12. Winkler CF, Mahr MM, Debandi H. Cisplatin and renal magnesium wasting (letter). *Ann Intern Med* 1979, **91**, 502–503.
13. Willox JC, McAllister EJ, Sangster G, *et al.* Effects of magnesium supplementation in testicular cancer patients receiving cisplatin: a randomised trial. *Br J Cancer* 1986, **54**, 19–23.
14. Mead GM, Arnold AM, Green JA, *et al.* Epileptic seizures associated with cisplatin administration. *Cancer Treat Rep* 1982, **66**, 1719–1722.
15. Macauley VM, Begent RHJ, Phillips ME, *et al.* Prophylaxis against hypomagnesaemia induced by cisplatin combination chemotherapy. *Cancer Chemother Pharmacol* 1982, **9**, 179–181.
16. Vokes EE, Mick R, Vogelzang NJ, *et al.* A randomised study comparing intermittent to continuous administration of magnesium aspartate hydrochloride in cisplatin-induced hypomagnesaemia. *Br J Cancer* 1990, **62**, 1015–1017.
17. Sartoric S, Nielsen I, Tassinari D, Rigoli F, Arcudl D, Abbasciario V. Changes in intracellular magnesium concentrations during cisplatin chemotherapy. *Oncology* 1993, **50**, 230–241.
18. Bell DR, Woods RL, Levi JA. Cis-diammine dichloroplatinum—induced hypomagnesaemia and renal magnesium wasting. *Eur J Cancer Clin Oncol* 1985, **21**, 287–290.
19. Scanion KJ, Newman EM, Lu Y, Priest DG. Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. *Proc Natl Acad Sci USA* 1986, **83**, 8923–8925.
20. Trave F, Rustum YM, Goranson J. Synergistic antitumor activity of cisplatin (DDP) and 5-fluorouracil (FUra) in mice bearing leukaemia L1210 cells. *Proc Am Assoc Cancer Res* 1985, **26**, 1270.
21. Evans TRJ, Glees JP, Knight MJ, *et al.* The use of a new combination chemotherapy: epirubicin, cisplatin and continuous infusion 5-fluorouracil (ECF) in upper gastrointestinal (GI) tumours (Abstr). *Ann Oncol* 1992, **3**, 53.
22. Findlay M, Mansi JL, Ford HT, Nash AT, Cunningham D. Epirubicin cisplatin and 5-fluorouracil (ECF) is highly effective in advanced gastric cancer. *Eur J Cancer* 1991, **27** (Suppl. 2), 399.
23. Jones AL, Smith IE, O'Brien MER, *et al.* Phase II study of continuous infusion 5-fluorouracil with epirubicin and cisplatin in patients with metastatic and locally advanced breast cancer: an active regimen. *J Clin Oncol* 1994, **12**, 1259–1265.
24. Parfitt AM, Klerekoper M. Clinical disorders of calcium phosphorous and magnesium metabolism. In Maxwell MH, Kleeman CR, eds. *Clinical Disorders of Fluid and Electrolyte Metabolism*. St. Louis, McGraw-Hill, 1980, 947–1151.
25. Levine BS, Coburn JW. Magnesium: the mimic/antagonist of calcium. *New Engl J Med* 1984, **310**, 1235–1253.
26. Rankin LI, Krous H, Whang R, *et al.* Effect of magnesium deficiency on gentamicin nephrotoxicity (Abstr). *Clin Res* 1981, **29**, 745.

Pergamon

European Journal of Cancer Vol. 31A, No. 2, pp. 178–183, 1995

0959-8049/95 $9.50+0.00

0959-8049(93)00421-8

Epidermal Growth Factor Receptor (EGFr) Expression in Non-small Cell Lung Carcinomas Correlates with Metastatic Involvement of Hilar and Mediastinal Lymph Nodes in the Squamous Subtype

G. Fontanini, S. Vignati, D. Bigini, A. Mussi, H. Lucchi, C.A. Angeletti, R. Pingitore, S. Pepe, F. Basolo and G. Bevilacqua

Epidermal growth factor receptor (EGFr) levels were evaluated in paraffin-embedded tumour specimens of non-small cell lung cancer (NSCLC) from 176 patients who underwent surgical resection. The EGFr expression was evaluated by immunocytochemical assay using a monoclonal antibody which recognises the external domain of the receptor. EGFr immunoreactivity was significantly higher in squamous than in non-squamous cell carcinomas ($P = 0.0009$). Hilar and/or mediastinal nodal involvement was found in 29 of 105 (27.4%) squamous cancers, and in this group of patients, the mean of EGFr positive cells was significantly higher than that of patients without nodal involvement ($P = 0.01$). No significant correlations were found between the expression of EGFr and other clinicopathological or biological parameters such as T-status, grading, proliferative activity. EGFR is suggested to represent a useful indicator of nodal metastasis in NSCLC.

