R1. R1 is an IgG_{2b} antibody directed against the extracellular portion of EGFR (Waterfield *et al.*, 1982). p185^{HER-2} was detected by the rabbit polyclonal antibody A485. This antibody was developed against a peptide sequence from the intracytoplasmic part of the human p185^{HER-2}.

Sections were washed with TBS and incubated for 30 min with the biotinylated secondary antibodies (anti-mouse E432 and anti-rabbit E433) (also previously titrated for optimal dilutions), followed by streptavidin–horseradish peroxidase (P397) (1:300 in TBS). After washing with TBS the peroxidase activity was visualised by incubation for 20 min in 0.04% 3-amino-9-ethylcarbazole solution containing 0.015% hydrogen peroxide, which gives a red-brown reaction product. After treatment, the sections were washed with distilled water, counterstained with haematoxylin and mounted with Aquamount. Human placenta and normal tonsil tissue were used as positive controls and included in each staining process. Negative control sections of the tumour tissue were immunostained under the same conditions, but omitting the primary antibody; reactions were negative in all cases.

Immunohistochemical assessment

After scanning and evaluation of the entire section under low power, a representative area was evaluated with high-power fields (×10 eyepiece, ×40 objective). Scoring of the immunohistochemical results was performed by one author (PP), who was blinded with regard to the clinical data.

The immunohistochemical staining was scored negative if membrane staining was absent. Weak but recognisable staining was classified as grade 1, moderate as grade 2 and strong as grade 3. When there were different intensities within the specimen, the highest grade was recorded. Furthermore, the percentage of positively reacting tumour cells was estimated using a semiquantitative scale ranging from 0% to 100%, with 10 per cent intervals. For further analyses, staining was graded as negative, moderate (<80%) or high (≥80%). The study was approved by the local ethics committee.

Statistical evaluation

Non-parametric statistics were applied. To compare the results of two or more subgroups, the Mann–Whitney (M–W) or Kruskal–Wallis (K–W) tests were used. Correlations between subgroups were assessed by Spearman's rank correlation coefficient (r_s) test. Survival curves were generated according to the Kaplan–Meier method and compared by the log-rank test. BMDP statistical software (BMDP/PC Release 7.01, 1993) was used.

Results

The 186 tumours were distributed as follows: 102 squamous cell carcinoma, 59 adenocarcinoma and 25 large-cell undifferentiated carcinoma (Table I).

EGFR and p185^{HER-2}

Heterogeneity of tumour staining was present in some biopsies. In addition, in some specimens, a clear difference

Table II Relationship between EGFR staining and histological classification in 186 patients with NSCLC

	No. of patients	EGFR staining None N (%)	Low N (%)	High N (%)
SQ	102	6 (6)	24 (23)	72 (71)
AD	59	7 (12)	30 (51)	22 (37)
LA	25	1 (4)	15 (60)	9 (36)
Total	186	14 (8)	69 (37)	103 (55)

SQ, squamous cell carcinoma; AD, adenocarcinoma; LA, large-cell carcinoma. SQ>AD: $P<0.001$. SQ>LA: $P = 0.003$.

Table III Relationship between p185HER-2 staining and histological classification in 186 patients with NSCLC

	No. of patients	p185HER-2 staining None N (%)	Low N (%)	High N (%)
SQ	102	22 (21)	66 (65)	14 (14)
AD	59	4 (7)	25 (42)	30 (51)
LA	25	3 (12)	17 (68)	5 (20)
Total	186	29 (16)	108 (58)	49 (26)

SQ, squamous cell carcinoma; AD, adenocarcinoma; LA, large-cell carcinoma. AD>SQ: $P<0.001$. LA>SQ: $P < 0.001$.

was observed in the immunostaining of EGFR or p185HER-2 between central and peripheral tumour cells, the peripheral cells being more often positive. The heterogeneity of immunohistochemical staining was not correlated with the tumour morphology as assessed by routine H&E staining or any other parameter. There was a highly significant correlation between staining intensity and the percentage of positive cells for EGFR as well as p185HER-2 (Spearman; $r_s=0.83$, $P<0.00001$). This indicates that both methods may be used in the immunohistochemical estimation of EGFR and p185HER-2 content; for the subsequent analyses we have chosen to use percentage of positive tumour cells. There was no association between EGFR and p185HER-2 status (Spearman; $r_s=0.004$).

