C-erbB-3 Expression in Non-small Cell Lung Cancer (NSCLC) Patients Treated by Erlotinib

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Abstract. *Background: The clinical impact of c-erbB-3 has seldom been assessed in patients with non small cell lung cancer (NSCLC). Patients and Methods: Forty-three NSCLC patients treated by erlotinib for c-erbB-3 and EGFR expression were investigated by immunohistochemistry analysis. Results: Two partial responses, one minor response, two stable diseases and twenty progressive diseases were observed at the first evaluation. Seventeen patients died before evaluation. Median EGFR expression was 70% of the cancer cells. Forty-two percent of the tumours co-expressed c-erbB-3 and EGFR without any difference according to histology or disease stage. There was no correlation between c-erbB-3 and EGFR expression. Median survival time was 2.6 months and the six months survival rate was 21%. There was no detectable impact of EGFR (p=0.94) or c-erbB-3 (p=0.93) expression on survival. Conclusion: In this small particular cohort of NSCLC patients receiving salvage therapy with erlotinib, there was no correlation between c-erbB-3 expression and clinical parameters, nor between cerbB-3/EGFR expression and outcome.*

In non-small cell lung cancer (NSCLC) patients, EGFR expression and mutation have been studied extensively; the role of c-erbB-2 has also been evaluated. The impact of other EGFR family members such as as c-erbB-3 and -4 has seldom been assessed. Koutsopoulos *et al*. (1) observed a marginally significant decreased survival when c-erbB-3 receptor is strongly overexpressed in NSCLC, while only 3.5% of the NSCLC specimens co-expressed c-erbB-1 and c-erbB-3. Fontanini *et al*. described up to 85% c-erbB-3 expression in stage I-IIIA NSCLC and co-expression with EGFR in 50% of the cases with no impact on death rate (16).

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Lai *et al*. evaluated the expression of the 4 EGFR family members by immunohistochemistry in 73 patients with stage I NSCLC: 66, 38, 64 and 78% of the patients expressed c-erbB-1, 2, 3 and 4, respectively (17). C-erbB-1 was co-expressed with c-erbB-2 in 26%, with c-erbB-3 in 49% and with c-erbB-4 in 58% of the patients. Patients with a non-well-differentiated tumour and an overexpression of c-erbB-3 had higher levels of recurrence. In some studies, patients with high c-erbB-3 expression survived for shorter periods than did those with low c-erbB-3-expression in stages III and IV, but not in stage I or II NSCLC (2, 3) but not in other publications (3).

The epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKI: gefitinib and erlotinib) have been extensively studied in clinical trials in recent years, especially in advanced NSCLC. *EGFR* mutation is a predictive factor for response to EGF-R TKI (4). EGF-R TKI sensitivity is not only directly dependent on EGFR but also influenced by other family members such as c-erbB-2 (5). Signalling through c-erbB-3 is different from that of the other members of the erbB family, since c-erbB-3 has no tyrosine kinase activity, but directly activates phosphatidyl inositol 3 kinase (PI3K). EGFR-mediated activation of Akt requires the activation of PI3K through a dimerisation of EGFR and c-erbB-3 (6). Preclinical data suggests that gefitinib inhibits cell proliferation by sequestration of c-erbB-2 and -3 in an inactive heterodimer configuration with EGFR (7). Moreover, the expression status of other erbB family members, such as c-erbB-3, seems to modulate the efficacy of EGFR targeting agents in NSCLC cell lines (8). For example, when Engelman *et al*. treated gefitinib-resistant cells (because of a focal amplification of the MET proto-oncogene) using monotherapy with gefitinib or PHA665752 (Pfizer), a MET inhibitor, there was no down-regulation of c-erbB-3/PI3K/AKT signalling and no suppression of cell growth. However, combined therapy with both gefitinib and PHA665752 resulted in a clear decrease in phosphorylation of c-erbB-3, AKT, and MET, with growth suppression (9). Therefore, as c-erbB-3 is kinase inactive, it is not a direct

target of kinase inhibitors and not an easy target for treatment, but c-erbB-3 may have a role in mediating resistance to inhibitors of EGFR and c-erbB-2. In light of these preclinical data, clinical trials directed at dual MET and EGFR inhibition are underway. In the same way, pan-erbB tyrosine kinase inhibitors, such as CI-1033, are under development (10).