Key words: EGFr, NSCLC, immunocytochemistry, prognosis
Eur J Cancer, Vol. 31A, No. 2, pp. 178–183, 1995

INTRODUCTION

Non-small cell lung cancer (NSCLC) represents both a biologically and histopathologically heterogeneous group of cancer. In patients with this type of cancer, TNM classification is the most important prognostic factor, in that it largely determines treatment [1]. For this reason, there is a need to identify parameters which can predict tumour behaviour and allow better prognostic evaluation and therapeutic approach.

Several reports have described the presence of epidermal growth factor receptor (EGFr) in many types of human malignancies [2–8]. Alterations in the expression of this receptor have been established as important features of the neoplastic process in several human cancers [9–17]. EGFr is a 170 000 kD protein which binds strongly with epidermal growth factor; this high-affinity binding induces cell proliferation and differentiation in several tissues by its tyrosine kinase activity [18]. EGFr is overexpressed in lung cancer cells compared with normal tissues [7, 19]. The high expression of EGFr has been related to poor prognosis and rapid progression in squamous lung cancer [17, 20, 21].

As EGFr is likely to play a role in human NSCLC progression, we evaluated the expression of this receptor in a large group of lung carcinomas, using an immunohistochemical method. The expression of this receptor was also correlated with the incidence of metastases at the time of surgery. Furthermore, since EGFr has a role in cancer growth control, we evaluated its expression with regard to tumour cell proliferation ability, using both immunohistochemical and flow cytometric analysis.

MATERIALS AND METHODS

Patients

Tumour samples obtained from 176 consecutive patients resected for NSCLC and previously untreated, were analysed. All patients were staged at the time of surgery. The histological classification of the tumours was based on the World Health Organization study [22]. The tumour stage (pTNM) was classified according to the guidelines of the AJCCS [1].

Immunohistochemistry

Freshly resected tissues from 176 consecutive NSCLC were collected for immunohistochemical analysis. The samples were immediately frozen in liquid nitrogen and stored in a −80°C freezer until sectioning. One portion of the same samples was fixed in 10% formalin and routinely processed for paraffin embedding. Monoclonal antibody (MAb) 31G7 (Triton Diagnostic-Alameda, California, U.S.A.) which reacts with the peptide backbone of the extracellular domain of the EGFr molecule, was used for the detection of EGFr. The proliferative activity of tumour samples was evaluated using two MAbs in both frozen and paraffin embedded samples: Ki-67 MAb (Dakopatts, Denmark) and PC10 (Novocastra Laboratoires Ltd, Newcastle, U.K.), respectively. The avidin biotin peroxidase method (Vectastain ABC Kit, Vector Laboratories, Burlingame, California, U.S.A.) was used for each antibody, after overnight incubation with each primary antibody at the different dilutions (1 : 100 for EGFr, 1 : 200 for PC10 and 1 : 50 for Ki-67, respectively).

Immunohistochemical evaluation

EGFr immunoreactivity was evaluated by a semiquantitative method, counting the number of positive cells in a minimum of 1000 neoplastic cells. The same method was used to evaluate PC10 and Ki-67 immunoreactivity.