EGFR In sections containing bronchial epithelium, we found membrane staining in the basal cells. The majority of tumours stained positively for EGFR; the extent of EGFR expression is shown in Table II. A total of 103 tumours (55%) were strongly positive (staining of $\geqslant 80\%$ of tumour cells), whereas only 14 (8%) were negative.

There was a definite relationship between EGFR status and histology (Table II). Higher expression of EGFR was found in squamous cell carcinomas of the lung than in large cell carcinomas and adenocarcinomas (K–W; $P=0.001$). EGFR content did not show any significant correlation with age, tumour size, lymph node involvement, stage or histological grading.

We found no correlation between EGFR staining and survival in patients with squamous cell carcinoma (relative risk 1.14; 95% I 0.79–1.66), adenocarcinoma of the lung, stage I and/or II, or in the entire group of patients with NSCLC (Figure 1). This was confirmed, whether we applied the cut-off points used in Table II (three groups), used median values (two groups), used quartiles (four groups) or related survival to intensity of staining.

p185HER-2 In the normal bronchial epithelium, membrane staining was most intense at the luminal border. In all, 49 tumours (26%) were strongly positive (staining of $\geqslant 80\%$ of tumour cells), whereas 29 (16%) were negative. p185HER-2 status was also related to histology (Table III), but in contrast to EGFR, the level of p185HER-2 staining was lower in squamous cell carcinomas than in adenocarcinomas and large cell carcinomas (K–W; $P<0.001$). p185HER-2 content was not correlated with age, tumour size, lymph node involvement, stage or histological grading.

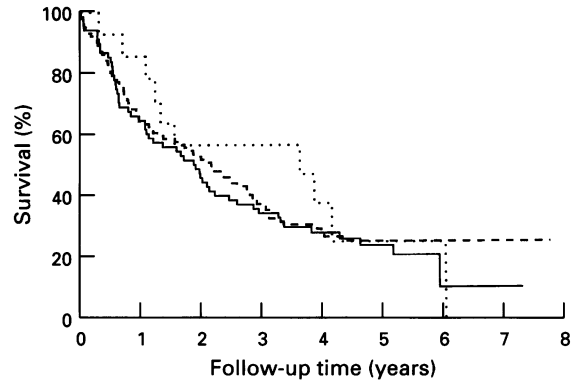

Figure 1 The survival of 186 patients with NSCLC categorised according to EGFR content ($P=0.9$). - - - -, None ($n=14$); ————, low ($n=69$); – – –, high ($n=103$).

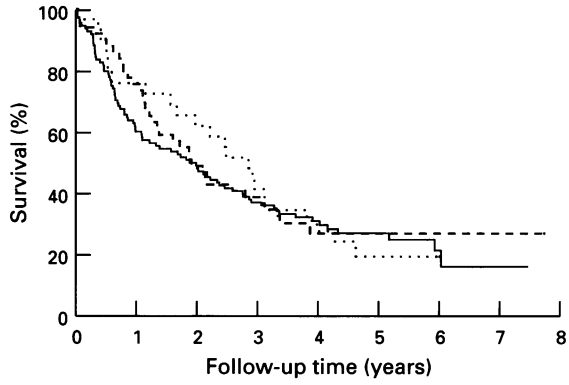

Figure 2 The survival of 186 patients with NSCLC categorised according to p185HER-2 content ($P=0.9$). - - - -, None ($n=29$); ————, low ($n=108$); – – –, high ($n=49$).

