

## EGFR, c-erbB-2 and Ki-67 in NSCLC and Preneoplastic Bronchial Lesions

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**Abstract.** *Background: The relationships between EGF-R and c-erbB-2 with other factors involved in tumour regulation are not well understood. The aim of this study was to correlate the expression of these markers with tumour proliferation. Materials and Methods: The presence of EGF-R, c-erbB-2 and Ki-67 was evaluated by immunohistochemistry in non-small cell lung cancer (NSCLC) and preneoplastic lesions. Results: Forty-two percent of the tumours were positive for EGF-R, 22% for c-erbB-2 and 97% for Ki-67. No statistically significant correlation was found between EGF-R and Ki-67, EGF-R and c-erbB-2 or between c-erbB-2 and Ki-67. With regards to Ki-67, a significant difference in survival was noted in favour of patients who did not express the marker. In preneoplastic lesions, most of the low-grade lesions showed neither EGF-R nor Ki-67 staining. In contrast, most of the high-grade lesions stained positively for these proteins. Conclusion: EGF-R and c-erbB-2 do not seem to be correlated with Ki-67 in NSCLC.*

The EGF family plays an important role in local tumour growth. This phenomenon requires growth regulating proteins, such as epidermal growth factor receptor (EGF-R) or c-erbB-2. The binding of one ligand to the extracellular region of one of those receptors implies its dimerisation with another member of the EGF family, resulting in autophosphorylation and activation of cytoplasmic signal proteins transmitting mitogenic signals (1). EGF-R also plays a role in cell motility, adhesion, invasion and angiogenesis (2). In non-small cell lung cancer (NSCLC), EGF-R overexpression is reported in 13 to 80% of tumours (24 to 89% of squamous cell cancer and 23 to 46% of

adenocarcinoma). The c-erbB-2 protein product, p185, is expressed in 20 to 30% of NSCLC. These two proteins seem to play an important role in NSCLC progression. However, their relationships with other factors involved in tumour regulation are still not well understood.

Based on those considerations, the present study was undertaken to correlate the EGF-R and c-erbB-2 protein expressions with tumour proliferation estimated by Ki-67 expression. The expressions of these markers were evaluated by immunohistochemistry in a series of resected NSCLC and in bronchial preneoplastic lesions.

### Materials and Methods

*Study population.* From November 1992 to July 2000, 129 consecutive patients were treated by wedge resection, lobectomy or pneumonectomy for lung carcinoma in our Department of Thoracic Surgery (CHU St Pierre, Brussels, Belgium). Out of these 129 patients, 25 had to be excluded because of lack of tissue samples available for the 3 immunostainings. After surgical resection, all tissues were fixed in 10% neutral buffered formalin and routinely embedded with paraffin.

Moreover, 124 eligible consecutive biopsies from 124 different patients, that had undergone laser-induced fluorescence (LIFE) bronchoscopy between February 1996 and October 2001, were reviewed. Out of those 124 eligible biopsies, 94 patients provided sufficiently large biopsies to evaluate EGF-R expression, 55 for Ki-67 and only 22 for both markers.

*Immunohistochemical studies.* All the reagents were of analytic quality and were used without any preliminary purification. Methanol, citric acid, sodium citrate, tris(hydroxymethyl)aminomethane (TRIS) and hydrochloridric acid were purchased from Merck (Darmstadt, Germany). Immunohistochemistry was performed according to a standard avidin-biotin-peroxidase complex.

For EGF-R, immunohistochemistry was performed using a mouse monoclonal antibody directed against the external domain of EGF-R (IgG2a; NCL – EGFR; clone EGFR.113) from Novocastra Laboratories (Newcastle-Upon-Tyne, UK) with a cellular membrane staining. For Ki-67 immunostaining, murine monoclonal antibodies directed against the Ki-67 antigen (clone MIB-1 from Neomarkers, Union City, USA) giving a nuclear staining were used. For c-erbB-2 immunostaining, a mouse

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monoclonal antibody directed to the internal domain of c-erbB-2 (clone CB11 from Novocastra) was used for labelling the cellular membrane.