Flow cytometry

Flow cytometry was performed on a nuclear suspension prepared from 50 μm sections of formalin fixed, paraffin-embedded tissue of primary lung cancers. In order to determine the percentage of tumour cells in the tissue analysed, adjacent 4 μm thick histological sections were cut before and after the 50 μm sections used for flow cytometry. A percentage of neoplastic cells between 25 and 30% was considered adequate. Briefly, the sections were dewaxed by xylene, rehydrated through a sequence of 90, 80, 70 and 50% ethanol, washed twice in double distilled water and minced in 1 ml of 0.5% pepsin (Sigma, St. Louis, Missouri, U.S.A.) in 0.9% NaCl (pH 1.5) at 37°C for 30 min. Samples were filtered through a 30 μm pore sized polyester filter and stained in a propidium iodide solution (PI 50 μg/l in PBS plus 0.1% v/v of Nonidet P40) for 30 min in the dark. Before analysis the samples were syringed 2–3 times through a 25G needle to avoid nuclear clumps. All samples were analysed by a FACScan flow cytometer (Becton Dickinson, San Jose, California, U.S.A.) coupled with a CONSORT 30 microcomputer (Becton Dickson and Hewlett-Packard, California, U.S.A.). For each sample, at least 35 000 events were acquired using the Cell Fit software (Becton Dickson). Cell cycle analysis was performed by the Dean method [23], with no background subtraction. The two major drawbacks of paraffin-embedded material as a source of nuclei for DNA-flow cytometry are the relative poor resolution [24] and the higher amount of cellular debris which might affect the real percentage of S-phase fraction. Despite these potential limitations, in our study, histogram resolution was good, with 5% as CV median value of diploid G0/G1 peak. Moreover, to reduce the possibility of missing some near-diploid tumours, histograms were considered interpretable only if the CV of the diploid G0/G1 peak was equal or less than 7%.

To every series of measurement, normal DNA fluorescence was adjusted to channel 200 by using non-neoplastic paraffin-embedded lung tissue. In each measurement session (12–15 samples), we found that all tumour samples contained a stemline with normal DNA-content. Aneuploidy was characterised by the presence of one or more additional stemlines, all with higher DNA content.

Statistical analysis

Statistical analysis was performed by StatView II software. The unpaired *t*-test and/or non-parametric test (Kruskall-Wallis test) were used to evaluate differences between groups. Linear regression coefficient and/or Spearman's Rank correlation coefficient were also calculated to relate EGFr and variables concerning proliferative activity.

RESULTS

EGFr expression in 176 NSCLC

A total of 176 tumours were analysed which consisted of 105 (59.66%) squamous cell carcinomas and 71 (40.34%) non-squamous carcinomas, including 59 (33.5%) adenocarcinomas

Correspondence to G. Fontanini.
G. Fontanini, S. Vignati, D. Bigini, R. Pingitore, F. Basolo and G. Bevilacqua are at the Institute of Pathology, via Roma 57, Pisa; A. Mussi, H. Lucchi and C.A. Angeletti are at the Service of Thoracic Surgery, University of Pisa, Pisa; S. Pepe is at the Department of Medical Oncology, University of Naples, Naples, Italy.
Revised 30 May 1994; accepted 14 Jul. 1994.

and 12 (6.8%) anaplastic large cells carcinomas. There were 160 males and 16 females with a mean age of 62.4 (range 36–79). 155 of 176 (88%) tumours showed EGFr immunoreactivity ranging from 0.2 to 90% of positive cells (mean 36.2 ± 25.4). EGFr positivity was mostly localised in the tumour cell membranes, but some positivity was also detected in the cytoplasm of cancer cells (Figures 1A, B). The characteristics of the patients according to clinicopathological parameters are summarised in Table 1. As shown in Table 2, the mean of EGFr positive cells was significantly higher in squamous carcinomas (42.1 ± 26.2) compared to adenocarcinomas (25.8 ± 21.5) and anaplastic large cell carcinomas (33 ± 21.1) ($P = 0.0009$). By contrast, no significant statistical association was found among EGFr expression and other clinicopathological parameters such as tumour size, tumour stage, nodal status and tumour differentiation (Table 3).

Correlation between EGFr immunoreactivity and nodal status in squamous lung tumours

As EGFr is highly expressed in the squamous subtype of NSCLC, we evaluated whether the EGFr immunoreactivity can be an indicator of advanced disease. 75 tumours (72%) without hilar and/or mediastinal nodal involvement showed a significantly lower EGFr expression compared with 29 tumours (28%) with nodal metastasis (Table 4; $P < 0.01$).