We found no correlation between p185HER-2 staining and survival in patients with squamous cell carcinoma, adenocarcinoma of the lung (relative risk 0.89; 95% CI 0.56–1.41), stage I and/or II, nor in the entire group of patients with NSCLC (Figure 2). This was confirmed, whether we applied the cut-off points for p185HER-2 from Table III (three groups), used median values (two groups), used quartiles (four groups) or related survival to intensity of staining.

In vitro studies (Kokai *et al.*, 1989) suggested that simultaneous overexpression of both EGFR and p185HER-2 acts synergistically. Consequently, we investigated whether patients with EGFR-positive *and* p185HER-2-positive tumours might have a poorer prognosis than patients with overexpression of either of the receptors or no overexpression at all. To analyse groups of comparable size we used the median value as cut-off point for both receptors. As for the individual receptor, overexpression of both receptors was of no prognostic significance ($P=0.3$; Figure 3).

Discussion

A detailed knowledge of prognostic and predictive factors can be essential for predicting patients' outcome and for proper selection of treatment, but also for optimal trial design and for comparison of results. New prognostic factors

may help to identify patients with poor prognoses, who might benefit from neoadjuvant (Roth *et al.*, 1994) or post-operative therapy (Holmes, 1993), and patients with good prognoses, who can be spared additional therapy. As new biological markers are constantly being uncovered and investigated, clinicians are faced with the problem: are these markers truly independent prognostic factors? Numerous studies investigating different factors, different cut-off points, different subsets of patients, and different end points are performed, often even without a prespecified hypotheses (Simon and Altman, 1994)

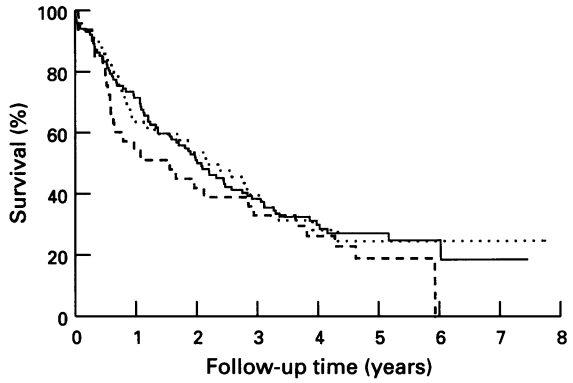

Figure 3 The survival of 186 patients with NSCLC categorised according to EGFR and p185^{HER-2} content ($P=0.38$). - - - -, high EGFR and high p185^{HER-2} ($n=50$); ————, high EGFR or high p185^{HER-2} ($n=103$); – – –, low EGFR and low p185^{HER-2} ($n=33$).

Simon and Altman (1994) have proposed a number of meaningful guidelines that should be fulfilled before definite statements are made concerning the prognostic significance of, for example, the EGF receptor family. In this regard, breast cancers are probably the best studied group of malignant tumours, with several large well-conducted studies which meet most of these guidelines; the majority of large well-conducted studies of breast cancer have found that overexpression of the EGF receptor family is associated with poor prognosis in patients with node-positive breast cancer although results on node-negative patients remain contradictory. This difference may be caused by a treatment effect, i.e. EGFR or p185^{HER-2} are predictive but not prognostic factors (Knoop *et al.*, 1994; Ravdin and Chamness, 1995). By contrast, prognostic studies of the EGF receptor family in NSCLC that include an adequate number of patients are very few.

EGFR and p185^{HER-2} are members of the type 1 (EGFR-related) family of growth factor receptors (Prigent and Lemoine, 1992). These receptors are important in the regulation of normal cell growth, they are connected to the carcinogenic process and they are potential prognostic factors in a variety of human malignant tumours (Gullick, 1991; Prigent and Lemoine, 1992).