Five- $\mu$ m-thick sections cut from paraffin-embedded tissues were deparaffinised in xylene and rehydrated in decreasing concentrations of ethanol.

For Ki-67 and c-erbB-2, the slides were submitted to antigen retrieval in 0.01 M citrate buffer pH 6 consisting of 3 x 5-min microwave treatments at 600 W for Ki-67 and 5 x 5-min treatments at 650 W for c-erbB-2. The slides were cooled for 25 min at room temperature and then rinsed twice in 0.005 M TRIS-HCl, pH 7.6, in 0.9% NaCl for 10 min. All the following steps were performed automatically at 37°C in the NexES system (Ventana Medical Systems, Tucson, AZ, USA). The endogenous peroxidases were quenched by incubation in 0.3% hydrogen peroxide in methanol for 4 min at room temperature. The antibodies anti-Ki-67 (dilution 1/50, final titration 1.9  $\mu$ g/ml) or anti-c-erbB-2 (dilution 1/30, final titration 0.9  $\mu$ g/ml) were deposited and incubated for 30 min.

For EGF-R, the endogenous peroxidases were quenched by incubation in 0.3% hydrogen peroxide in methanol for 30 min at room temperature and the slides were rinsed twice in 0.005 M TRIS-HCl pH 7.6 in NaCl 0.9% for 10 min. They were then submitted to antigen retrieval in 0.01 M citrate buffer pH 7 consisting of 3 x 5-min microwave treatments at 650 W. The slides were cooled for 25 min at room temperature. The antibodies anti-EGF-R (dilution 1/20, final titration 5  $\mu$ g/ml) were incubated for 60 min at 37°C. All the following steps were performed automatically at 37°C in the NexES system (Ventana Medical Systems).

The protein-antibody complex was fixed with 0.05% glutaraldehyde in 0.9% NaCl. The secondary biotinylated antibody was incubated for 8 min. The slides were stained with a diaminobenzidine (DAB) detection kit (Ventana Medical Systems) and counterstained with haematoxylin.

The negative controls were carried out by omitting the primary antibody and also by substituting normal mouse immunoglobulin (Ig G2a for EGF-R or Ig G1 for Ki-67 and c-erbB-2 for the primary antibody). The positive controls were known positive lung carcinoma and placental tissue for EGF-R and known positive breast cancer for Ki-67 and c-erbB-2.

*Evaluation of the staining.* Four observers (A.P.M., J.M.V., B.M., C.M.) evaluated the results of the immunohistochemical staining without any knowledge of the clinical data.

The level of positivity was expressed as the percentage (0-100%) of tumour cells showing membrane (for EGF-R) or nuclear (for Ki-67) staining and the slides were considered as positive for the marker when  $\geq 1\%$  of cells were stained (3).

For c-erbB-2, the classification applied in breast cancer and accepted by the FDA was used. The immunoreaction was determined as weakly positive (2+) if more than 10% of the tumour cells showed weak to moderate complete membrane staining, or as strongly positive (3+) if a strong complete membrane staining was observed in more than 10% of the tumour cells. All other staining patterns were interpreted as negative (0 - 1+).

For preneoplastic lesions, the bronchial lesion staining was scored as follows for EGF-R (4): 0 = no staining or staining of the basal cell layer only; 1+ positive = staining of cells in the basal and suprabasal regions (maximum the lower third of the epithelium); 2+ positive = staining of cells in the lower two-thirds of the

epithelium; 3+ positive = staining cells in all the epithelium. For Ki-67, as described previously, the presence of Ki-67-positive nuclear clusters was assessed (5). A cluster was defined as a group of at least 2 contiguous cells showing strongly Ki-67-positive nuclei located in the upper third of the pathological epithelium.