EGFr expression and proliferative activity

The proliferative activity was evaluated in all cases for proliferating cell nuclear antigen expression (PCNA) with PC10 MAb and in 108 out of 176 for Ki-67 expression. We also evaluated the flow cytometric S-phase fraction in 47 out of 176 paraffin-embedded samples. PC10 and Ki-67 immunoreactivity was confined to the nuclei of neoplastic cells (Figures 1C, D), ranging from 0.1 to 80% and 0.1 to 60% respectively. As shown in Table 5 and Figure 2, no correlation was found between inherent proliferative activity data and EGFr expression, although the S-phase mean in EGFr positive tumours was higher than in EGFr negative tumours.

DISCUSSION

The most commonly used criteria to determine the prognosis of NSCLC are tumour size and extension and nodal status [25]. However, the outcome of patients with the same clinical stage and histological type may diverge considerably. Efforts are thus necessary to identify additional factors to be evaluated at the time of presentation, and which can predict outcome and help in choosing the best type of treatment.

EGFr overexpression has been reported to be a frequent feature in breast [2], gastric [4], colorectal [5], genito-urinary [6, 26], laryngeal [14] and thyroid [16] cancers. This alteration is also recognised by several authors in the majority of NSCLC [3, 7, 8, 9, 19, 27, 28]. In this study, the EGFr expression in 176 formalin-fixed and paraffin-embedded lung tumour samples was evaluated by a new MAb. The high level of EGFr found in this group of tumours was in agreement with the data reported by other authors who employed either immunohistochemical [3, 7, 29] or ligand binding assays [19]. In some cases, EGFr immunoreactivity was revealed not only in the neoplastic cells but also in the basal layer of the normal bronchial epithelium. This finding, already reported by more than one group [19, 29], suggests that these stem cells require EGF for normal mucosal cell proliferation and differentiation.

The significantly higher EGFr expression in our series of squamous cell carcinomas, confirms data from other groups [7, 17, 29, 30]. In our series of NSCLC, 89.5% squamous cancers were positive for EGFr. This higher expression in squamous than in non-squamous carcinomas is an expected result, EGF mainly being a factor which promotes proliferation and differentiation of epidermal-like tissues.

The EGFr overexpression has been found to be associated either with degree of invasion and poor differentiation, for example in genito-urinary carcinomas [6, 26, 31], or with bad prognosis and short survival, as reported in mammary [11, 13, 15], head and neck [14], gastric, and colonic [4] cancers. In contrast to this series of investigations, other authors [19] found no correlation between EGFr expression and clinical stage, although EGFr concentrations were higher in neoplastic than in normal tissues. Our study is the first report in which a large number of NSCLC have been evaluated immunohistochemically for the EGFr expression in routine material. We found that in the squamous subtype, the percentage of immunopositive neoplastic cells was significantly higher in tumours with nodal metastatic involvement (mean 47.8 ± 26.1) than those without this type of involvement (mean 33 ± 27.4). The mechanism by which tumours with high levels of EGFr more frequently metastasise is not well understood. However, one possible explanation is that tumour cell clones expressing more EGFr may be selected for growth, invasion and metastasis. As suggested, EGFr may be involved in tumour progression through an autocrine mechanism whereby tumour cells expressing receptors secrete the ligand which interacts with the receptor to further stimulate the growth [32]. The increased growth potential after supplementation of EGF both *in vivo*, using lung cancer cell lines [33], and *in vivo*, after implantation in nude mice [34] of human squamous cancers, reinforced this hypothesis. In addition, while definitive experimental proof is still lacking that EGFr is involved in spontaneous metastasis, there are some indications that EGFr activation can influence the interaction of cells with defined matrix component [35], or its activity may also directly influence cell–cell contact, another critical parameter known to define epithelial invasiveness [36].

Relationship between EGFr expression and proliferative activity

To date a significant correlation between EGFr expression and proliferative activity has been found in breast [12] and in prostate cancer [25]. In both studies, the authors reported that tumours expressing EGFr had a higher proliferative activity, analysed either immunohistochemically using Ki-67 MAb, or by studying cells in S-phase using flow cytometric analysis. In our series of lung cancers, no differences were observed in positive or negative EGFr tumours regarding the proliferative activity studied by immunohistochemical and flow cytometric S-phase analysis. However, EGFr expressing tumours showed a higher proportion of cells in S-phase, although the statistical difference was not reached. The discrepancy between our and Visakorpi's study may be due to the different organs, but in particular to the small number of tumours analysed for S-phase. Moreover, the EGFr expression does not always provide information on proliferation. Indeed, not all cell types demonstrate a biological response to the growth factor [37]. In some cases, EGFr expression is probably more related to the specific stage of differentiation.