Table IV Immunohistochemical detection of EGFR and survival in patients with NSCLC

Reference	*No. of patients*	*Histological subtype* SQ	AD	LA	Unknown	*Prognosis*	*Comments*
Dazzi *et al.* (1989)	152	97	31	7	17	NS	Patients with 'positive tumours'
% positive		55	45	43	29		tended to have improved survival
Tateishi *et al.* (1990)	131	131				NS	
% positive		42					
Volm *et al.* (1992)	81	81				S	Examined 11 potential 'prognostic
% positive		79					factors'
Volm *et al.* (1993)	121	121				S	Stage, EGFR, fos and jun were
% positive		83					prognostic factors
Volm and Mattern (1993)	88	88				S	Evaluated 9 potential 'prognostic
% positive		77					factors'
Rusch *et al.* (1993)	57	19	31	3	4	NS	IH was correlated to mRNA, which
% positive		?					had no impact on survival

IH, immunohistochemistry; S, significant; NS, not significant.

Table V Immunohistochemical detection of p185^{HER-2} and survival in patients with NSCLC

Reference	*No. of patients*	*Histological subtype* SQ	AD	LA	Unknown	*Prognosis*	*Comments*
Kern *et al.* (1990)	55	16	29	10		S for AD	Significant by multivariate analysis
% positive		31	34	0			
Tateishi *et al.* (1991)	203	84	119			S for AD	
% positive		2	28				
Volm *et al.* (1992)	81	81				NS	Examined 11 potential 'prognostic
% positive		36					factors'
Volm *et al.* (1993)	121	121				NS	Examined five potential 'prognostic
% positive		46					factors'
Volm and Mattern (1993)	88	88				NS	Examined nine potential 'prognostic
% positive		35					factors'
Kern *et al.* (1994)	46		46			S	Significant by multivariate analysis
% positive			34				
Bongiorno *et al.* (1994)	29		29			NS	
% positive			96				

IH, immunohistochemistry; S, significant; NS, not significant

We designed this study to test the hypothesis that EGFR and p185HER-2 are of prognostic value in patients operated on for NSCLC, and we attempted to meet as many of the above-mentioned guidelines as possible. Furthermore, patients were not treated with adjuvant cytotoxic therapy even, with the exception of 2 patients, when recurrent disease was diagnosed. Thus, we had the possibility of conducting a plain prognostic study.

In agreement with most other studies, we found expression of EGFR in all subtypes of NSCLC, but most frequently in squamous cell carcinomas (Veale *et al.*, 1987; Hendler and Ozanne, 1984; Sobol *et al.*, 1987; Kaseda *et al.*, 1989; Berger *et al.*, 1987) and no correlation between EGFR and the size of the primary tumour, lymph node status or stage (Kaseda *et al.*, 1989; Dittadi *et al.*, 1991; Veale *et al.*, 1989; Di Carlo *et al.*, 1993; Bolufer *et al.*, 1993; Rusch *et al.*, 1993; Dazzi *et al.*, 1989; Volm and Mattern, 1993).

However, when expression of EGFR or p185HER-2 has been correlated with outcome, conflicting results are presented. In some studies, overexpression of EGFR has been an indicator of bad prognosis (Volm *et al.*, 1992, 1993; Hendler and Ozanne, 1984; Veale *et al.*, 1987); in some studies expression of EGFR had no impact on outcome (Gorgoulis *et al.*, 1992; Scagliotti *et al.*, 1993; Rusch *et al.*, 1993), whereas still others indicated that patients with EGFR-positive tumours may survive longer than patients without EGFR expression (Dazzi *et al.*, 1989; Veale *et al.*, 1993).

The first immunohistochemical studies to investigate the content of EGFR in patients with NSCLC were based on cryosections (Cerny *et al.*, 1986; Berger *et al.*, 1987; Veale *et al.*, 1987; Sobol *et al.*, 1987). However, almost all subsequent studies used paraffin-embedded tissues. Table IV summarises published immunohistochemical studies that correlate EGFR with outcome in NSCLC. Three studies (Dazzi *et al.*, 1989; Tateishi *et al.*, 1990; Rusch *et al.*, 1993) failed to find a correlation between EGFR expression and prognosis, whereas three other studies, which included patients with squamous cell carcinoma, showed that a high EGFR content was correlated with poor prognosis. However, these three studies, which were all published by Volm *et al.* (Volm *et al.*, 1992, 1993; Volm and Mattern, 1993), do not state how patients were selected, and these different studies might at least partly include the same patients.