*Statistical analysis.* Statistical analysis was performed with non-parametric tests. The rate of positivity between two binary variables was compared by Mc Nemar tests. The correlation between continuous variables was assessed by Spearman rank correlation coefficients. The values of the distributions of the biological variables were compared according to histology by the Mann-Whitney test. Survival was measured from the date of surgery and survival distribution was estimated by the Kaplan-Meier method. Survival comparisons were performed by two-sided log rank tests. The criterion for statistical significance was  $p < 0.05$ .

## Results

The patient population with resected tumours consisted of 86 males (83%) and 18 females (17%), with a median age of 62 years (range: 27 - 86 years). The histology, according to the new World Health Organisation (WHO) 1998 classification (6), was adenocarcinoma in 49 patients, squamous cell carcinoma in 47 and other NSCLC in 8. Sixty-two patients had stage I disease (59%), 26 stage II (25%), 15 stage III (14%) and 1 stage IV (due to the presence of 2 tumours in the same lung) (1%), according to the 1997 International Staging System (7).

The median expression of EGF-R was 0% (range 0 - 100%) and was higher ( $p < 0.001$ ) in squamous cell cancer (7%, range 0-100%) than in adenocarcinoma (0%, range 0 - 100%). Forty-two percent of the tumours were positive for EGF-R.

Twenty-two percent of the tumours were positive for c-erbB-2 with a statistical difference between adenocarcinoma and squamous cell carcinoma (30% versus 15%;  $p = 0.05$ ).

The median expression of Ki-67 in NSCLC was 20% (range 0 - 92%) and was higher ( $p < 0.001$ ) in squamous cell carcinoma (33.3%, range 0 - 90%) than in adenocarcinoma (8.3%, range 0-92%); 97% of the tumours were positive for Ki-67.

If we consider the variables as continuous, no correlation between EGF-R and Ki-67 ( $R = 0.0026$ ;  $p = 0.97$ ) was found. In addition, if we consider the markers as binary variables, discordances between EGF-R and Ki-67 ( $p < 0.001$ ), between EGF-R and c-erbB-2 ( $p < 0.001$ ) and between c-erbB-2 and Ki-67 ( $p < 0.001$ ) were found with the Mc Nemar tests.

The median follow-up was 48 months. Among the 104 patients, 62 had died at the time of analysis. The median survival time (MST) was 53 months. No significant differences in terms of survival distribution according to EGF-R (49 vs. 55 months MST for EGF-R-positive vs. -negative patients;  $p = 0.42$ ) or c-erbB-2 (18 vs. 55 months MST for c-erbB-2-positive vs. -negative patients;  $p = 0.76$ )

were observed. However, a significant difference was found for Ki-67 expression (54 vs. 95 months MST for Ki-67-positive vs. -negative patients;  $p < 0.005$ ).

Among the preneoplastic lesions, 2 metaplasia, 4 mild dysplasia, 6 severe dysplasia and 10 carcinoma *in situ* were assessable for both EGF-R and Ki-67. In the metaplastic lesions, no cell was found positive for EGF-R or for Ki-67 in the upper third of the epithelium. In the 4 mild dysplasia, 1 patient had Ki-67 clusters in the upper third of the epithelium but none showed EGF-R-positive cells. In severe dysplasia, 5 out of the 6 lesions had Ki-67 clusters, but only 3 of them had EGF-R-positive cells in the upper third of the epithelium (2 with Ki-67 clusters and 1 without Ki-67 clusters). Finally, in carcinoma *in situ*, all the lesions had Ki-67 clusters and 8 out of 10 had EGF-R-positive cells in the upper third of the epithelium.

## Discussion

The present results confirm a higher expression of EGF-R (8) and Ki-67 (9) and a lower expression of c-erbB-2 (10) in squamous cell carcinoma as compared to adenocarcinoma. No correlation was observed between EGF-R expression in NSCLC and tumour proliferation index (estimated by expression of Ki-67), or with another member of the EGF-R family (c-erbB-2). These results were the same regardless of the method of assessing the expressions of the different markers (as dichotomic variables or as continuous variables), suggesting that there was no bias due to the choice of the cut-off.