EGF is an important growth promoting agent binding with the external domain of its receptor results in the initiation of a series of biochemical reactions which lead to DNA synthesis and cell growth [38]. The prominent reactions are the receptor

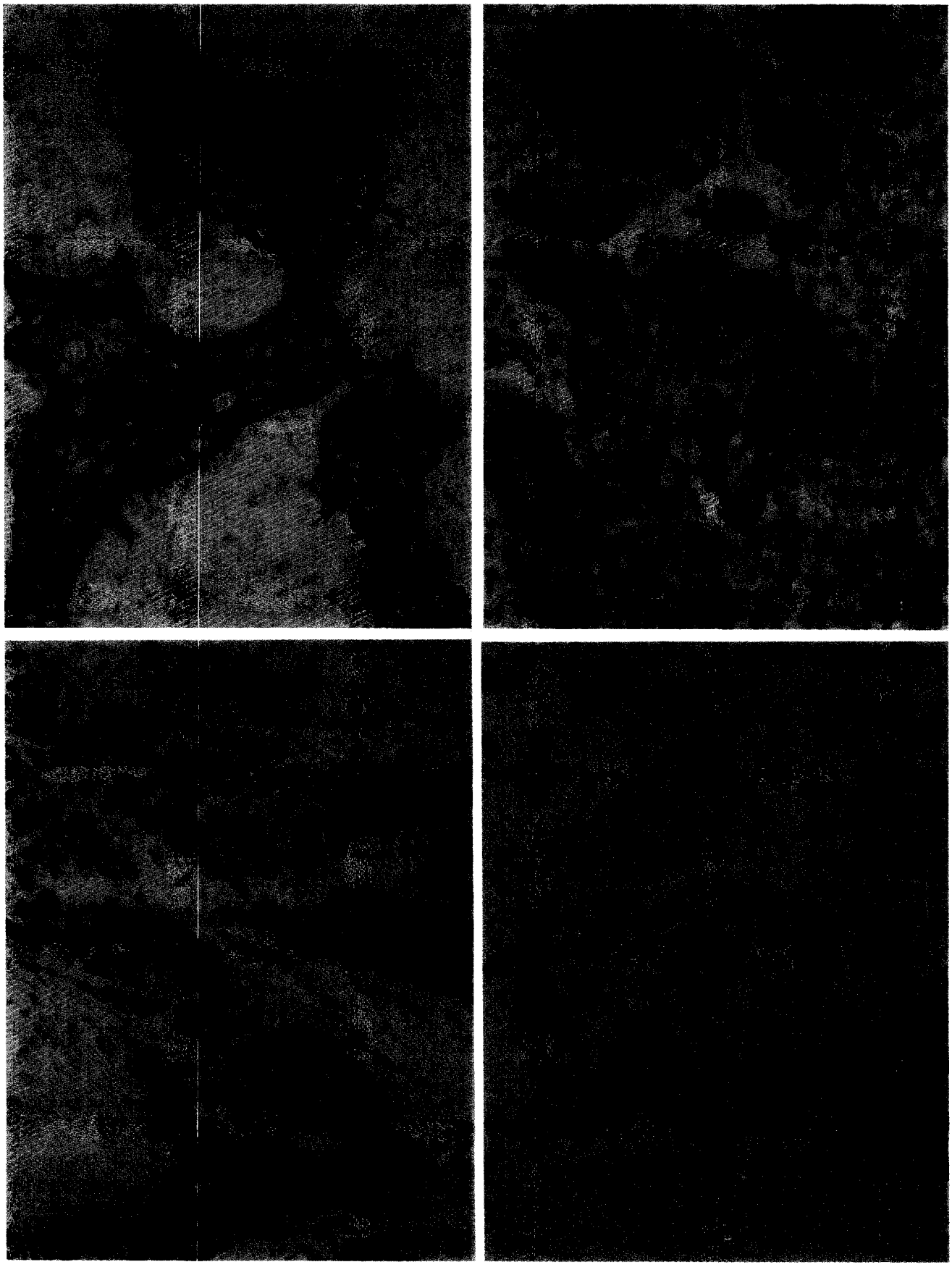

Figure 1. Differentiated squamous cell carcinoma of the lung, intense 31G7 immunoreactivity in the membrane (A, arrows) and in the cytoplasm (B, arrow) of neoplastic cells. Nuclear staining with PC10 (C) and Ki-67 (D) monoclonal antibodies, which recognise cell cycle-related antigens.