The oncogene *HER*-2 and its gene product p185HER-2 have also been studied in NSCLC. In accordance with most studies, we found that p185HER-2 staining was more prominent in adenocarcinomas than in squamous cell carcinomas and large cell carcinoma (Kern *et al.*, 1990; Tateishi *et al.*, 1991; Shi *et al.*, 1992). In agreement with most studies, we also found no relationship between p185HER-2 and the extension of the disease (Volm *et al.*, 1992, 1993; Shi *et al.*, 1992; Bongiorno *et al.*, 1994). Most authors agree that p185HER-2 staining is not correlated with prognosis in patients with squamous cell carcinoma (Kern *et al.*, 1990; Volm *et al.*, 1992, 1993; Tateishi *et al.*, 1991). However, a higher content of p185HER-2 is correlated with a diagnosis of adenocarcinoma of the lung, and p185HER-2 may be a prognostic factor in these adenocarcinomas (Kern *et al.*, 1990, 1994; Tateishi *et al.*, 1991).

Table V summarises published immunohistochemical studies that correlate p185HER-2 with survival in NSCLC. Kern *et al.* (1990) examined the expression of p185HER-2 in 55 patients with NSCLC and operated on between 1982 and 1985. They found that p185HER-2 expression was associated with shortened survival in a subgroup of 29 patients with adenocarcinoma (Kern *et al.*, 1990). In a later study, Kern *et al.* (1994) evaluated the prognostic significance of p185HER-2 expression and *ras* gene mutations in 46 patients with pulmonary adenocarcinoma. By univariate and multivariate analysis they showed that p185HER-2 expression was associated with shortened survival, whereas K-*ras* mutation approached significance as a poor prognostic indicator. The impact of both p185HER-2 expression and a K-*ras* mutation on survival was additive and highly significant. However, these 46 patients with adenocarcinomas were operated on during the same period as the 29 patients previously mentioned (Kern *et al.*, 1990) and this indicates some selection bias. Tateishi *et al.* (1991) examined p185HER-2 in 203 patients with NSCLC and found 5-year survival rates of p185HER-2-positive and -negative patients of 30% and 52% respectively; they concluded that the expression of p185HER-2 may serve as a prognostic indicator in adenocarcinoma of the lung.

Bongiorno *et al.* (1994) used cryosections to evaluate the content of p185HER-2 in 29 patients with adenocarcinomas but found no correlation with survival. Except for the study by Bongiorno *et al.* (1994) and ours, all other studies in Table V were based on paraffin-embedded tissue.

It is becoming apparent in breast cancer at least (Ravdin and Chamness, 1995) that overexpression of these receptors is related to response to therapy (i.e. it is a predictive factor), and this may explain, at least to some extent, the conflicting results summarised above.

EGFR and p185HER-2 are expressed in NSCLC at a higher level than that of normal bronchial or premalignant tissue (Di Carlo *et al.*, 1993), but without correlation with extension of disease once an invasive tumour has developed. These facts suggest an important step during preinvasive carcinogenesis. It might provide the potential tumour cell with the ability to proliferate when the supply of growth factors is restricted and/or escape terminal differentiation. In addition, if these growth factor receptors and their ligands are important in tumorigenesis, the data suggest that adenocarcinomas, squamous cell carcinomas and large cell carcinoma probably have some carcinogenetic mechanisms in common, regardless of progenitor cell or anatomical location (Weiner *et al.*, 1990).

In conclusion, the present study failed to demonstrate that overexpression of EGFR and p185HER-2 is prognostic in systemically untreated patients with NSCLC. Instead EGFR and p185HER-2 may be associated with the development of the primary malignant tumour, but without impact on further tumour progression, invasion or development of metastasis. Most studies of EGFR and p185HER-2 have used different monoclonal or polyclonal antibody directed toward different epitopes of the receptor and before results are incorporated in the clinical situation, standardisation of the immunohistochemical analyses is therefore indispensable.

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