The clinical impact of c-erbB-3 in NSCLC patients treated by erlotinib has not been well evaluated. We investigated c-erbB-3 expression by immunohistochemistry and its correlation with EGFR expression, and its predictive value response and prognostic role in NSCLC patients treated by erlotinib.

Patients and Methods

Study population. Forty-three patients with advanced NSCLC who had been treated with erlotinib (150 mg/day *per os*) at the Jules Bordet Institute and for whom biopsies were available for c-erbB-3 assessment were recruited into the study. Erlotinib was administered at 150 mg per day *per os* until progression, death or unacceptable toxicity. Complete work-up was carried out every three months to assess response.

Immunohistochemistry. 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 obtained from Merck (Darmstad, Germany). Immunohistochemistry was performed according to a standard avidin-biotin-peroxidase complex.

For c-erbB-3 immunostaining, the slides were deparaffinised in xylene (2×10 min) and rehydrated in ethanol (100%, 95% and 70%). They were then submitted to antigen retrieval in EDTA buffer pH 8, with two microwave treatments of 10 min duration at 650 W. The slides were cooled for 20 min at room temperature. The endogenous peroxidases were quenched by incubation in 3% hydrogen peroxyde in water for 10 min at room temperature. A total volume of 100 μl of goat serum (from Sigma, St Louis, USA; dilution 1/10) was incubated on each slide for 1 h at 4˚C. Blocking reagents (kit from Ventana, Medical Systems, Tucson, AZ, USA) were used for 5 min at 37˚C. The mouse monoclonal antibody RTJ.2 (from SantaCruz Biotechnology Inc., Santa Cruz, USA) mapping within the cytoplasmic domain of c-erbB-3 (100 μl/slides; dilution 1/50) was incubated on the tissue for 60 min at room temperature. The complex between the protein and its antibody was fixed with glutaraldehyde NaCl 0.9%. The secondary biotinylated antibody was incubated for 8 min. The slides were stained with 100 μl diaminobenzidine (DAB) detection kit (DAKO, Glostrup, Denmark) for 5 min and counterstained with haematoxylin for 5 min. Negative controls were carried out by omitting the primary antibody. The positive external control was renal parenchyma.

For EGFR immunostaining, 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 TRIS-HCl 0.005 M NaCl 0.9% pH 7.6 for 10 min. They were then submitted to antigen retrieval in citrate buffer 0.01 M pH 7 consisting of three microwave treatments of 5 min at 650 W. The slides were cooled for 25 min at room temperature. The mouse monoclonal antibody directed against the external domain of EGFR (clone EGFR.113 from Novocastra Laboratories, Newcastle-Upon-Tyne, UK) giving a cytoplasmic and membrane staining (dilution 1/20, final titration 5 μg/ml) was incubated for 60 min at 37˚C. All the next steps were performed automatically at 37˚C in a NexES system (Ventana Medical Systems, Tucson, AZ, USA). The complex between EGFR and its antibody was fixed using glutaraldehyde NaCl 0.9%. The secondary biotinylated antibody was incubated for 8 min. The slides were stained with DAB detection kit (Ventana Medical Systems, Tucson, AZ, USA) and counterstained with haematoxylin. Negative controls were performed by omitting the primary antibody and also by substituting normal mouse immunoglobulin G2α for the primary antibody. The positive controls were known EGFR-positive lung carcinoma.

Three observers (APM, CM, IT) independently evaluated the slides. The results were compared and discordant interpretations were resolved by review of the specific slides by the three observers at a multihead microscope. Results were expressed as a percentage of stained tumour cells (semi-quantitative measure). A tumour was considered positive for EGFR and/or c-erbB-3 when more than 10% of the tumour membrane cells were stained.