Table 1. Characteristics of 176 patients with NSCLC according to clinico-pathological parameters

Variables	Number of cases	Percentage
Sex		
Males	160	91
Females	16	9
Histology		
Squamous	105	60
Adenocarcinoma	59	34
Large cells	12	7
Tumour size		
≤3 cm	52	29.5
>3 cm	124	70.5
pNodal status		
N0	117	66
N1–2	59	34
pTumour status		
T1	38	21.5
T2	120	68
T3	18	10.2
pStage		
S1	109	62
S2	16	9
S3	51	29

Table 2. Relationship between EGFr expression and histology in 176 cases of NSCLC

Variable histology	Number of cases	EGFr Percentage of positive cells (mean ± SD)	P*
Squamous	105	42.1 (±26.2)	
Adenocarcinomas	59	25.8 (±21.5)	0.0009
Large cell anaplastic	12	33.0 (±21.1)	

*Kruskall-Wallis test.

Table 3. Relationship between EGFr immunoreactivity and clinico-pathological parameters in 176 cases of NSCLC

Variables	EGFr immunoreactivity Number of cases	Mean (±SD)	P*
Tumour size			
≤3 cm	52	30.6 ± 27.5	
			ns
>3 cm	124	32.4 ± 26.3	
pTumour stage			
S1	109	30.2 ± 26.6	
			ns
S2–3	67	34.5 ± 26.5	
pNodal status			
N0	117	29.3 ± 26.1	
			ns
N1–2	59	36.8 ± 27.0	

*Unpaired t-test.

Table 4. EGFr expression in squamous cell carcinomas (n = 105) according to nodal status

Variables	EFGr expression Number of cases	Percentage	Mean (±SD)	P†
pNodal status				
N0	75*	72.1	33 (±27.4)	
				0.01
N1–2	29	27.9	47.8 (±26.1)	

*In one case no data were available concerning lymph nodes status; †unpaired t-test.

Table 5. Proliferative activity according to EGFr expression in NSCLC

Proliferative activity	EGFr expression Positive	Negative	P†
Ki-67			
Number of cases	87	21	
Percentage of positive cells*	16.8 ± 14.3	21.1 ± 16.3	ns
PCNA			
Number of cases	155	21	
Percentage of positive cells*	30.9 ± 20.9	34.2 ± 22.6	ns
S-Phase			
Number of cases	40	7	
Percentage of positive cells*	23.3 ± 10.5	18 ± 5.3	ns

*The data are expressed as mean±SD; †unpaired t-test.

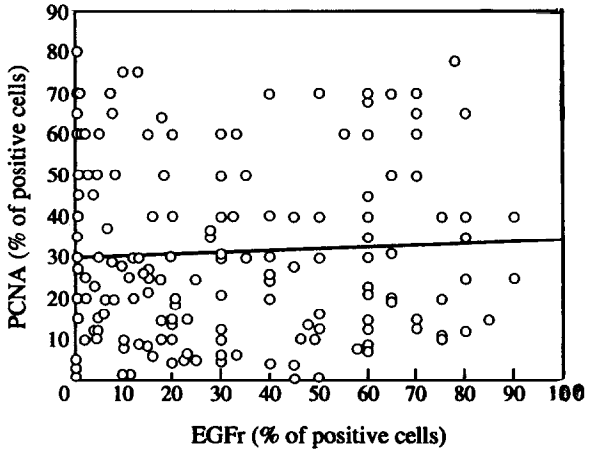

Figure 2. Linear regression analysis between the percentage of EGFr and PCNA immunoreactive cells. No correlation was found between the two variables ($r = 0.05$; $P = 0.4$).

autophosphorylation and cytosolic protein phosphorylation. Activation of the protein tyrosine kinase activity of the EGFr is believed to trigger this complex cascade of biochemical reaction [39].