These results also suggest a lack of direct interaction of EGF-R and c-erbB-2 with cellular proliferation when evaluated by Ki-67. One potential bias has, nevertheless, to be considered: Ki-67 estimates a measure of cellular proliferation index, but this index is not the only parameter determining tumour proliferation, since the duration of the cellular cycle and the cell death rate are other important factors for tumour growth. Ki-67 only evaluates the intrinsic cellular proliferation. Other methods which assess proliferation, such as the study of thymidine kinase 1 (TK1), a key enzyme involved in the synthesis of DNA precursors and thus cell proliferation-dependent, could be a more reliable marker (11). In a previous study, Dazzi *et al.* (12) showed that tumours with a low proliferation index (as evaluated by flow cytometry) were significantly more often EGF-R-positive. On the other hand, EGF-R and PCNA (the proliferating cell nuclear antigen) expressions were found to be similar in squamous cell carcinoma in the study by Yoneda (13). Ki-67 expression does not seem to reflect EGF-R-driven proliferation. Moreover, the implication of EGF-R on angiogenesis, cell motility and invasion favouring tumour proliferation is not evaluated by Ki-67 immunostaining. The impact of EGF-R on cellular proliferation could be more

important and more directly associated in the first steps of lung carcinogenesis, its other role predominant in later stages. In terms of preneoplastic lesions, most of the low-grade lesions (metaplasia and mild dysplasia) showed no EGF-R or Ki-67 staining. In contrast, most of the high-grade lesions (severe dysplasia and carcinoma *in situ*) showed Ki-67 (15/16) and EGF-R staining (11/16). However, the concordance was not perfect. No correlation was performed with c-erbB-2, since we had previously demonstrated that there was no c-erbB-2 expression in preneoplastic lesions (14).

The lack of correlation between EGF-R and c-erbB2 has already been described by other authors. According to Giatromanolaki *et al.* (15), 18% of cases with EGF-R reactivity overexpressed c-erbB-2, 22% of EGF-R-negative cases were positive for c-erbB-2 and 17% were negative for both markers without significant correlation. Similarly, in the Reinmuth *et al.* (16) and Kanematsu *et al.* (17) studies, no significant correlation was noted between c-erbB-1 and c-erbB-2. Franklin *et al.* (18) found 22% of NSCLC with high c-erbB-1 and high c-erbB-2, 18% of NSCLC with low c-erbB-1 and c-erbB-2 and 49% of NSCLC with high c-erbB-1 and low c-erbB-2. For Tateishi *et al.* (19), lung cancer patients whose tumours overexpressed EGF-R and c-erbB-2 seemed to have a worse survival than patients with only c-erbB-1 overexpression: this co-expression occurred in 13% of their 119 adenocarcinoma and was greater in patients with metastasis. On the other hand, overexpression of both receptors was not a prognostic factor in the study of Pfeiffer *et al.* (20), including chemotherapy-naïve patients with stage I-IV NSCLC. In 73 stage I NSCLC, Lai *et al.* (21) carried out immunohistochemical analyses of the expression of the 4 EGF-R family members: 66 and 38% of the patients expressed c-erbB-1 and 2, respectively. C-erbB-1 was co-expressed with c-erbB-2 in 26% of the patients.

In terms of survival, our analysis did not detect any significant difference for EGF-R or c-erbB-2. This could be explained, in part, by the small number of patients included in this study. Indeed, meta-analyses of the literature, allowing for the analysis of a large number of cases, have shown that EGF-R (22), c-erbB-2 (23) and Ki-67 (24) could be negative prognostic factors for survival in NSCLC. The need for a meta-analysis to demonstrate this impact and the fact that the hazard ratios were not high may show that the prognostic role of these factors are probably not very important. Moreover, the impact of Ki-67 on survival should be interpreted with caution, since only 3% of the patients were Ki-67-negative.

In conclusion, our data suggest that the regulation of tumour growth by EGF-R and c-erbB-2 does not seem to be directly dependent on the cellular proliferation estimated by Ki-67.

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