Statistical methods. c-erbB-3 was analyzed both as a continuous and binary (positive or negative expression) variable. Bilateral chi-square tests were used to compare c-erbB-3 expression according to histology and disease stage or other baseline categorical covariates. Fisher test was used to evaluate the relationship between response to erlotinib and c-erbB-3 expression. Spearman test was used to study the correlation between EGFR and c-erbB-3 as continuous measures. Survival distributions were estimated by the Kaplan-Meier method and compared by the logrank test. Survival was estimated from the first day of erlotinib treatment. A p-value ≤5% was considered as significant.

Results

The 43 eligible patients consisted of 26 men and 17 women. The median age was 60 (range: 38-84) years. Nine patients had squamous cell carcinoma, 29 adenocarcinoma and 5 other types of NSCLC. Disease stages were I/III/IV in 1, 12 and 30 patients, respectively. Karnofsky performance status was 50/60/70/80/90 in 8/14/9//11/1 patients, respectively. Twenty-four of the patients were smokers, 15 ex-smokers and 4 non-smokers. Erlotinib was administered in second/third or fourth line treatment in 22/18 and 3 of the cases. Median duration of treatment was 47 days (range: 20-144).

As required in Belgium for erlotinib treatment, all the patients had an EGFR-positive tumour. The median percentage of tumour cells expressing EGFR was 70% (ranging from 10 to 100%, mean 63%, SD 36%). Fifteen tumours were positive for c-erbB-3 expression. The median c-erbB-3 expression was 0% (mean 20%, SD 32%; range 0 - 100%). There was no difference in terms of c-erbB-3 expression according to gender, age, performance status, stage, histology or smoking status (Table I). Forty-two percent of the tumours co-expressed c-erbB-3 and EGFR but there was no linear correlation detected between EGFR and c-erbB-3 expressions (R=0.09; p=0.55) (Figure 1).

At the first evaluation, there were two cases of partial response, one case of minor response, two cases of stable diseases and twenty cases of progressive diseases. Seventeen patients died before evaluation (16 early deaths due to malignancy and 1 intercurrent death). One patient stopped treatment due to skin toxicity. The characteristics of the five non-progressive patients are given in Table II. We found no statistical difference in term of response/stabilization of the disease between patients with or without c-erbB-3 (p=0.64) or EGF-R (p=1) expression.

At time of analysis, all but one patient were dead. Median survival was 2.6 (95% CI: 1.7-3.5) months. 6-month survival rate was 21% (95% CI: 9%-33%). There was no impact of EGFR (p=0.94) nor c-erbB-3 expression (p=0.93) on survival.

Table I. *c-erbB-3 positivity according to clinical characteristics.*

Karnofsky score		
≤70	29%	p=0.29
≥80	50%	
Gender		
Male	35%	p=1
Female	35%	
Median age (years)		
62	Tumor negativity	p=0.35
57	Tumor positivity	
Cancer stage		
Non metastatic	46%	p=0.32
Stage IV	30%	
Smoking history		
Non smoker	25%	p=1
Ex-smoker or smoker	36%	
Histology		
Adenocarcinoma	31%	p=0.51
Other NSCLC	43%	

Discussion

In this small particular cohort of heavily treated NSCLC patients receiving salvage therapy with erlotinib, 42% of the tumours had c-erbB-3 expression. Moreover, there was no correlation between c-erbB-3 expression and clinical parameters, no linear correlation between cerbB-3/EGFR expression, response to erlotinib and other outcomes (non progression to erlotinib and survival).