In conclusion our results suggest that the EGFR over-expression may provide further information on NSCLC behaviour, although these must be confirmed by additional perspective studies in patients with complete follow-up data.

1. Mountain CF. The new international staging system for lung cancer. *Surg Clin North Am* 1987, **67**, 925–935.
2. Sainsbury JRC, Nicholson S, Angus B, Farndon JR, Malcolm AJ, Harris AL. Epidermal growth factor receptor status of histological sub-types of breast cancer. *Br J Cancer* 1988, **58**, 458–460.
3. Dazzi H, Hasleton PS, Thatcher N, *et al.* Expression of epidermal growth factor receptor (EGF-r) in non-small cell lung cancer. Use of archival tissue and correlation of EGF-r with histology, tumor size, node status and survival. *Br J Cancer* 1989, **59**, 746–749.
4. Yasui W, Sumiyoshi H, Hata J, *et al.* Expression of epidermal growth factor receptor in human gastric and colonic carcinomas. *Cancer Res* 1988, **48**, 137–141.
5. Kaklamanis L, Gatter KC, Mortensen N, Harris AL. Interleukin-4 receptor and epidermal growth factor receptor expression in colorectal cancer. *Br J Cancer* 1992, **66**, 712–716.
6. Smith K, Fennelly JA, Neal DE, Hall RR, Harris AL. Characterization and quantitation of the epidermal growth factor receptor in invasive and superficial bladder tumors. *Cancer Res* 1989, **49**, 5810–5815.
7. Hendler FJ, Ozanne BW. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J Clin Invest* 1984, **74**, 647–651.
8. Veale D, Kerr N, Gibson GJ, Harris AL. Characterization of epidermal growth factor receptor in primary human non-small cell lung cancer. *Cancer Res* 1989, **49**, 1313–1317.
9. Cerny T, Barnes DM, Hasleton P, *et al.* Expression of epidermal growth factor receptor (EGF-R) in human lung tumors. *Br J Cancer* 1986, **54**, 265–269.
10. Gargoulis V, Aninos D, Mikou P, *et al.* Expression of EGF, TGF-alfa and EGFR in squamous cell lung carcinomas. *Anticancer Res* 1992, **12**, 1183–1188.
11. Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ, Harris AL. Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet* 1987, **20**, 1398–1402.
12. Toi M, Nakamura T, Mukaida H, *et al.* Relationship between epidermal growth factor receptor status and various prognostic factors in human breast cancer. *Cancer* 1990, **65**, 1980–1984.
13. Nicholson S, Richard J, Sainsbury C, *et al.* Epidermal growth factor receptor (EGFr); results of a 6-year follow-up study in operable breast cancer with emphasis on the node negative subgroup. *Br J Cancer* 1991, **63**, 146–150.
14. Maurizi M, Scambia G, Benedetti Panici P, *et al.* EGF receptor expression in primary laryngeal cancer: correlation with clinicopathological features and prognostic significance. *Int J Cancer* 1992, **52**, 862–866.
15. Bolla M, Chedin M, Colonna M, Marron J, Rostaing-Puissant B, Chambaz E. Prognostic value of epidermal growth factor receptor in a series of 303 breast cancers. *Eur J Cancer* 1992, **28**, 1052–1054.
16. Akslen LA, Myking AO, Salvensen H, Varhaug JE. Prognostic impact of EGF-receptor in papillary thyroid carcinoma. *Br J Cancer* 1993, **68**, 808–812.
17. Volm M, Drings P, Wodrich W. Prognostic signifiance of the expression of c-fos,c-jun and c-erbB-1 oncogene products in human squamous cell lung carcinomas. *J Cancer Res Clin Oncol* 1993, **119**, 507–510.
18. Hunter T, Cooper JA. Epidermal growth factor induces rapid tyrosine phosphorylation of protcins in A-431 human tumor cells. *Cells* 1981, **24**, 741–752.
19. Dittadi R, Gion M, Pagan V, *et al.* Epidermal growth factor receptor in lung malignancies. Comparison between cancer and normal tissue. *Br J Cancer* 1991, **64**, 741–744.
20. Veale D, Kerr N, Gibson JK, Kelly PJ, Harris AL. The relationship of quantitative epidermal growth factor receptor expression in non-small cell lung cancer to long-term survival. *Br J Cancer* 1993, **68**, 162–165.
21. Volm M, Efferth T, Mattern J. Oncoprotein (c-myc, c-erb-B1, c-erb-B2, c-fos) and suppressor gene product (p53). Expression in squamous cell carcinomas of the lung. Clinico-pathological correlations. *Anticancer Res* 1992, **12**, 11–20.
22. World Health Organization: The World Health Organization histologic typing of lung tumours. *Am J Path* 1982, **77**, 123–136.
23. Dean PN. Data analysis and cell cycle kinetics research. In Gray JW, Darzynkiewicz Z, eds. *Techniques in the Cell Cycle Analysis*. Clifton, New Jersey, Human Press, 1987, 386–398.
24. Hedley DW, Friedlander ML, Taylor IW. Application of DNA flow cytometry to paraffin-embedded archival material for the study of the aneuploidy and its clinical significance. *Cytometry* 1985, **6**, 327–333.
25. Naruke T, Goya T, Tsuchiya R, Suemasu K. Prognosis and survival in resected lung carcinomas based on the new international staging system. *J Thorac Cardiovasc Surg* 1988, **96**, 440–447.
26. Visakorpi T, Kallionemi OP, Kalvula T, Harvey J, Isola J. Expression of epidermal growth factor receptor and ERB-B2 (HER-2/Neu) oncoprotein in prostatic carcinomas. *Mod Pathol* 1992, **5**, 643–648.
27. Berger HS, Gullick WJ, Greenfield C, Evans S, Addis BJ, Waterfield MD. Epidermal growth factor receptor in lung tumours. *J Pathol* 1987, **152**, 297–307.
28. Hwang DL, Tay J, Lin SS, Lev-Ran A. Expression of epidermal growth factor receptors in human lung tumors. *Cancer* 1986, **58**, 2260–2263.
29. Veale D, Ashcroft T, Marsh C, Gibson GJ, Harris AL. Epidermal growth factor receptor in non small cell lung cancer. *Br J Cancer* 1987, **55**, 513–516.
30. Gargoulis V, Giatromanolaki A, Karameris A, *et al.* Epidermal growth factor receptor expression in squamous cell lung carcinomas: an immunohistochemical and gene analysis in formalin-fixed, paraffin-embedded material. *Virchows Arch A Pathol Anat* 1993, **423**, 295–302.
31. Neal DE, Marsh C, Bennett MK, *et al.* Epidermal growth factor receptors in human bladder cancer: comparison of invasive and superficial tumours. *Lancet* 1985, **16**, 366–368.
32. Sporn MB, Todaro GJ. Autocine secretion and malignant transformation of cells. *New Engl J Med* 1980, **303**, 878–880.
33. Singletary SE, Baker FL, Spitzer GG, *et al.* Biological effect of epidermal growth factor on the *in vitro* growth of human tumors. *Cancer Res* 1987, **47**, 403–406.
34. Ozawa S, Ueda M, Ando N, Abe O, Hirai M, Shimizu N. Stimilation by EGF of the growth of EGF receptor-hyperproducing tumor cells in athymic mice. *Int J Cancer* 1987, **40**, 706–710.
35. Basson MD, Modlin IM, Madri JA. Human enterocyte (Caco-2) migration is modulated *in vitro* by extracellular matrix composition and epidermal growth factor. *J Clin Invest* 1992, 15–23.
36. Behrens J, Frixen U, Schipper J, Weidner M, Birchmeier W. Cell adhesion in invasion and metastasis. *Semin Cell Biol* 1992, **3**, 169–178.
37. Carpenter G, Zandegui JG. Epidermal growth factor, its receptor and related proteins. *Exp Cell Res* 1986, **164**, 1–5.
38. Carpenter G, Cohen S. Epidermal growth factor. *Ann Rev Biochem* 1979, **48**, 193–196.
39. Lokeshar VB, Huang SS, Huang JS. Protamine enhances epidermal growth factor (EGF)-stimulated mitogenesis by increasing cell surface EGF receptor number. *J Biol Chem* 1989, **264**, 19318–19326.

Acknowledgements—This work is supported by the Italian Association for Cancer Research (A.I.R.C.); Dr D. Bigini is an A.I.R.C. Fellow. We are indebted to Mr Ascolo Vannuzzi for his invaluable technical assistance.