NSCLC patients harboring *EGFR* mutation are highly responsive to TKI therapy (11). The acquisition of TKI resistance in these patients appears to be correlated with the development of other *EGFR* mutations (12). Characterization of NSCLC with TKI-sensitive *EGFR* mutations reveals that PI3K/Akt pathway in these tumours is dependent on c-erbB-3 signalling (8). Tyrosine kinase inhibitor therapy of TKI-sensitive lung cancers inactivates c-erbB-3 signalling and downstream PI3K/Akt signalling, but c-erbB-3 signalling in TKI-resistant lung cancers appears to be uncoupled from EGFR and resistant to inactivation by TKIs (8). The development of the *EGFR* T790M mutation in lung cancer confers drug resistance and is associated with persistent activation of c-erbB-3/PI3K/Akt signalling (13). The constitutive activation of c-erbB-3 signalling in TKI-resistant lung cancer can also be mediated through the amplification of *MET*, recently identified in certain TKI-resistant sub-clones of lung cancer cells (9). The potential importance of c-erbB-3 in response and resistance to TKI therapies identifies it as a target for newer anticancer agents. However, c-erbB-3 is a considerably challenging target because it lacks kinase activity and its signaling functions cannot be inhibited by TKIs.

In this study, there was no correlation between c-erbB-3 and EGFR expression and survival or response to erlotinib in the studied population. High polysomy or amplification of c-erbB-3 did not correlate with gefitinib sensitivity in advanced NSCLC patients (5). To the best of the Authors' knowledge, this is the first study of immunohistochemical evaluation of c-erBb-3 expression in NSCLC patients treated by erlotinib. One study assessed the impact of erbB family members including c-erbB-3 in 42 lung adenocarcinomas treated by gefitinib (14). erbB-3 expression was higher in tumours from patients who achieved an objective response or stabilization of the diseases than in those with progressive disease (14). Reinmuth *et al*. (15) demonstrated that c-erbB-3 expression is not correlated with *EGFR* mutational status but Kawano *et al*. (3) found, among patients with *EGFR* mutation, that *erbB-3* mRNA levels were significantly higher than in those without *EGFR* mutation.

The small cohort of patients presented in this study was very homogeneous: all patients were Caucasian and the tumours expressed EGFR. But these facts do not allow an extrapolation of the results to a larger population of NSCLC patients.

In this study, there was no correlation between c-erbB-3 expression and clinical parameters (performance status, gender, age, stage, smoking history or histology). In contrast, Yi *et al*. (2) observed that squamous cell carcinoma showed the greatest rate of high c-erbB-3 positivity (34/119; 28.6%), followed by adenocarcinoma (41/256; 15.9%) and large cell carcinoma (7/66; 10.6%). Kawano *et al*. (3) described a tendency toward more c-erbB-3 protein-expressing tumours in smokers than in non-smokers, but also *erbB-3* mRNA expression levels were significantly higher in female, non-smoker and adenocarcinoma patients. In that study, Kawano *et al*. also observed that the *erbB-3* mRNA expression levels were related to the protein expression.

Although in this study 42% of the tumours co-expressed c-erbB-3 and EGFR, there was no linear correlation between cerbB-3/EGF-R expressions. Koutsopoulos *et al*. (1) observed also that only 3.5% of the NSCLC tumours had a co-expression of c-erbB-1 and c-erbB-3. Fontanini *et al*. (16)

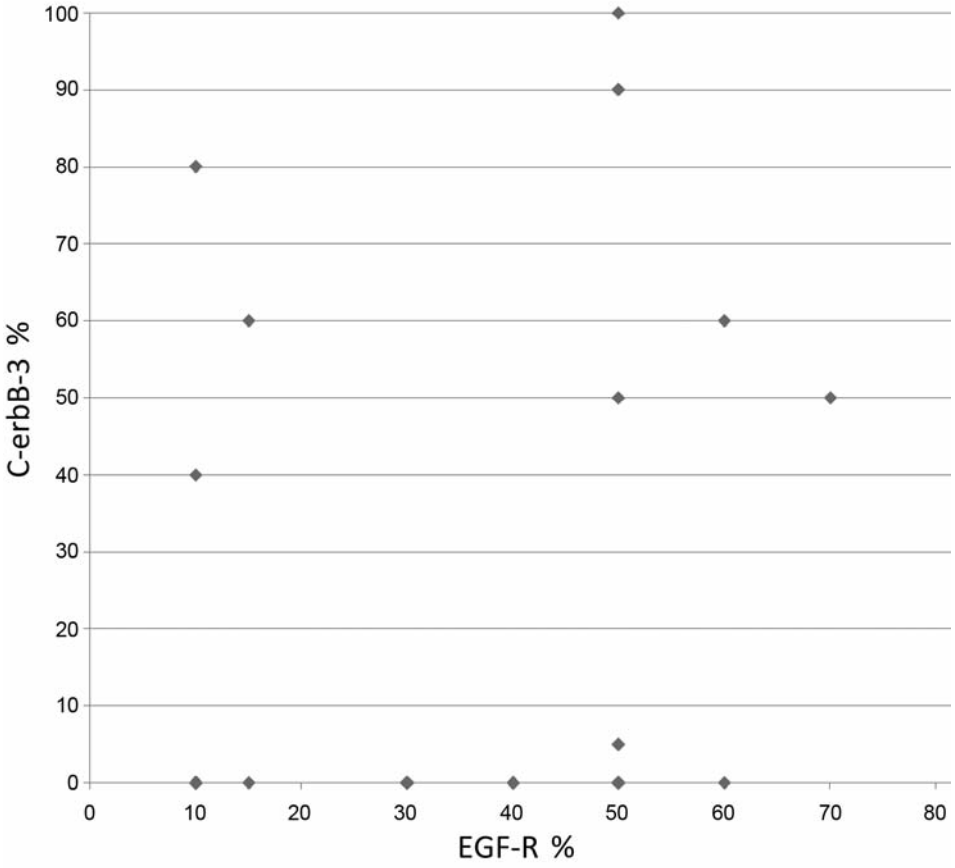

Figure 1. *Correlation between the percentage of EGFR- and c-erbB-3-stained cells: p=0.55, R=0.09.*

Table II. *Characteristics of the five non-progressive patients.*

Gender	Smoking history	PS	Histology	No. line	EGF-R (% of positive cells)	c-erbB-3 (% of positive cells)	Response
Male	Ex-smoker	60	Adeno	3	40	0	NC
Female	Never smoker	90	Adeno	2	10	0	NC
Female	Never smoker	80	Adeno	2	15	0	MR
Male	Ex-smoker	80	SQCC	3	100	10	PR
Male	Smoker	80	Other	2	90	0	PR

Adeno: Adenocarcinoma; SQCC: squamous cell carcinoma; NC: no change; MR: minor response; PR: partial response; PS: performance status.

described, in stage I-IIIA NSCLC, a co-expression with EGFR in 50% of the cases. Lai *et al*. (17) observed in 73 stage I NSCLC patients that c-erbB-1 was co-expressed with c-erbB-3 in 49% of the cases.

These discrepancies between studies can be explained, at least in part, by the different immunohistochemistry techniques used. The advantage of immunohistochemistry is its simplicity and low cost. However, numerous potential problems can be linked to immunohistochemistry techniques. No standard immunohistochemical protocol has been developed for lung cancer. Variability in tissue fixation and processing, variable sensitivity and specificity of antibodies and difference in scoring may alter the test results. The particular antibody used might change the results obtained. Koutsopoulos *et al*. used a rabbit polyclonal antibody against N-terminus of c-erbB-3 protein whereas Fontanini also used a mouse monoclonal antibody (RTJ2) (16). The cut-off used to determine the positivity of a tumour could also affect the

results. For EGFR, this study used 10% of EGFR-positively stained cells as a cut-off, as already used in previous works and required by the Belgian law for erlotinib refunding. Whether cytoplasmic, membranous or even nuclear staining should be taken into account in lung cancer remains unclear. Nuclear positivity of c-erbB-3 was observed in some specimens by Koutsopoulos *et al*. (1), however, they only took into account membranous staining. Further investigation is needed to evaluate the biological significance of nuclear staining. No nuclear staining was found in this study.

Further studies on large sample sizes will help to determine if c-erbB-3 is a clinically relevant marker to define patients who will benefit from EGF-R TKI.

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